

Circadian Rhythms

Methods and Protocols

Edited by

Ezio Rosato

Circadian Rhythms

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Circadian Rhythms

Methods and Protocols

Edited by

Ezio Rosato

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Preface

Rhythmicity is a pervasive feature of life. Most organisms, from bacteria to humans, have the ability to interpret and predict the daily cycles of our world, which indicates the presence of a timing device, a circadian (from the Latin *circa diem*, “about a day”) clock, able to synchronize the endogenous functions with the external environment. Furthermore, the ability to manipulate the temporal dimension offers ground to complexity, as the organisms have the opportunity to separate competing or even incompatible functions within the same cell. Thus, it is not surprising that natural selection is operating on the circadian clock, an additional reminder of the importance of this regulatory pathway. Selection has been shown directly by competition experiments between clocks with different periodicities, and indirectly by studying the molecular evolution of clock genes.

In the last 20 years, the molecular mechanisms underlying the functioning of the circadian clock have been actively investigated for several model systems. It has emerged that circadian timing affects every kind of organism and, in multicellular organisms, many different cell types. Basic and specialized cell functions are regulated by the clock through multiple molecular events. Furthermore, although the major divisions of life use different molecular cogs in the building of the pacemaker, there is a common design based on interlocked negative feedback loops. Many components and molecular functions can feed into the loops at different levels, making the architecture of the clock intrinsically robust and open to a wide range of interactions with other major regulatory pathways. This has become even more apparent after microarray studies have shown that key regulators of metabolic pathways, cell cycle components, ion channels, and immuno-response genes are all transcribed in a rhythmic fashion. Further developments have extended the description of the interconnection between the circadian and cell cycles and sketched a role for clock dysfunctions in cancer development. Although we have begun to understand the basic mechanisms of the clock, we still do not have a definitive answer to many questions. We still ask ourselves how the clock generates rhythmic phenotypes in the model systems we have studied for so long. Moreover, we start asking with more insistence how the circadian clock is regulated in other organisms, especially those also showing robust rhythmicity in other temporal domains.

To answer those questions, we have at our disposal a large arsenal of methodologies. These range from a whole organism approach, analyzing physiology and behavior, to a more reductionist attitude using genetics, molecular and cellular biology, and post-genomics technologies. The power of this multilevel approach is visible in the huge progress achieved by the chronobiology field in the last 20 years. However, the variety of methods, further multiplied by the peculiarities of each model system, and the hitches added by the temporal dimension, might have a hard impact on the novice.

The aim of *Circadian Rhythms: Methods and Protocols* has been to provide a resource that can be adopted by several types of users: those who are new to circadian biology, those who are already active in the field but are interested in learning new techniques, and researchers who are considering moving to a new model system or undertaking comparative studies and would like to consult protocols applied to different organisms before starting the study of new species. This task has been achieved by collecting a full range of methods, many provided by leading experts in the field, that should satisfy the needs of the novice, by illustrating procedures that have been recently introduced in circadian studies, and by presenting, for many basic techniques, variations to take into account the peculiarities of different model systems.

Finally, I would like to express my gratitude to the contributors who have shared their protocols and experience with the community, making the realization of *Circadian Rhythms: Methods and Protocols* possible.

Ezio Rosato

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I _____

OVERVIEWS

Light, Photoreceptors, and Circadian Clocks

Russell G. Foster, Mark W. Hankins, and Stuart N. Peirson

Summary

Research over the past decade has focused increasingly on the photoreceptor mechanisms that regulate the circadian system in all forms of life. Some of the results to emerge are surprising. For example, the rods and cones within the mammalian eye are not required for the alignment (entrainment) of circadian rhythms to the dawn–dusk cycle. There exists a population of directly light-sensitive ganglion cells within the eye that act as brightness detectors; these regulate both circadian rhythms and melatonin synthesis. An understanding of these “circadian photoreceptor” pathways, and the features of the light environment used for entrainment, have been and will continue to be heavily dependent on the appropriate use and measurement of light stimuli. Furthermore, if results from different laboratories, or species, are to be compared in any meaningful sense, standardized methods for light measurement and manipulation need to be adopted by circadian biologists. To this end, we describe light measurement in terms of both radiometric and photometric units and consider the appropriate use of light as a stimulus in circadian experiments. In addition, the construction of action spectra has been very helpful in associating photopigments with particular responses in a broad range of photobiological systems. Because the identity of the photopigments mediating circadian responses to light are often not known, we have also taken this opportunity to provide a step-by-step approach to conducting action spectra, including the construction of irradiance response curves, the calculation of relative spectral sensitivities, photopigment template fitting, and the underlying assumptions behind this approach. The aims of this chapter are to provide an accessible introduction to photobiological methods and explain why these approaches need to be applied to the study of circadian systems.

Key Words: Radiometry; photometry; light; action spectra; photoentrainment.

1. Introduction

Until recently, light has been used as a gross stimulus to elicit a response from the circadian clock. In such experiments organisms are usually exposed to “bright” artificial light controlled by a simple timer that regulates exposure

by turning lights either on or off. These conditions bear little resemblance to the natural photoperiod, and may actually confuse our understanding of circadian mechanisms. This approach is analogous to using a hammer to drive in a screw, an action that is quick and easy but entirely inappropriate. The recent general interest in the action of light on the circadian system makes it all the more important for circadian biologists to adopt the standardized approaches of photobiology. This will be critical if experimental results from different laboratories, or even species, are to be compared in any meaningful way.

In the first part of this chapter we will illustrate the finding that the photoreceptor systems involved in clock regulation are quite distinct from the photoreceptor pathways associated with image formation. Following this brief introduction, the discussions will then focus on the use of different light stimuli in circadian experiments and the appropriate methodologies for the measurement and manipulation of light. The final section details how action spectroscopy can be used to define the photopigments underlying circadian responses to light.

2. Mammalian Photoentrainment

Until recently, discussion that the eyes of humans and other mammals might contain a novel photoreceptor mechanism generated either bewilderment or hostile rebuttal by most researchers. It seemed impossible that something as important as another group of light-sensing cells could have been missed. The rationale was that the eye has been the subject of serious study for some 150 yr, and in broad terms we understand how the eye functions. Photosensory rods and cones of the outer retina transduce light, and the cells of the inner retina provide the initial stages of signal processing before topographically mapped signals travel down the optic nerve to specific sites in the brain for advanced visual processing. All responses to light were ascribed to this basic mechanism. However, an interest in how circadian rhythms are regulated by light led to the discovery of an entirely new form of ocular light sensor that has little to do with image detection.

The circadian timing system fine-tunes physiology and behavior to the varying demands of activity and rest and is synchronized (entrained) primarily by the systematic daily change in the gross amount of light (irradiance) at dawn or dusk. This daily adjustment to the light cycle has been called “photoentrainment” (1). The classic example of a mismatch between biological and environmental time is jet lag. We ultimately recover from jet lag as a result of exposure to the light environment in the new time zone. Our circadian pacemaker, or “master clock,” resides in the suprachiasmatic nucleus (SCN) (2). This small paired nucleus resides in the anterior hypothalamus; destruction of the SCN abolishes 24-h rhythmicity. Light information reaches the SCN

through a dedicated pathway (the retinohypothalamic tract), which originates in the retina (3,4). Eye loss in every mammal, including humans, confirms that photoentrainment originates within the eye (5). However, studies during the 1990s in mice with hereditary retinal disorders produced some very puzzling results. Despite the fact that most of the rods and cones had been lost in these mice, and no visual light perception was detected, photoentrainment to the light–dark cycle still occurred. It seemed extraordinary that the sensitivity of the circadian system to light did not parallel the loss of either rod or cone photoreceptors, or the loss of visual function (6). This work paved the way for the development of a transgenic mouse model (*rd/rd cl*) that was engineered to lack all functional rods and cones. Despite the ablation of the classical photoreceptors, both circadian entrainment and the regulation of pineal melatonin remained intact in these animals (7,8). There had to be another light-sensing mechanism within the eye. Furthermore, studies on *rd/rd cl* mice showed that a number of other physiological and behavioral responses to environmental brightness are either intact or retained at some level in the absence of the rods and cones. Such responses include pupil constriction (9) and the direct modification of behavioral responses to light, such as masking behavior (10). This suggests that novel photoreceptors might contribute to many more aspects of mammalian physiology and behavior than previously suspected. For example, light level modulates sleep, cortisol, heart rate, alertness, performance, and mood. Whether these irradiance responses are also influenced by non-rod, non-cone ocular photoreceptors is the subject of ongoing studies.

The cellular localization of the non-rod, non-cone ocular photoreceptors has been based on a number of different lines of evidence (11). The most comprehensive approach has employed the isolated rodless and coneless *rd/rd cl* mouse retina in combination with calcium (Ca^{2+}) imaging techniques. Approximately 1% of the neurons in the retinal ganglion cell layer responded to light directly (12). Detailed analysis showed that there exists a heterogeneous coupled syncytium of intrinsically photosensitive neurons in the ganglion cell layer of the mouse retina that detects environmental brightness (12). The use of action spectrum approaches (see **Heading 5**) has shown that these photoreceptors employ a previously uncharacterized opsin/vitamin A-based photopigment with peak sensitivity in the blue part of the spectrum near 480 nm (opsin photopigment [OP]⁴⁸⁰) (9,13). Furthermore, behavioral studies in humans suggest that we also possess an ortholog of mouse OP⁴⁸⁰ (14–16). Currently the gene for this photopigment awaits unambiguous identification, but *Opn4* or melanopsin is a very strong contender (13,17–19).

It is important to note that although rod and cone photoreceptors are not required for the regulation of the circadian system, this does not mean that these photoreceptors play no role. Indeed, the data emerging suggest that there

is a complex interaction among rods, cones, and novel photoreceptors in the regulation of circadian responses to light. For example, *rd/rd cl* mice fail to entrain to dim light–dark cycles with a normal phase, initiating their activity several hours before congenic wild-type controls (20). In addition, action spectra for phase-shifting circadian rhythms in wild-type mice suggest the involvement of rods and/or cones (Thompson, S., et al., unpublished data).

Why multiple photopigments seem to mediate the effects of light on temporal physiology remains a mystery but must surely relate to the task of extracting time-of-day information from dawn and dusk (1). During twilight, the quality of light changes in three important respects: (1) the amount of light; (2) the spectral composition of light; and (3) the source of light (i.e., the position of the sun). These photic parameters all change in a systematic manner and in theory could be used by the circadian system to detect the phase of twilight. For example, at twilight there are very precise spectral changes, primarily an enrichment of the shorter wavelengths (<500 nm) relative to the mid-long wavelengths (500–650 nm) overhead (21). If the circadian system was capable of utilizing multiple photopigments to ratio changes in the relative amounts of short- and long-wavelength radiation, and couple this information with irradiance levels, then the phase of twilight could be determined very accurately (1).

The photosensory task of entrainment is likely to be very complex. On the basis of what we know about other sensory systems, we would predict that the photic inputs regulating temporal physiology will be both specialized and complex. Indeed, we have strong evidence for both novel (specialized) and multiple (complex) inputs regulating temporal physiology in mammals and many other species (22). For example, even unicellular organisms such as *Gonyaulax* employ multiple photopigments for photoentrainment (23). An understanding of both the photoreceptor systems regulating the clock and their sensory task is dependent on the appropriate use and measurement of light. Outlined below are some of the approaches required for these measurements.

3. The Measurement of “Light” or Radiant Energy

Technically light and radiant energy are not the same. Radiant energy is considered to be all electromagnetic energy, whereas historically, light refers to the part of the electromagnetic spectrum that is visible to the human eye (between the wavelengths of 380 and 750 nm). Thus, the strict definition of light is based on human sensitivity to radiant energy. However, the term “light” is generally used to define radiant energy detected by physiological systems in humans and nonhumans, and the term “light” will be used interchangeably with the term “radiant energy” in this chapter. An understanding of light (or radiant energy) as a stimulus is essential if biological responses to light are to be understood.

The measurement of light can be undertaken using a variety of techniques and expressed in several different units. This diversity has led to considerable confusion about what different light units represent and which units are most appropriate for a given experimental situation. We can start by dividing light measures into two broad categories, radiometric or photometric measures. These terms can be further subdivided and are associated with specific units. The terms and units discussed in the following subheadings are summarized in [Table 1](#).

3.1. Radiometry

Radiometry is the measurement of radiant energy in the form of electromagnetic radiation, which includes ultraviolet radiation, visible light, and infrared radiation. An ideal radiometric detector has a near flat spectral response ([Fig. 1A](#)), and the remaining irregularities may be compensated for by calibration. A range of radiometric quantities and units is used; the two most commonly used in biology are irradiance and radiance ([Fig. 2A,B](#)).

Irradiance is a radiometric measure of the amount of light falling on a known surface area. The unit of measure of irradiance is W/m^2 . However, most detectors are calibrated in W/cm^2 . Biologists use this measure to quantify the incident light coming from all directions over a 180° field of view ([Fig. 2A](#)). Note that many irradiance detector heads do not provide a complete 180° field of view. Instead of using a cosine diffuser (which integrates light from an entire 180° field), detector heads are constructed so that light from a broad field of view passes through an aperture and falls on the detector surface, commonly of 1 cm^2 . The most frequently used irradiance units are $\mu\text{W}/\text{cm}^2$ and $\text{photons}/\text{cm}^2/\text{s}$.

Radiance is a measure of the amount of light in a specific region of space ([Fig. 2B](#)). The key to understanding radiance is that it refers to the amount of light as viewed from a specific direction. Radiance detectors are usually constructed by using an aperture and a positive (convex) lens in front of the detector, which allows light from a defined region of space (unit of solid angle or steradian [sr]) to fall onto the detector surface. Thus, radiance is expressed in terms of energy per unit area, per unit time, per sr. The most commonly used units are $\mu\text{W}/\text{cm}^2/\text{sr}$ and $\text{photons}/\text{cm}^2/\text{s}/\text{sr}$.

3.2. Irradiance or Radiance?

Even with the definitions above, the appropriate application of irradiance or radiance measures is not always clear. The rule is that if the direction or position of the light source is important, then radiance measures are used. If the position of the light source is not important, then irradiance measures should be collected. A few examples below may help clarify this issue further.

Table 1
Units of Radiometry and Photometry

Radiometric measures	
Measurement of electromagnetic energy within the optical spectrum, which includes ultraviolet radiation, visible light, and infrared radiation. An ideal radiometric detector has a “flat” spectral response.	
Irradiance	Radiance
• Measure of radiant energy from all directions over a 180° field of view. • Common units: – erg/s/cm² – μW/cm² – photons/cm²/s	• Measure of radiant energy viewed from a specific direction or region in space. • Common units: – erg/s/cm²/sr – μW/cm²/sr – photons/cm²/s/sr
Photometric measures	
Measuring human visual responses to radiant energy. A measurement of visible light that falls between the wavelengths of 400–700 nm. The spectral response of a photometric detector is not flat but attempts to reproduce that of the average human eye. Two average human-eye responses are used: a <i>photopic response</i> (maximum sensitivity at a wavelength of 555 nm), and a <i>scotopic response</i> (maximum sensitivity at a wavelength of 507 nm). By convention, photometric measurements are considered photopic unless otherwise stated.	
Illuminance (illumination)	Luminance
• Measure of light from all directions over a 180° field of view. • Common units: – lux (lx) – lumen (lm)/m² – phot (ph)= lm/cm² – footcandles (fcd) = lm/ft²	• Measure of light viewed from a specific direction or region in space. • Common units: – candela (cd) – lumen (lm)/sr – cd/m² = lm/m²/sr – lm/m²/sr = lx/sr – footlamberts (fL) = fcd/sr

A radiance measure would be appropriate to determine the amount of light passing through a hole in the forest canopy and falling onto a basking lizard. However, having said that radiance is good for measuring the amount of light in a particular direction of space, this measure is not an appropriate unit for characterizing point sources of light (e.g., light emitted from a laser) because the field of collection of the detector usually extends outside the area of the

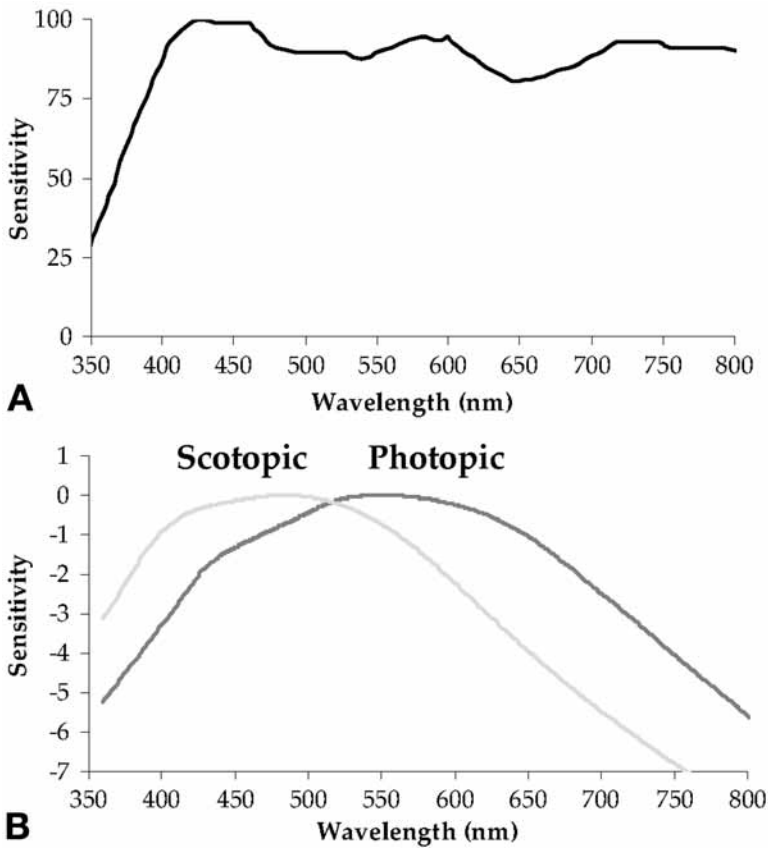


Fig. 1. The “ideal” spectral responses of (A) a radiometric detector that has a relatively flat spectral response (sensitive to all wavelengths equally) and (B) the sensitivity of a photometric detector (e.g., luxmeter) that has a spectral response approximating the spectral range of human photopic color vision. Human scotopic sensitivity is also shown for comparison (*see Subheading 3.4.* and [Table 1](#) for details).

point source. For this reason it is better to use an irradiance detector and direct the point source 90° to the collection surface.

A common problem in circadian rhythms research is the characterization of a phase-shifting light stimulus. For example, when a light pulse is delivered to a rodent in circadian experiments, it is often placed into a chamber designed to provide near-uniform lighting (such a device is shown in [ref. 6](#)). Light usually originates above the animal, and is scattered using a frosted glass screen (an “opal”screen). As a result, the light falling on the animal is uniform and occupies an extended area. In this example the light should be characterized using a

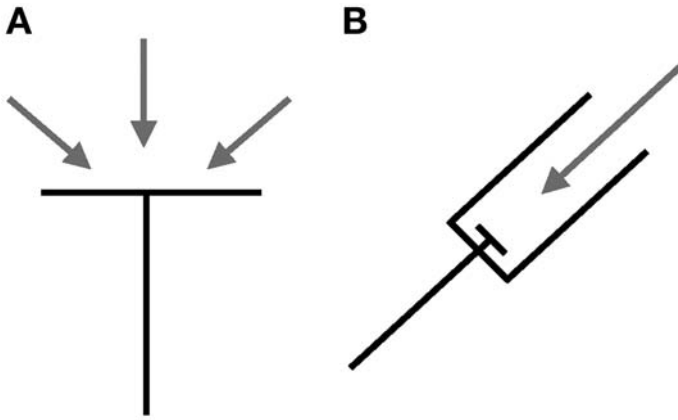


Fig. 2. Representation of the arrangement of the photocell used for the detection of (A) irradiance/illuminance and (B) radiance/luminance. Irradiance/illuminance measures collect radiant energy from all directions over a 180° field of view, whereas radiance/luminance measures collect radiant energy viewed from a specific direction or region in space (see Table 1). In most cases a lens is used to determine the collection angle for a radiance detector; however, a narrow aperture tube can be substituted (as shown in B). Figure based on that shown in ref. 21.

measure of irradiance with the detector head placed either on the opal screen or at the level of the animal. A final example would be the measurements of sunlight. Sunlight within the environment varies in its direction, spectral quality, and flux (21). For example, if a measure of all the available light arriving on the ground is required, then irradiance measures are appropriate. By contrast, if measures of sunlight at the horizon are required (the viewing direction is clearly important), then radiance measures are appropriate. In this case the radiance measure would ignore much of the scattered light above the horizon.

3.3. The Photon

It is critical to remember that photopigments absorb energy as photons, and hence act as photon counters. Thus all measures of light used in biological experiments should ideally be expressed as a photon flux. The energy of a photon is proportional to the reciprocal of its wavelength ($1/\lambda$). This means that high-frequency short-wavelength light (blue light) has photons of higher energy than red light (see Note 1). Nonetheless, because photopigments act as photon counters, the biological effectiveness of light is unrelated to these differences in energy. Thus if the biological effectiveness of 450-nm light (blue light) and 620-nm light (red light) are to be compared, the stimuli must contain the same number of photons (photon flux). Because the standard radiance/irra-

diance measures are assessments of energy, if the stimuli are calibrated to contain an equal radiometric irradiance (same number of $\mu\text{W}/\text{cm}^2$), then the blue-light stimulus would contain fewer photons than the red-light stimulus. In these experiments the apparent sensitivity of the photopigment will be erroneously shifted to longer wavelengths. When comparing the effect of different wavelengths of light, it is necessary to convert measures of radiant energy into photons. An example of such a calculation is shown below:

To convert energy flux (e.g., W/cm^2) to photon flux you first need to know the amount of energy contained in a photon: $E = hn$

Where

E is the energy in a photon ($\mu\text{W}\cdot\text{s}^2/\text{photon}$)

h is Planck's constant ($6.626 \times 10^{-34} \text{ W}\cdot\text{s}^2$)

n is the frequency of the wave. Remember that: $n = c/\lambda$

Where

c equals the speed of light in a vacuum ($3.00 \times 10^{17} \text{ nm/s}$)

λ is wavelength in nanometers (nm).

Therefore, for 1 photon of 500-nm light:

$$E = (6.626 \times 10^{-34} \text{ W}\cdot\text{s}^2) \times (3.00 \times 10^{17} \text{ nm/s}) / 500 \text{ nm} =$$

$$3.976 \times 10^{-19} \text{ W}\cdot\text{s}^2/\text{photon or}$$

$$3.976 \times 10^{-13} \mu\text{W}\cdot\text{s}^2/\text{photon}$$

The conversion of a light value from watts into photons proceeds as follows: If, for example, the irradiance of the stimulus was $3 \times 10^{-2} \mu\text{W}\cdot\text{s}^2$ at a λ of 500-nm light then one would need to divide the measured value in $\mu\text{W}\cdot\text{s}^2$ by the energy of a 500 nm photon in $\mu\text{W}\cdot\text{s}^2$. Thus $= 3 \times 10^{-2} \mu\text{W}\cdot\text{s}^2 / 3.975 \times 10^{-13} \mu\text{W}\cdot\text{s}^2/\text{photon} = 7.5 \times 10^{10} \text{ photons}/\text{cm}^2/\text{s}$. You may want to repeat this calculation using the same irradiance ($3 \times 10^{-2} \mu\text{W}\cdot\text{s}^2$) but substitute different λ values. By doing this you will appreciate the relationship between watts and photons, and that *an equal number of watts does not contain the same number of photons*.

An alternative unit of photon flux is the Einstein ($\text{E}/\text{m}^2/\text{s}$) or microEinstein ($\mu\text{E}/\text{m}^2/\text{s}$), and is the measure of light most often used by plant physiologists: 1 Einstein = 1 mol of photons at a specific wavelength (6.022×10^{23} photons); 1 μE = 1 μmol of photons at a specific wavelength (6.022×10^{17} photons).

3.4. Photometry

In contrast to radiometry, photometry is concerned with measuring human visual responses to radiant energy and deals only with the measurement of visible light that falls between the wavelengths of 400 and 700 nm. In addition, the spectral response of a photometric detector is not flat (like a radiometer) but attempts to reproduce that of the average human eye (**Fig. 1B**).

A complication in photometry is the use of two average human-eye responses. During the hours of daylight, the cone photoreceptors of the retina provide the “photopic response,” with a maximum sensitivity at a wavelength of 555 nm (**Fig. 1B**). Under low levels of light the rod photoreceptors of the retina provide the “scotopic response,” with a maximum sensitivity at a wavelength of 507 nm (**Fig. 1B**). By convention, photometric measurements are considered photopic unless otherwise stated. A more detailed discussion of photometric units, is provided below and in **Table 1** (24).

The basic energy unit for the photometric unit is the lumen (lm), which is defined with reference to a truly monochromatic (single wavelength) light source in the following way: for scotopic measurements, 1 W = 1700 scotopic lm at 507 nm. For photopic measurements, 1 W = 683 photopic lm at 555 nm. However, note that because photometric measures do not have a flat spectral response, and because the emission spectrum for most light sources is varied (*see Heading 4*) converting photometric units into radiometric units is difficult. Indeed, this is possible only if the precise emission spectrum of the light source is known (*see Note 2*). If both photometric and radiometric units need to be measured or reported it is much simpler (and probably more accurate) to use dedicated detectors.

A critical point is that photometric units provide a measure of visual brightness as it would appear to a human observer. Of course, the spectral responses of other organisms differ from that of humans. Therefore it is inappropriate to use photometric measurements when studying nonhuman photoreceptor systems. Moreover, because we know that the photoreceptors that mediate human circadian responses to light are different from the rods and cones and appear to involve a novel opsin with a λ_{max} of 460 to 480 nm (15,16,22), there is no justification for the use of photometric measures in human circadian studies either.

The only reason for reporting photometric light measures in circadian papers is that historically many studies have used these units, and thus these measures can provide a point of comparison with earlier studies. Finally, a critical point to remember is that because photometric measures do not have a flat spectral response (**Fig. 1B**) they should never be used to calibrate colored (monochromatic) light sources. For example, equal photometric measures of “red” and “green” light will have a very different photon content, and would not be comparable light stimuli.

Having rejected photometric measures as unsuitable for circadian studies, let us now, for completeness, consider these measures in some detail. A range of photometric units are used, which in the first instance may be separated into those associated with illuminance or luminance (**Table 1**).

3.5. Illuminance (Illumination)

Illuminance is a measure of the amount of light falling on a surface of defined area. It is analogous to the radiometric measure of irradiance except that detectors do not have a flat spectral response and depend on the subjective human sensitivity to light (**Fig. 1B**). One lumen of light falling on one square foot is termed a *footcandle* (fcd). The metric equivalent, 1 lm/m^2 , is called a lux (lx; $1 \text{ lx} = 10.76 \text{ fcd}$) (**Table 1**). Traditionally the lux has been the most commonly used measure of light in circadian experiments. To a large extent this is owing to the low cost and availability of luxmeters in comparison with other detectors.

Luminance is also known as photometric brightness. Luminance is analogous to the radiometric term “radiance” except for its dependence on the subjective human sensitivity to light. Like measures of radiance, luminance detectors have a defined collection angle for light, expressed in sr. In most cases a lens is used to determine the collection angle. A great variety of different units are used to express luminance (**Table 1**), the most common being lx/sr. Note that the term “intensity” is often used as a general term to mean an amount of light. Technically, however, it has a very specific meaning. Intensity is a measure of the radiant or luminous energy from a given direction in space. Units for intensity are energy (luminous or radiant) per sr per unit time. To avoid confusion, the term “intensity” should not be used as a general term for a quantity of light.

4. Light Stimuli

Deciding which light source is appropriate for any circadian experiment will depend on a large number of factors, including compatibility with previous experiments, cost, availability, and the nature of the experimental question. Emission spectra for a number of commonly used light sources are shown in **Figs. 3** and **4**, and illustrate the big differences that occur in the spectral output of these lights. In general, light stimuli can be divided into white or monochromatic light sources.

4.1. “White” Light

A very large number of “white”—or, more accurately, broad-spectrum—light sources have been used in circadian experiments. Many sources are dependent on a heated element, and as such will exhibit temperature dependence. Such sources exhibit “black-body” (or cavity) radiation, in that the output of radiation at any given wavelength is dependent on the temperature of the radiator (**Fig. 3**). Commonly available light sources include tungsten–halogen incandescent lights, which contain relatively low levels of short-wavelength/blue light, are rich in long-wavelength and infrared light, and whose spectral emis-

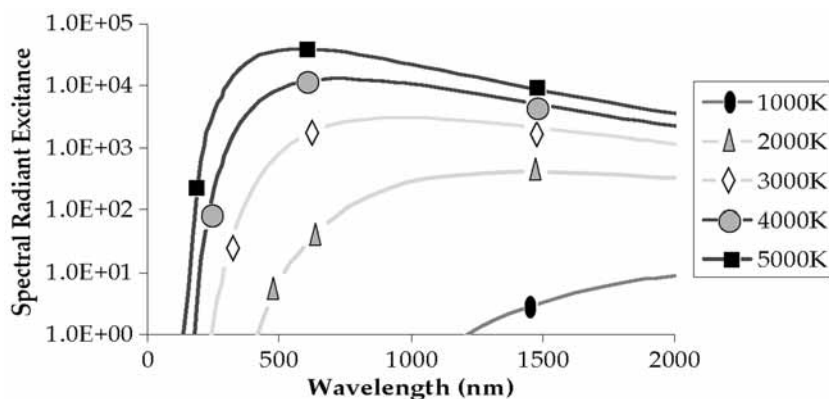


Fig. 3. Black-body or cavity radiation at a range of temperatures from 1000 K to 5000 K. The wavelength of maximum emission moves to shorter wavelengths as temperature increases.

sion is profoundly affected by voltage (**Fig. 4A**; *see Note 3*); a huge variety of fluorescent lights, most of which contain strong emission peaks or lines in the blue/ultraviolet (UV) portion of the spectrum (**Fig. 4B**); high-pressure xenon arc lamps, which provide one of the most uniform broad-spectrum light sources (**Fig. 4C**); and sunlight, the spectral composition of which varies both with the time of day and other features of the environment (**Fig. 4D**) (21). For a detailed description of the emission spectrum of these and other broad spectrum light sources *see* **ref. 24**. The point to emphasize is that although these “white” light sources seem superficially similar, they vary greatly in their emission spectra, as illustrated in **Fig. 4**.

4.2. “Monochromatic” Light

There are five ways to produce narrow spectrum or monochromatic light.

1. Absorption filters are made of glass, gelatin, or liquids in which colored agents are dissolved or suspended. Gelatin filters are perhaps the most versatile type of filter and are made by mixing organic dyes in gelatin. They have the advantage of being relatively inexpensive and can be made very large, but are not stable over time (they can change their transmission spectrum with use and age) and tend to have fairly broad transmission spectra (**Fig. 4E**). Also be aware that short-wavelength (blue) filters often have a second long-wavelength (red) transmission that is frequently not reported by the manufacturer.
2. Cutoff filters essentially transmit or block light above or below defined cutoff wavelengths. These can be particularly useful in the generation of narrow-band UV light sources when combined with fluorescent lights that have strong emission peaks in the UV (e.g., **ref. 25**).

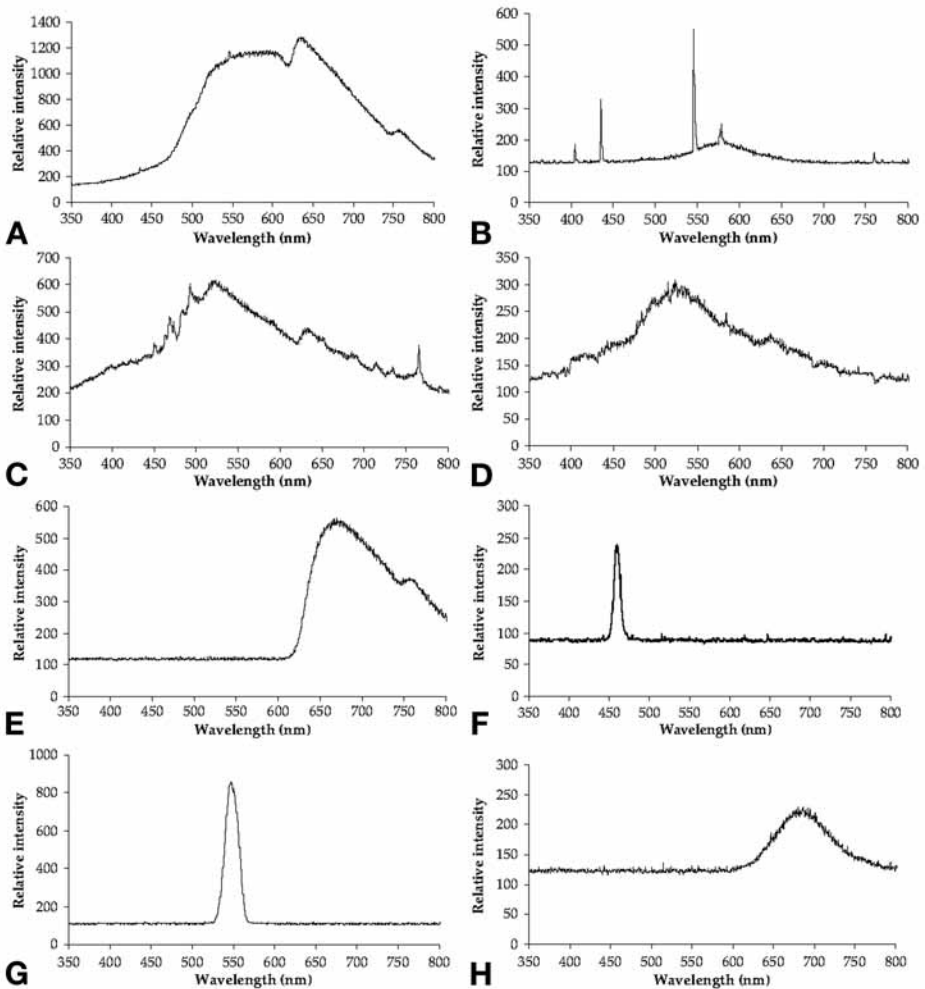


Fig. 4. Emission spectra for a variety of commonly encountered white-light (A–D) and monochromatic (E–H) light sources. (A) Tungsten–halogen bulb. (B) Fluorescent tube. (C) Xenon-arc light. (D) Sunlight (cloudy day in London). (E) Gel filter (Kodak safelight). (F), 460-nm interference filter. (G) Diffraction grating monochromator set to 550 nm. (H) “Red” light-emitting diode. All measurements made using an USB2000 fiberoptic spectrometer (Ocean Optics).

3. Interference filters produce high-quality monochromatic light using an arrangement of highly reflective surfaces that ensure that only a narrow band of wavelengths are transmitted (*see Note 4*). These filters are made by successively evaporating dielectric and silvered films on glass. Interference filters are defined on the basis of their wavelength of maximum transmission (λ_{max}) and on the

basis of their half-maximal bandwidth ($\Delta\lambda$)0.5 (**Fig. 4F**). An important point to note is that the angle of incidence of the light falling on these filters greatly modifies their transmission spectra; it is critical that the incident light strike the filter at 90° to the surface (thus the source beam needs to be collimated). Also note that the mirrored surface should face the light source. Although they are able to produce higher-quality monochromatic light than gelatin filters, interference filters are expensive, and because of their small size, cannot be used to bathe large areas in monochromatic light. Interference filters, coupled with powerful tungsten-halogen (or similar) bulbs and high-quality optic fibers for light delivery, have formed the basis of most monochromatic light sources for circadian experiments (**6**).

4. Monochromators or diffraction gratings are designed to disperse incident light into its spectral components—from which any desired narrow band of wavelengths can be isolated (**Fig. 4G**). Although very versatile, they are expensive, produce relatively little monochromatic light, and, like interference filters, cannot be used to irradiate large areas.
5. Light-emitting diodes (LEDs) emit monochromatic light of high purity, although their half-maximal bandwidth is usually broader than interference filters. The early LEDs had a relatively low irradiance confined to a fairly narrow portion of the spectrum, usually in the yellow, red, or infrared (**Fig. 4H**). However, the latest generation of LEDs can produce large amounts of monochromatic light at wavelengths that span the whole spectral range. Thus, for some applications, LEDs may provide a suitable substitute for interference filters.

4.3. Changing Emission Level (Photon Flux) of Light Source

There are two common methods for controlling the irradiance of a light source. The voltage applied to the light source can be varied and/or the light source can be screened using neutral density filters. In almost all cases, changing the voltage applied to the light source will change its color temperature, and hence the spectral emission of the bulb (as described by black-body radiation; *see Fig. 3*). In this case an associated change in spectral composition may invalidate comparisons at different irradiances. Any experimental effects might result from changes in the light spectrum rather than changes in the photon flux. By contrast, if good-quality interference filters are being used to generate monochromatic light, changes in the spectral composition of the light source will not present a problem. In any case, the preferred way of changing the flux of a stimulus is through use of neutral density filters, which ideally reduce transmission at all wavelengths equally. A great range of neutral density filters exist, varying in both quality and price, ranging from black plastic trash bags to glass neutral density filters carefully calibrated to reduce radiant flux by specific log unit amounts. Other methods for controlling photon flux include the use of crossed polarizers, and movement of the light source away from the exit aperture of the device.

4.4. White Light or Monochromatic Light as Stimulus?

It is difficult to make generalizations, but ideally, a “standard” monochromatic light stimulus, selected to coincide with the maximum (or near maximum) spectral response of the photosystem under examination, should be used for entrainment experiments. Monochromatic light has the advantage that it can be precisely defined and reproduced. Monochromatic light also avoids the complication of photoreversal, the biological phenomenon in which light at one wavelength drives a response in one direction, whereas light at another wavelength drives it in the reverse direction. Photoreversal has been reported in a number of photoreceptor systems and may even be important in photoentrainment (26,27).

Despite the advantages of monochromatic light, most researchers use artificial broad-spectrum light as a general entraining stimulus. This is largely because white light is inexpensive to produce and can be used to irradiate large areas. It is also true that we do not know which, or how many, photopigments mediate photoentrainment in most organisms. Without this knowledge it is impossible to select a “standard” monochromatic light stimulus. The ease of use and cost advantages of white light will undoubtedly ensure its continued use as a stimulus in circadian experiments; however, it is important to be aware of the problems that are inherent in this approach. For example, the varied types of fluorescent and incandescent light sources have widely different emission spectra. As a result, comparison of the effects of white light treatments are valid only when exactly the same light source has been used (remember that the emission spectrum from different types of fluorescent bulbs can be very different, and that the emission spectrum of a bulb will change with age). In addition, radiometric light detectors are sensitive to radiant energy in both the visual spectrum and infrared parts of the spectrum. If a light source is rich in infrared light (e.g., any incandescent lamp such as a tungsten-halogen bulb) then a significant portion of the flux recorded will be derived from energy that is not typically perceived as light. This would greatly complicate comparisons between results using different white-light sources, particularly when comparing incandescent and fluorescent light sources, because fluorescent lights produce relatively low levels of infrared. It is more difficult to change the levels of radiant energy produced by a white-light source uniformly across the spectrum. Note that neutral density filters are often “neutral” only across certain regions of the optical spectrum. This problem is often observed when attempting to produce very low irradiances of light using several layers of neutral density filters. There comes a point when adding additional layers of filters has little effect in lowering the amount of “light” detected by the radiometer. This occurs because some plastic neutral density filters are not effec-

tive at blocking infrared light, and radiometers are sensitive in the infrared part of the spectrum. Finally, white-light sources, and particularly incandescent sources, have an additional problem for the circadian researcher in that they generate heat and thus could provide a thermal zeitgeber (an entraining agent).

5. Action Spectra

Action spectra provide a means of establishing the spectral sensitivity of a biological response by determining the amount of light of different wavelengths required to elicit the same response.

5.1. Why Action Spectra?

Absorption spectroscopy has been and continues to be an important approach to studying photopigments (28). The first law of photochemistry states that only the radiation absorbed by a substance can produce a photochemical effect, and therefore a measurement of the absorption spectrum of a pigment provides the most direct measurement of the spectral sensitivity of a photopigment. The problem, however, is that it is often difficult to isolate sufficient photopigment to get reliable spectra, and extracts may contain a mixture of photopigments. As a result, this approach is suitable only if the photopigment occurs in high abundance (e.g., rhodopsin from retinal rod photoreceptors [29]), or can be functionally expressed at high concentration (e.g., some of the cone photopigments [30,31]). Microspectrophotometry can provide a partial solution to this problem. In this case a narrow monochromatic beam of light is passed through the photopigment-containing part of a photoreceptor cell to obtain the absorbance of the pigment. The advantage of this approach is that it enables photopigments to be characterized within their native photoreceptors, and enables multiple intact, isolated photoreceptors to be analyzed within a single preparation. (For further details on microspectrophotometry, *see refs. 32 and 33.*)

Unfortunately, these approaches cannot often be applied to the study of circadian photopigments, where the location of the photoreceptor itself may be unknown, and even if known, the photopigment concentration may be too low for such absorption techniques to be feasible. Action spectra provide an indirect approach to analyzing such photopigments. Action spectra determine the spectral sensitivity of a biological response at different wavelengths. If a high-photon flux at a particular wavelength is required to produce a biological effect, then this implies a weak absorption by the photopigment. Conversely, a lower photon flux at a specific wavelength to evoke the same response implies a high absorption. If the reciprocal of the light response is plotted against wavelength (λ), then the action spectrum should mirror the absorption spectrum of the pigment mediating the response.

A step-by-step approach to conducting an action spectrum is detailed in the following subheadings, including all necessary calculations. Equations are not provided using full mathematical notation simply to facilitate understanding.

5.2. Biological Response

The biological response to light should be investigated using a series of monochromatic light stimuli, and for each wavelength, a range of irradiances should be used. These data are used to construct irradiance response curves (IRCs). The precise number of wavelengths, irradiance levels, and replicates per irradiance level will be a compromise based on such factors as the variability in response and the length of time it takes to undertake the experiments. The most important consideration, however, is the resolution of the IRCs. It is not worthwhile to investigate a large number of wavelengths if each of the IRCs is poorly resolved. It is better to use the same resources to generate a smaller number of well-resolved IRCs. Another consideration relates to the use of short wavelength stimuli. Many light sources produce relatively little short-wavelength light (*see Heading 3*), and many organic molecules and biological pigments absorb in the UV. In addition, short-wavelength light is scattered to a much greater extent than long-wavelength light (Rayleigh scatter). Because responses to short-wavelength stimuli are often modified by some or all of the above, the responses measured often provide less-reliable measurements of relative sensitivity. As a result, try to bias the analysis to the longer-wavelength limb of the sensitivity curve.

The first task is to gain an accurate measure of the photon flux of the stimulus. Irradiance is most often measured using a power meter, typically in $\mu\text{W}/\text{cm}^2$. As the energy per photon (W/s) = hc/λ , the number of photons at a specific wavelength λ (in nm) can be calculated as in **Eq. 1**.

$$\text{Energy per photon (W/s)} = hc/\lambda \quad (1)$$

where h is Planck's constant ($6.625 \times 10^{-34} \text{ W/s}^2$) and c equals the speed of light in a vacuum ($3.00 \times 10^{17} \text{ nm/s}$). The photon flux may then be calculated (**Eq. 2**) by dividing the irradiance ($\mu\text{W}/\text{cm}^2$) by the energy per photon:

$$\text{Photon flux (Photons/cm}^2\text{/s)} = \frac{\mu\text{W/cm}^2}{\mu\text{W/cm}^2} = \frac{\text{irradiance}}{\text{energy/photon}} \quad (2)$$

For example, for monochromatic light at 500 nm, the energy per photon is $3.98 \times 10^{-19} \text{ W/s}$, or $3.98 \times 10^{-13} \mu\text{W/s}$. For an irradiance of $0.03 \mu\text{W}/\text{cm}^2$, this would give a photon flux of $7.44 \times 10^{10} \text{ photons/cm}^2\text{/s}$ (*see Subheading 3.3.*).

5.3. Constructing and Using Irradiance Response Curves

For each wavelength, plot the IRC as log photon flux (x-axis) vs response magnitude (y-axis) as shown in **Fig. 5A**. The EC_{50} is then calculated from this

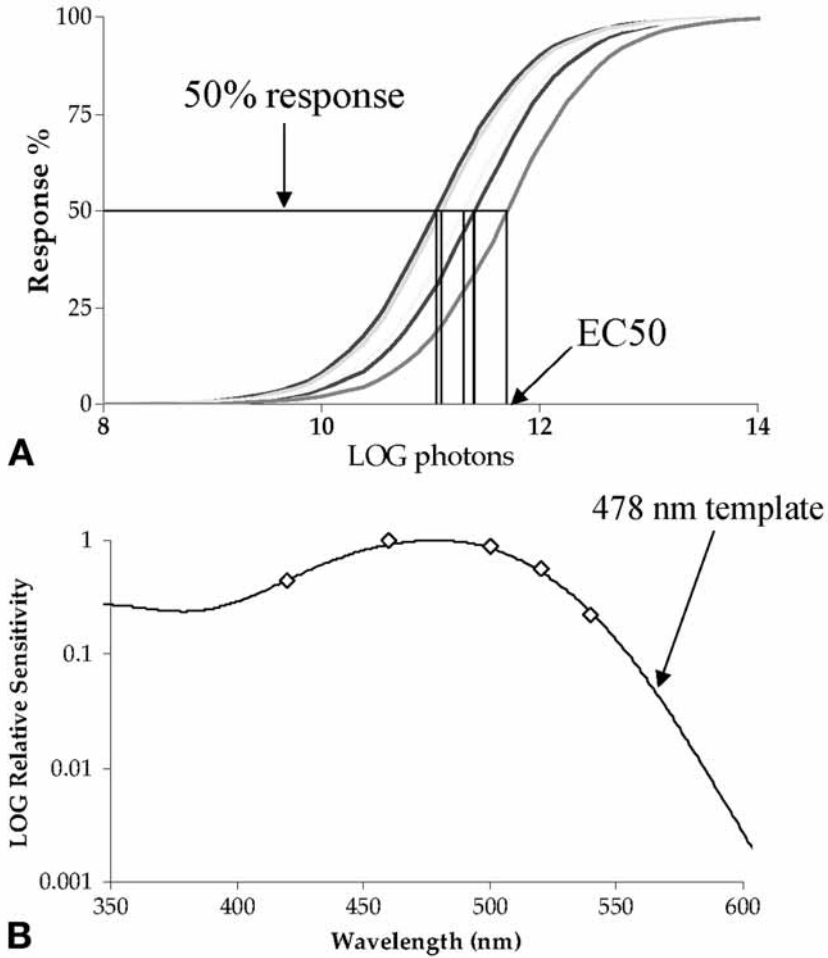


Fig. 5. Principles of constructing action spectra. **(A)** Irradiance response curves are first constructed, plotting the logarithm of light dose against biological response. A sigmoid model is then fitted to the data. **(B)** Relative sensitivity is then calculated from the photon dose required to elicit a 50% response at each wavelength (EC₅₀). This is plotted on a logarithmic scale and fitted against a visual pigment template (*see Subheading 5.5.* for details).

IRC by fitting a sigmoid function to the IRC. A sigmoid curve may be defined by four parameters (**Eq. 3**):

$$\text{Response} = \frac{(\text{Top} - \text{Bottom})}{1 + 10^{((\text{EC}_{50} - n) \times k)}} \quad (3)$$

where “*Top*” and “*Bottom*” correspond to the maximum saturating response and baseline response, respectively; the EC_{50} is the photon flux necessary to elicit a 50% maximum response (around which the curve rotates); and k is the Hill slope of the curve. Ideally, one should obtain a saturating response for every wavelength, and use a four-parameter model for every IRC. However, in practice this is often not possible, as it may not be feasible to produce a sufficient photon flux to produce a maximal response from the available light source. If the saturating response is known, then this value may be used as the maximum for all IRCs. In many biological systems no detectable response is obtained with a low photon flux, and thus the baseline can be set to 0. This leaves just two remaining parameters to fit, in what is often termed a “global” model (ref. 34; and see Note 5). Unlike linear regression, the best fit of a non-linear model such as a sigmoid curve cannot be solved analytically, and numerical methods are therefore required. This requires an iterative approach to minimize the sum of squares of the model (SS_{model}), which is equal to the sum of the difference between the data and the sigmoid model, squared (so as to become sign-independent) at each point on the IRC, i.e., $(data - model)^2$ (Fig. 6A). This is compared with the total sum of squares (SS_{total}), which assumes a straight line through the mean (Fig. 6B). The measure of fit, R^2 , is a value between 0 and 1, and may be thought of as the fraction of the variance explained by the model, and is derived from $1 - (SS_{model}/SS_{total})$. When $R^2 = 0$, then the model is no better than a straight line through the data, and when $R^2 = 1$, the model describes the data perfectly (R^2 is normally used to denote the fit of nonlinear regression, whereas r^2 is used to denote linear regression).

Most statistical packages (e.g., GraphPad) will perform these dose–response curves to give the best possible fit. However, the above is provided to enable researchers to have a better understanding of the calculations these packages are actually performing; they may be emulated simply within Excel using the Solver Add-in (Microsoft) for those without access to such statistical software (see Note 6). In addition, the same protocol is required for fitting the resulting action spectra with a photopigment template.

5.4. Calculate Relative Sensitivity

Once IRCs have been constructed and the EC_{50} calculated for each wavelength, the relative sensitivity may be calculated. As sensitivity is based on the photon flux required to elicit the same response, a low-photon flux is indicative of high sensitivity to this wavelength, and vice versa (i.e., there is a reciprocal relationship between photon flux required to elicit a 50% response and sensitivity). To calculate relative sensitivity, divide the *lowest* photon flux obtained by the photon flux obtained at each wavelength (on a linear scale,

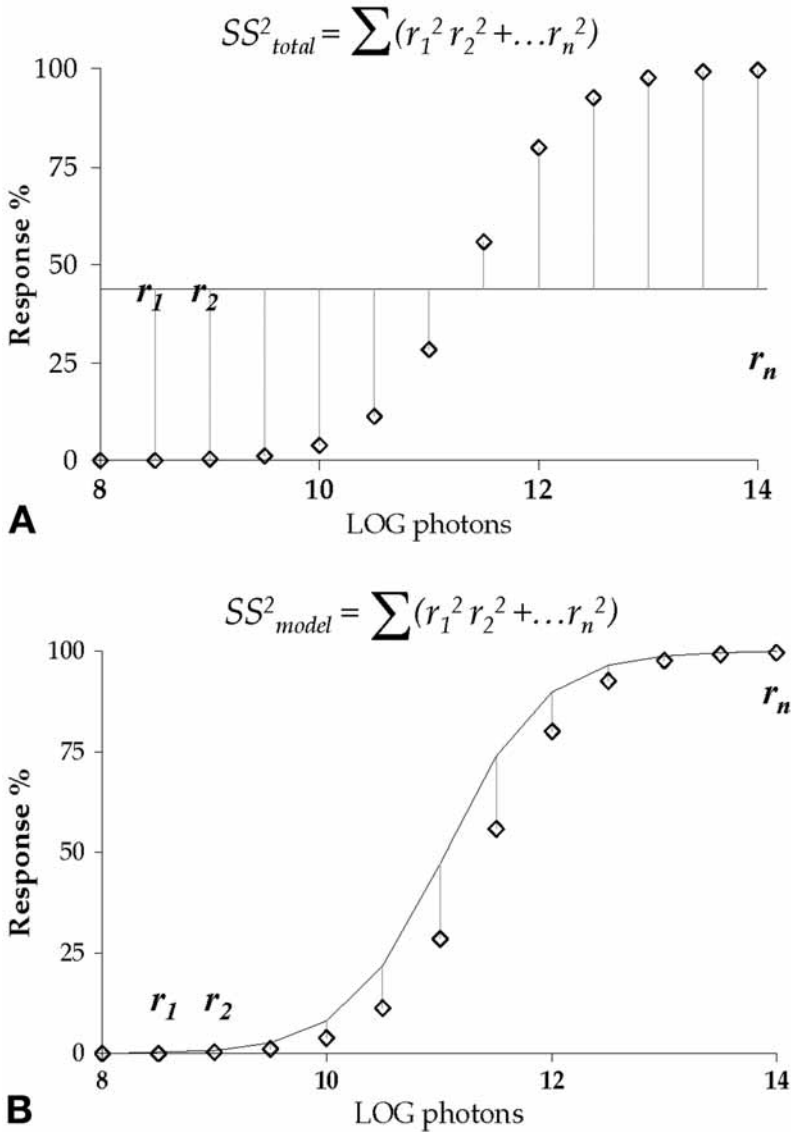


Fig. 6. Fitting of nonlinear models is based around minimizing the sum of squares between data and model. **(A)** The total sum of squares (SS_{total}) is calculated by plotting a straight line through the mean of the data points and then calculating the sum of the squared difference of each point from this line. **(B)** The fit of a mathematical model to the data is then calculated from the sum of the squared differences from each data point to the model (SS_{model}). The fit is termed R^2 , and equals $1 - (SS_{total} / SS_{model})$, representing the proportion of the variance explained by the regression.

not in log units). This will produce a value between 0 and 1, with the wavelength of maximum sensitivity having a value of 1 and higher photon fluxes possessing values below 1. The action spectrum is simply the plot of relative sensitivity (y-axis) against wavelength (x-axis), as shown in **Fig. 5B**.

5.5. Fitting a Template

Photopigments have well-characterized absorption spectra. In 1953, H. J. A. Dartnall demonstrated that vitamin A-based photopigments have a characteristic absorption profile, and when expressed on a frequency scale (wavenumber rather than wavelength), the shape of this profile is constant (**35**). Although the wavelength of maximum sensitivity ($\lambda_{\max}$) may vary, the shape of the absorption spectrum is constant. In short, a single curve defined by the $\lambda_{\max}$ will provide the entire absorption profile of a vitamin A-based photopigment. The discovery of an A₂ form of retinal in amphibia, teleosts, and some reptiles resulted in a second slightly differing visual pigment template, which is proportionally broader (**36**). Several improvements to the template first produced by Dartnall have been made by Partridge and De Grip (**37**) and Govardovskii et al. (**38**).

Wavenumber (traditionally measured per cm²) may be calculated simply by $1/\lambda \times 10^6$. For example, 500 nm is equivalent to 20,000 waves/cm². The wavenumber separations from the peak for both A₁ and A₂ visual pigments are provided in **Table 2**; for more advanced templates the reader is directed to the original publications.

When calculating the fit of a visual pigment template, calculations should be conducted on the logarithm of relative sensitivity. The reason for this should become apparent when one considers the above calculations for R^2 . As the SS^2_{model} is calculated from the squared difference between data and model, a difference of 10% near the wavelength of maximum sensitivity will contribute proportionally more to the SS^2_{model} than a 10% difference at any other wavelength. When calculated on a logarithmic scale, a 10% difference would give the same contribution to the SS^2_{model} at all wavelengths. This is important in that it ensures that all data points contribute equally to the fit of the photopigment template. Fitting a photopigment template relies on the same nonlinear protocol as that for fitting the IRC. The only variable here is the $\lambda_{\max}$, and the SS^2_{model} is minimized by changing this parameter to provide the best fit, with a resulting R^2 value as above.

5.6. Action Spectra and Potential Problems

The construction of action spectra is dependent on several basic assumptions; if these assumptions are violated then the resulting action spectra will be

Table 2
Absorption Spectra for Visual Pigment

Absorbance %	Wavenumber separation from peak	
	Rhodopsin tA ₁)	Porphyrropsin (A ₂)
30	+3736	–
40	+2940	+3866
50	+2442	+2905
60	+2020	+2353
70	+1620	+1880
80	+1224	+1445
90	+796	+985
95	+545	+670
100	0	0
95	–520	–321
90	–732	–869
80	–1031	–1213
70	–1275	–1476
60	–1492	–1707
50	–1705	–1924
40	–1930	–2142
30	–2178	–2372
20	–2460	–2638
10	–2850	–2999

To convert wavelength, λ (nm) wavenumber (cm^{–1}), simply take $1/\lambda \times 10^7$.

distorted and not reflect the absorption spectrum of the photopigment. Problems occur in the generation of action spectra when (1) responses fail to show univariance; (2) there is a lack of reciprocity; and (3) when screening pigments modify the light reaching the photopigment.

Univariance is evident when the driving mechanism is the same at all wavelengths. This is apparent in a family of IRCs when they all show a similar slope (*k* value). If IRCs demonstrate markedly different slopes, then this suggests a complex set of interactions, as might occur if different photopigments contribute to the same biological response.

The law of reciprocity states that photochemical action is dependent on the product of light intensity and duration of exposure. Thus, exposure to low irradiances for longer durations should yield the same effect as higher irradiances for shorter durations. Reciprocity may break down when saturating stimuli are applied, as the addition of extra photons cannot initiate any further response (hence the need to use the EC₅₀).

Screening of the photopigment by a biological pigment may also occur and modify the apparent spectral sensitivity of the biological response. Several factors may affect both the wavelength and amount of light reaching the photopigment, including increased scattering of short-wavelength light (Rayleigh scatter), absorption by hemoglobin in overlying tissues (39), and other biological pigments such as melanin (40). In addition, the study of biological responses that in themselves modify the light reaching the photopigments are also a problem, as in pupil and melanophore constriction, and these effects should be taken into account.

6. Conclusions

The problem of providing an appropriate light stimulus in circadian experiments is greatly exaggerated because the photopigments that mediate photoentrainment have not been defined in most organisms. This issue is compounded by our poor understanding of the features of the light environment that provide the entrainment signal. In the absence of this information, circadian researchers should attempt to use light stimuli that can be accurately reproduced. For the reasons outlined above, light stimuli should be monochromatic and defined in radiometric units. This will enable different research groups to compare and collate their data, and will simplify the interpretation of results.

Although techniques based on absorption spectroscopy provide an ideal means of studying classical photoreceptors, these techniques are often not suited to the study of novel photoreceptors, such as those mediating circadian responses to light. Action spectra provide an ideal indirect method of investigating the sensitivity of biological responses to different wavelengths of light. By investigating the photon dose required to elicit the same response at different wavelengths, it is possible to determine the relative spectral sensitivity of the response, even if the photoreceptor mediating this response is unknown. Such action spectra approaches have provided much of the information we have regarding the photoreceptor systems mediating circadian responses to light.

7. Notes

1. Short-wavelength light possesses high-energy photons. If these photons contain sufficient energy they may damage biological systems, stripping electrons from molecules (i.e., ionizing radiation such as UV light, X-rays, and γ -rays). Conversely, long-wavelength photons (e.g., infrared light, microwaves, and radio waves) are of a lower energy and any energy they impart to a biological system is through the effects on the vibrational or rotational energy of electrons, which act to elevate temperature.

2. By calculating the photometric output for a light source, and the proportion of this output around 555 nm, it is then possible to approximate this photometric emission to power as $1 \text{ W} = 683 \text{ photopic lm}$.
3. Flame sources such as candles provide a similar spectral output.
4. The transmission spectrum (transmittance [T]) of an interference filter. Such filters are classified on the basis of their wavelength of maximum transmission (λ_{max}) and on the basis of their half-maximal bandwidth $(\Delta\lambda)_{0.5}$. High-quality interference filters have a half-maximal bandwidth close to 10 nm (Fig. 4F). Note that the spectral T of an interference filter depends on the angle of incidence of light. Light should always strike the filter surface at 90° . Also note that the T spectrum of interference filters tend to be symmetrical, whereas the T spectrum of gelatin filters are often very asymmetrical (Fig. 4E).
5. It is in fact possible to define one complete IRC and then use a single nonsaturating irradiance at other wavelengths. This makes the assumption that univariance holds for all wavelengths.
6. The starting point for nonlinear regression is also important. A reasonable approximation must be used, or a local minimum may be reached, at which point the iterative procedure will stop. This is most easily visualized by thinking of the best fit as the lowest point of a landscape formed by the parameters being fitted, with the height of the landscape being defined by the SS_{model} . The fitting protocol changes the parameters to try to reach the lowest point of the landscape (metaphorically, trying to reach the valley bottom). However, regions may exist within the landscape with a low SS, but not the *lowest* possible SS (these may be thought of as higher valleys or dips in the landscape). These local minima will lead to the search for the minimal SS being terminated—often yielding a poor fit. As such, nonlinear regression cannot be conducted from an arbitrary starting point—a reasonable estimate must be provided as a starting point.

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Statistical Analysis of Biological Rhythm Data

Harold B. Dowse

Summary

The author has developed an ensemble of digital signal analysis techniques applicable to biological time series containing circadian and ultradian periodicities that is of very high resolution and functions well even in the presence of extreme noise and trend. A method for quantifying the significance, strength, and regularity of the rhythmic process is included. To illustrate these techniques, the author presents analyses of artificial periodic data containing varying amounts of noise, trend, and multiple periodicities. The periods and amplitudes of circadian and, where included, ultradian periodicities, and all other components of the test signals are known exactly. Analyses are illustrated in a step-by-step manner and the results are compared with the known input parameters. Trends are removed; spectra, autocorrelation functions, and rhythmicity indices are produced and discussed. References covering theory and details of all analyses are supplied. All programs employed are available from the author free of charge.

Key Words: Autocorrelation; biological rhythms; circadian; maximum entropy spectral analysis; MESA; noise; rhythmicity index; RI; spectral analysis; trend; ultradian.

1. Introduction

Extraction of reliable information on period, phase, and signal robustness from biological rhythm data is central to interpretation of the data. Advances in digital signal analysis in the fields of engineering, geology, and astrophysics have yielded a good selection of high-resolution algorithms, a number of which are applicable to analysis of biological time series. A combination of the older autocorrelation analysis with the modern technique of maximum entropy spectral analysis (MESA), adapted for the short, noisy, nonstationary time series typical of biological systems, has proven especially useful. MESA provides an extremely reliable estimate of period with arguably the highest resolution and sensitivity available, whereas autocorrelation can be used to test for the statistical significance of any periodicity in the data and, at the same time, provides

a useful measure of the strength of the signal being analyzed. In order to maximize the reliability and sensitivity of these analyses, it is common to remove digitally any nonstationary trends, as well as other periodicities not of interest. As an example of the latter, ultradian periodicity in deep body temperature in the hourly (circchoral) range may be obscured by the much stronger circadian cycle. Removal of the near 24-h period enables analysis of the ultradian oscillations (1–14).

2. Materials

1. PC running Windows® operating system capable of executing programs in a DOS window, or any system that can run MATLAB® (see **Note 1**) or similar software.
2. Text data editor with sufficient capacity to handle the required data stream. MS Word in text mode is convenient in Windows OS.
3. Any plotting system with sufficient capacity for large files. MATLAB is used here throughout.
4. SIGGEN: This program can create data sets containing up to two periodicities with sinusoidal, square, triangle, or sawtooth wave forms with any desired percentage of white noise added (see **Note 2**).
5. HRMES: This is a version of MESA adapted for biological rhythms by the author. It has a low-pass filter that can optionally be activated at run time, which digitally removes noise in the very short period range (see **Note 3**).
6. AUTOCO: This is a straightforward autocorrelation analysis program written by the author that contains the same optional digital filter as that found in MESA.
7. FILCON (see **Note 4**): This routine is used here for high-pass filtering to eliminate long-range trends or for removal of circadian periodicities to accentuate ultradian rhythms.
8. CROSSCO: A variation on autocorrelation that allows an objective estimate of phase and, additionally, a test of whether two data sets have periodicity in common.
9. AUTPEAK: A routine to find the height of the third autocorrelation peak to yield the rhythmicity index (RI; see **Note 5**). RI is an objective measure of how robust and regular a periodic signal is.
10. MESPEAK: A Turbo Basic routine to find the height of the three most prominent peaks in a MESA spectrum (see **Note 5**).

3. Methods

The methods outlined below describe the editing/formatting, preconditioning, analysis, and plotting of biological rhythm data in the circadian and ultradian range. The author has used similar programs to analyze *Drosophila melanogaster* heartbeat as well, but this aspect is not covered here (see **Note 6**). For this work, it was considered desirable to use artificial data, as periodicity, signal-to-noise ratio, amplitude, and waveform are controlled and hence are known exactly beforehand.

3.1. Editing and Preparation of Data Vector

3.1.1. Creation of Data Sets to Be Analyzed

The program SIGGEN was employed to create data sets similar to biological data. A simple sinusoid was chosen, but other waveforms can be produced by this program. A circadian period of 24.75 h was chosen for all data sets. This noninteger value provides a realistic test of the capabilities of the analysis programs. In all examples, the amplitude was set at unity and was positive everywhere except as noted. Both the MESA and autocorrelation routines in the suite always fit a straight line to the data by regression and subtract it to eliminate any linear trend. This has the added benefit of adjusting the mean of the series to zero. Eliminating this so-called “DC offset” emphasizes the periodic portion of the signal in the subsequent analysis. In general, amplitude will affect spectral power, but not RI. All data sets consisted of 10 d of readings with a sampling rate at twice per hour with one exception, as noted (SET III). A more rapid sampling rate has been shown to be unnecessary for periodicities in the circadian range and very high rates may actually be undesirable if behavioral data are being summed into “bins” (15).

In SET I, the data were produced with amounts of white noise added in the following percentages: 10, 20, 30, 40, 50, 60, 70, and 80. The raw data set generated with 80% noise is shown in **Fig. 1** (see **Note 7**).

In SET II, data were produced identically to SET I, except that a long-range sinusoidal trend was added with a period of 40 d, with the phase angle adjusted so that the data have an approximately monotonically increasing envelope from $t = 0$ to 10 d. The amplitude of this sinusoid was set at 10-fold that of the circadian periodicity. **Figure 2** depicts data containing 80% noise with the long-period trend added.

In SET III, data were produced as in SET I and additionally had a 1-h circchoral ultradian component added. The peak-to-peak amplitude of the circadian rhythm was 10-fold that of the ultradian, which was unity. Sampling rate was set to 12/h (i.e., at 5-min intervals) to characterize more adequately this relatively high-frequency component (7). Such circchoral rhythms have been uncovered in human deep-core body temperature using the sensitive methods outlined here (14). **Figure 3** shows the data containing the standard circadian periodicity along with an ultradian periodicity of 1 h. In this example 80% noise was added.

For SET IV, a series of files created with data sampled twice per hour and with a 24.75-h periodicity was generated. In these examples, phase was delayed by 0, 2, 4, 6, 8, and 22 h, respectively, to show the output of CROSSCO.

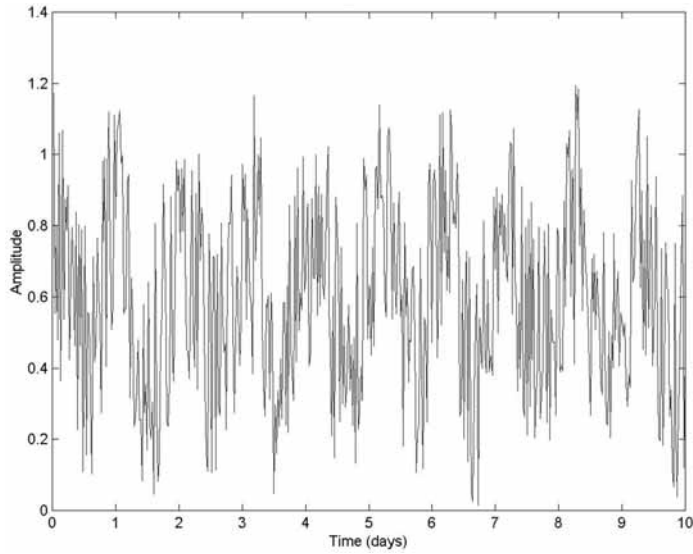


Fig. 1. An artificial data set consisting of a unit amplitude sinusoid and periodicity of 24.75 h with an added 80% white noise. Data were sampled at half-hour intervals over the equivalent of 10 d.

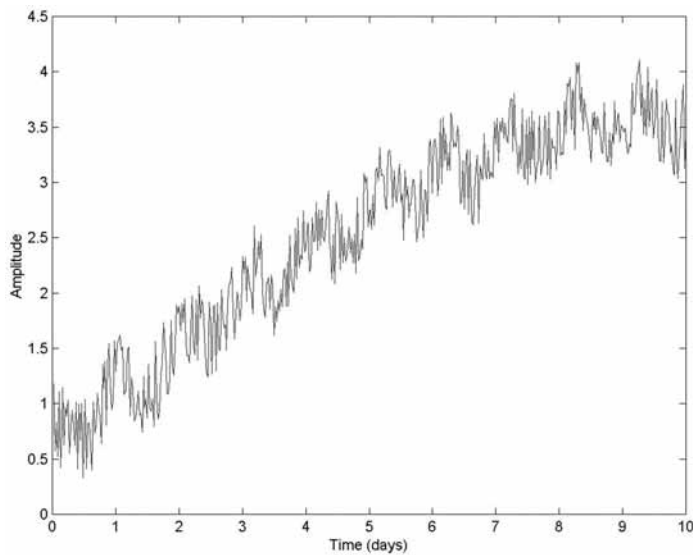


Fig. 2. The data set depicted in Fig. 1, but in this case with a monotonic trend added with amplitude functionally five times that of the sinusoid. Period and amplitude of the signal remain in Fig. 1.

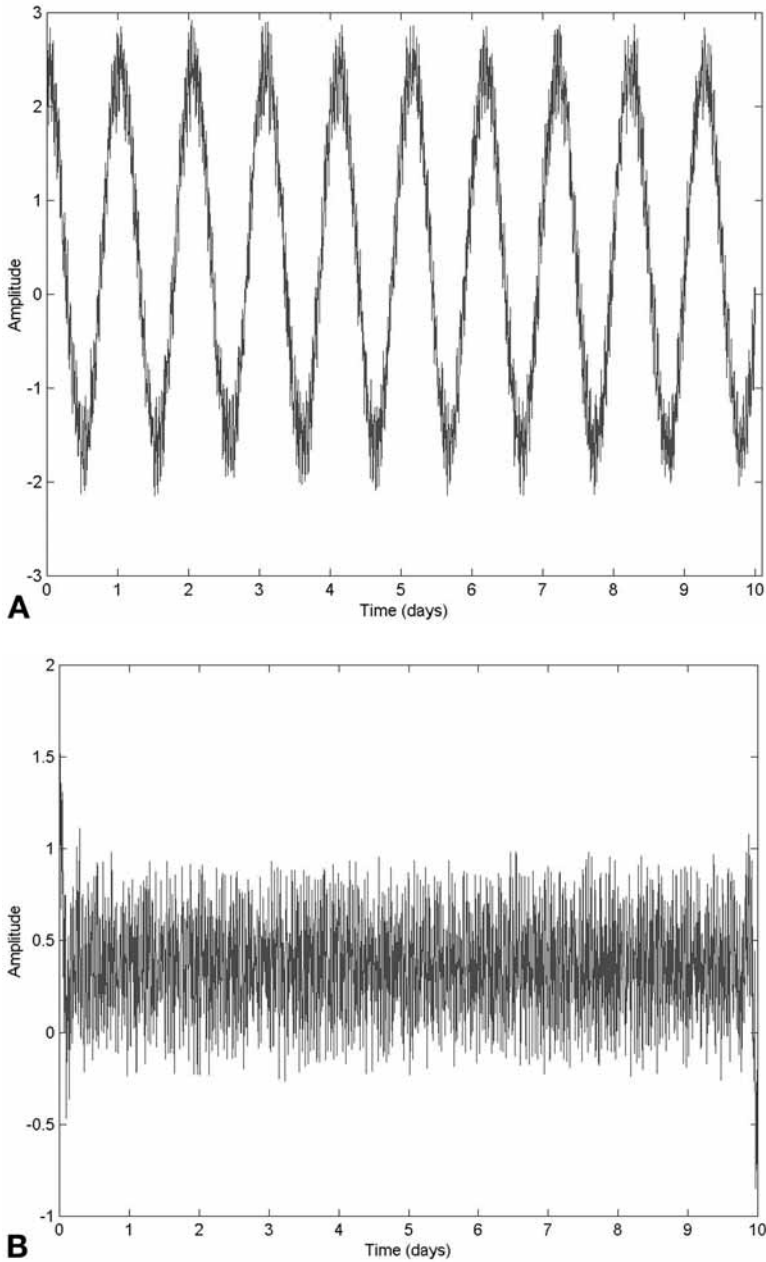


Fig. 3. (A) Data as Fig. 2, containing a periodicity of 24.75 h and 80% noise, but with a unit amplitude ultradian rhythm having a period of 1 h added. This cannot be seen in the unfiltered data. (B) The same data set after removal of the circadian range periodicity using FILCON.

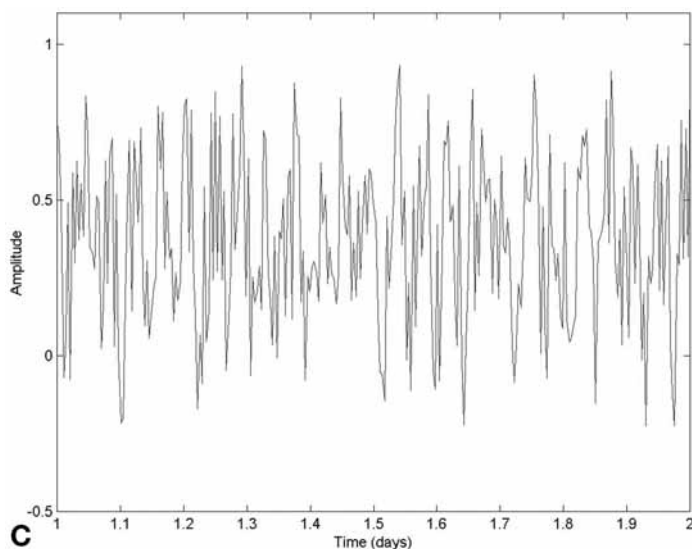


Fig. 3. (*continued*) (C) Here, a 1-d interval of the data is shown at a magnified scale to illustrate the noisy hourly rhythm.

3.1.2. Format of Data

The analysis software is configured to allow batch processing of concatenated files. Input data are placed in a single column. Each separate file is given a descriptive, reasonably short title (here, fewer than 15 characters) at the start of the vector. The end of each individual file is signaled by appending -5000. Batch processing is terminated with the word "END." Output of both spectra and autocorrelations is written file by file in two columns, with either spectral power or autocorrelation values in the first column and either period or lag in the second. In autocorrelation output, the 95% confidence value indicating "significant rhythmicity" is given below the title (*see Note 8*). For actual rhythmicity data, the columns of input are concatenated and formatted as above using an editor. Output for individual spectra and correlograms excised from the concatenated file were plotted using MATLAB (*see Note 9*). An example of the raw data format follows:

```
I80
1.173287
5.549589E-01
8.012864E-01
4.805624E-01
1.060405
```

```
...
3.837983E-02
2.867427E-01
4.349197E-01
6.987450E-01
8.844982E-01
1.210237E-01
4.635225E-01
-5000.000000
END
```

3.2. Estimating Periodicity and Strength of Rhythmicity

3.2.1. Estimates of Period

To estimate period, the concatenated data sets were analyzed using MESA and the results plotted using MATLAB software. Data were analyzed both with and without the use of a 4-h-cutoff low-pass two-pole Butterworth digital filter (see **Note 10** and **ref. 16**). To report the best estimate, the three highest spectral peaks were read from the output files with MESPEAK. These results were then checked against the output plots and the autocorrelations to ensure that the peak best representing the periodicity was chosen.

The output of MESA analysis for the data file from SET I, containing 80% noise with no low-pass filtering, is shown in **Fig. 4**. The period, as retrieved from the output data file, is 24.62, corresponding closely to the known programmed-in value. Despite the very low signal-to-noise ratio in the raw data, the MESA spectral peak is sharp and little of the noise appears in the region of the circadian periodicity. **Table 1** contains the results for periodicity in SET I.

For the data in SET II, with the long-period trend, the data were either run as is, with and without the low-pass filter, or after first being conditioned by removal of all periodicity greater than 2 d using FILCON, set to null out the coefficients for periodicities greater than 2 d. Analysis proceeded as usual from that point on. **Table 1** summarizes the output for these data. An improvement in the estimates is notable.

For SET III (with the circalunar component), the data were analyzed first untreated, and then again after conditioning with FILCON set to remove all periods greater than 10 h. **Figure 5** depicts the spectrum derived from the data containing a circadian period of 24.75 h, a 1-h ultradian periodicity of much lower amplitude, and 80% noise after de-trending with FILCON. **Table 1** shows the periodicities extracted by MESA before and after removal of the circadian periodicity. For data before filtering, both the primary and secondary peaks found are reported.

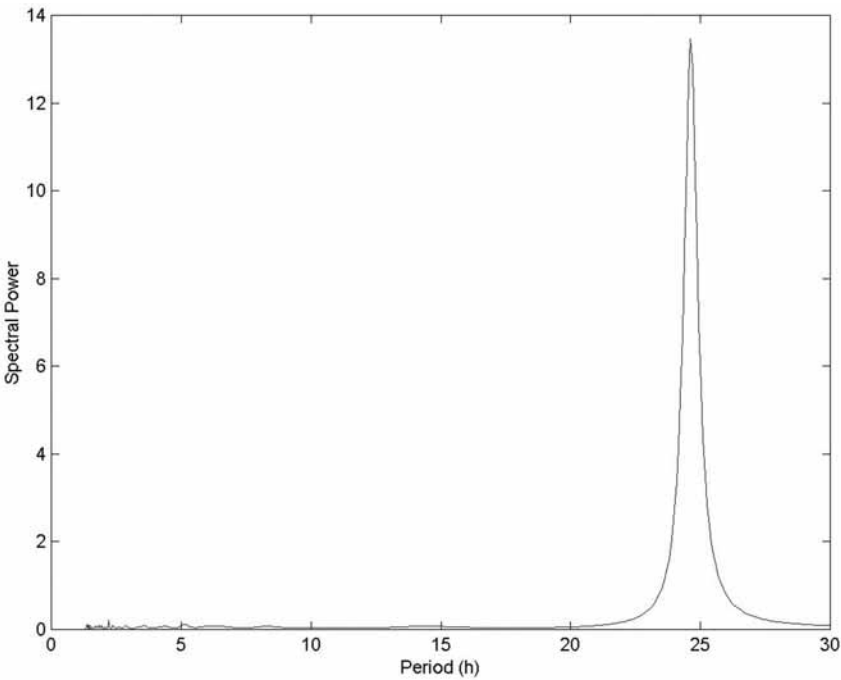


Fig. 4. The MESA spectrum from the data depicted in Fig. 1. No filtering was done, yet the spectrum is extremely clean, even given the high proportion of noise and attendant low signal-to-noise ratio.

Table 1
Primary Periods Found by MESA for Each Level of Noise

% Noise	I	II U	II F	III U 1°	III U 2°	III F
10	24.78	25.26	24.81	24.8	1.00	1.00
20	24.78	25.34	24.81	24.72	1.00	1.00
30	24.78	25.34	24.88	24.72	1.00	1.00
40	24.78	25.43	24.88	24.80	1.00	1.00
50	24.78	25.43	24.88	24.72	1.00	1.01
60	24.78	25.51	24.97	24.80	1.00	1.01
70	24.78	25.43	24.97	24.80	1.00	1.01
80	24.62	25.10	24.88	24.80	1.00	1.01

The data set analyzed is noted by set number (*see Subheading 3.2.1.*). U = Raw (1° and 2° indicate primary and secondary peaks uncovered), and F = output after program FILCON was run on the data. Note that even before the strong circadian rhythm was removed, MESA found the hourly peak even in 80% noise.

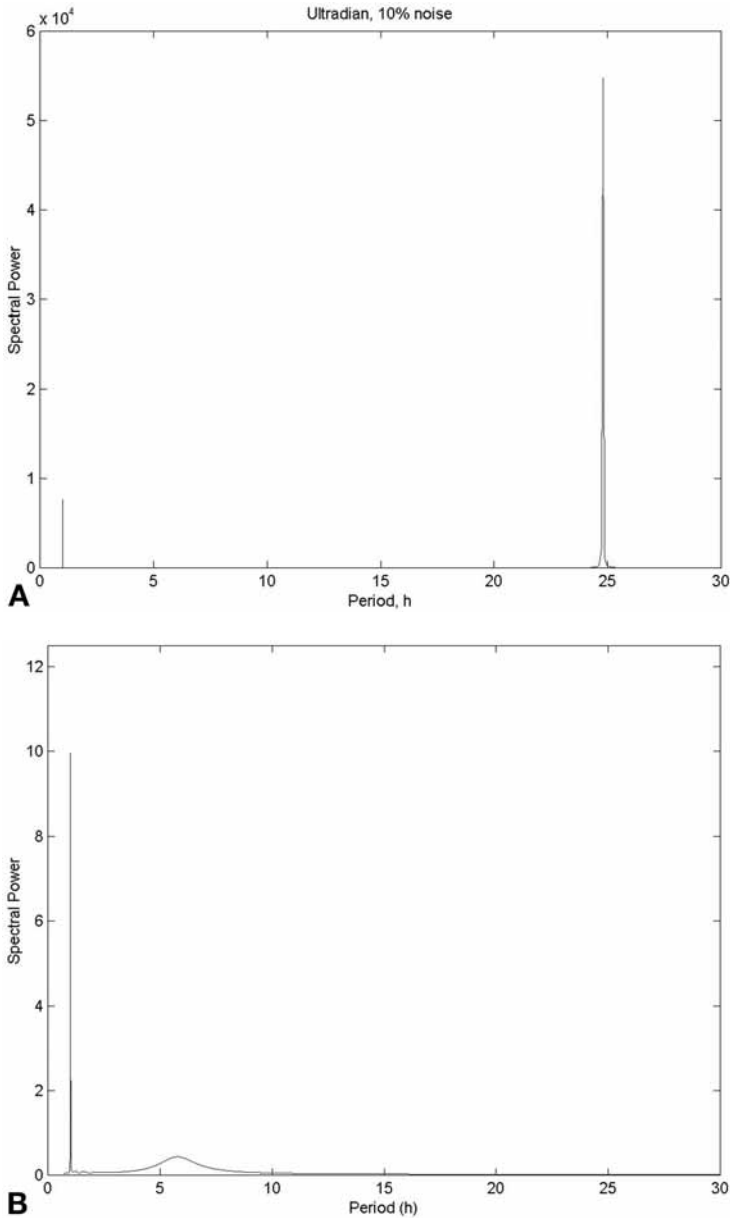


Fig. 5. MESA plot produced from the data shown in [Fig. 3 \(A\)](#) before removal of the circadian component with FILCON and [\(B\)](#) after removal. The large circadian peak has been entirely eliminated, and the remaining ultradian periodicity is clear, constituting the only peak in the spectrum. The small bump at approx 6 h is an artifact of the sharp cutoff of the Fourier filtering.

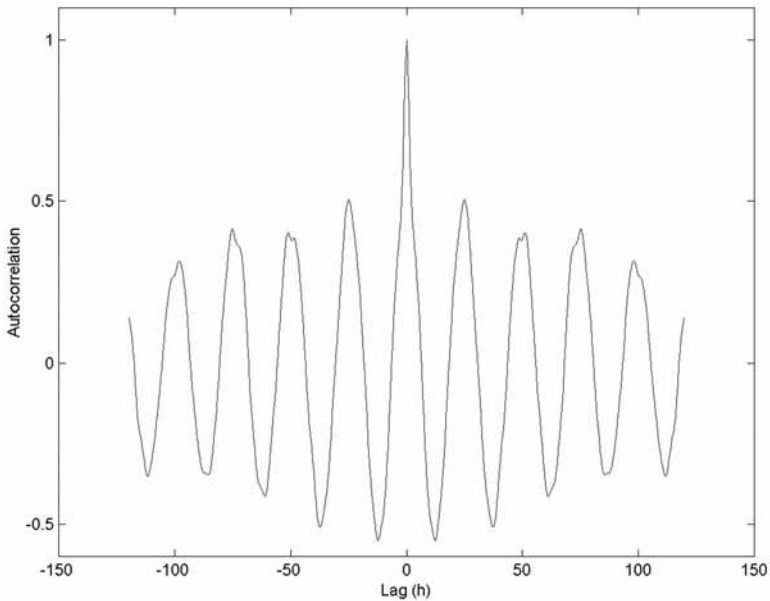


Fig. 6. Autocorrelation plot from the data set depicted in [Fig. 1](#). The decay of the envelope in the function is a result of the large amount of noise added. Nonetheless, strong rhythmicity is evident and corroborates the periodicity reported by MESA.

3.2.2. Autocorrelation Analysis

To test for significance of periodicity and provide a crosscheck on the period estimates, the program AUTOCO was run on all data as above. All data were treated as had been done with MESA with regard to filtering and conditioning with FILCON. Plotted output was compared with the spectral analysis results to check for agreement. The autocorrelogram was shown in [Fig. 6](#), computed for the same data set that was analyzed by MESA in **Subheading 3.2.1**. For all figures in this section, correlograms shown will be for the same files as the MESAs in the previous section. Note that the peaks of autocorrelation are robust and repeat regularly, clearly verifying the periodicity reported by MESA in [Fig. 4](#).

The autocorrelation for the data initially containing a trend are shown before and after operation of FILCON ([Fig. 7](#)). Note that the peaks of autocorrelation are superimposed on the strong trend, but that this disappears after its removal. It is substantially easier to verify the MESA peak using correlograms from de-trended data.

An autocorrelation of the data from SET III, containing the ultradian rhythm in the presence of the strong circadian rhythm and 80% noise, is depicted in

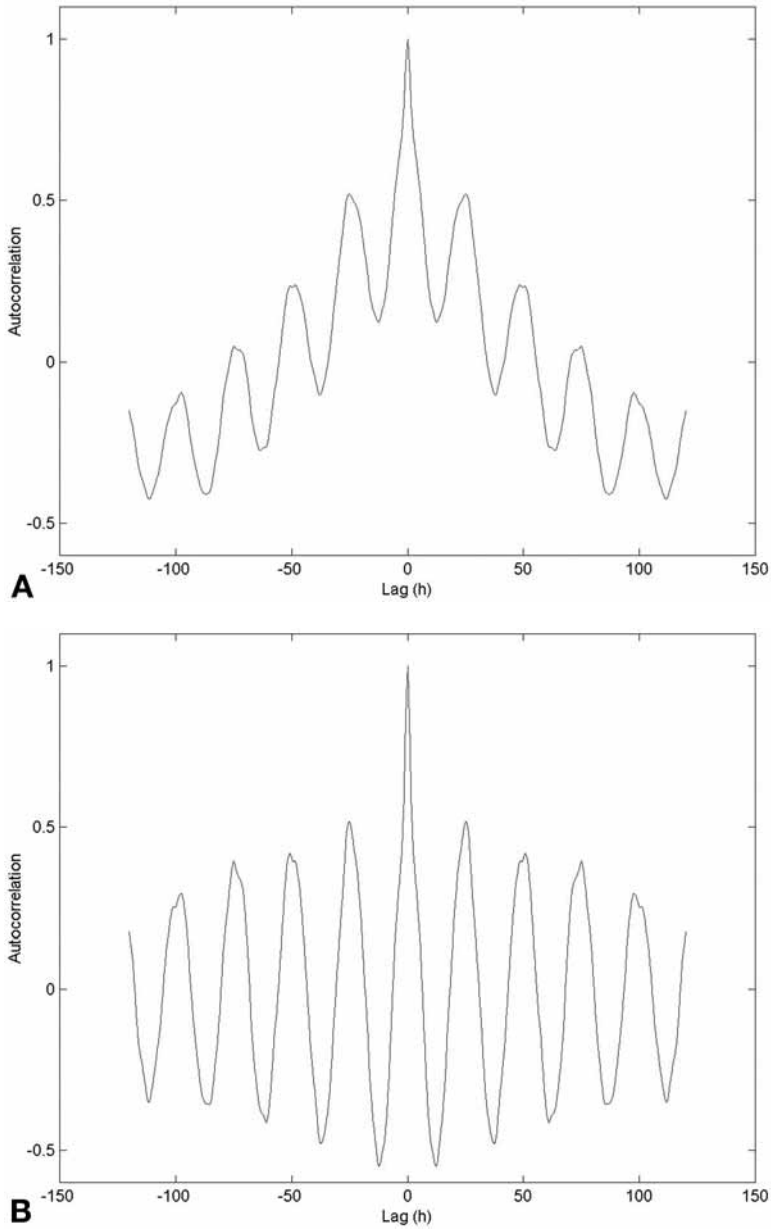


Fig. 7. Autocorrelation analysis of the data depicted in [Fig. 2](#) with a strong monotonic trend shown before (**A**) and after (**B**) de-trending with FILCON.

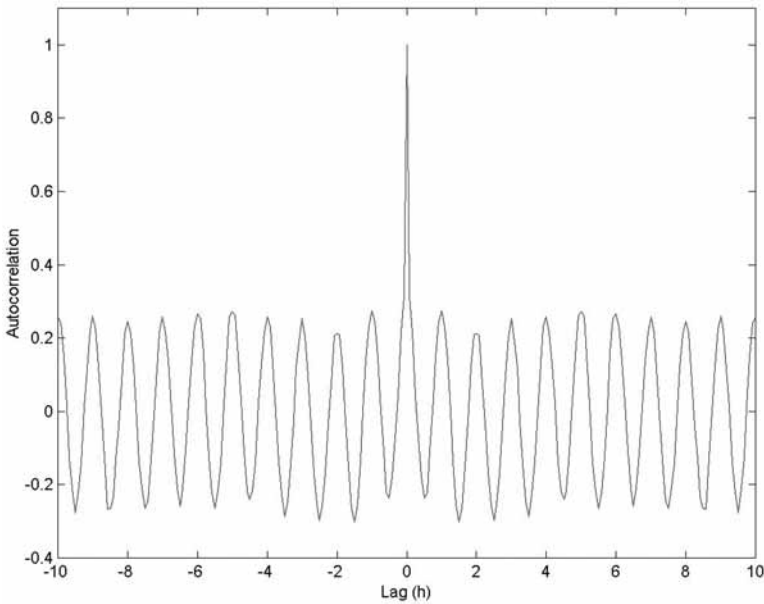


Fig. 8. Autocorrelation produced from data containing a 1-h ultradian periodicity, as depicted in Fig. 3, after the circadian component had been removed. Before removal, there was no visible evidence of this period. The scale has been shortened to ± 10 h of lags. This corroborates the MESA spectrum for both the filtered and unfiltered data.

Fig. 8. Here, the correlograms before and after removal of the circadian rhythm are shown to illustrate the dramatic change in the character of the signal. The hourly rhythm is unequivocally verified by the regular peaks in the autocorrelation function (*see Note 11*).

3.2.3. Assessing Robustness of Rhythmicity

The output files from the autocorrelation analysis were further scrutinized for RI with a BASIC program set to find the height of the third peak (counting the peak at lag zero as 1). As has been discussed elsewhere (7–11), the decay envelope of the autocorrelation function is a measure of the long-range regularity of the rhythmicity, as well as its robustness. These numbers could then be compared with the known (programmed-in) signal-to-noise ratios.

Table 2 contains the extracted RIs for SETS I through III. Note that the RI covaries with the amount of noise added. Filtering the data produces a striking increase in the RI at the higher noise levels. For the data containing the ultradian component, the RIs before filtering are representative of the circadian periodicity, whereas numbers after filtering are for the remaining ultradian rhythm.

Table 2
Rhythmicity Index (RI) for Each Percentage of Noise

% Noise	SET I	SET II U	SET II F	SET III U	SET III F
10	0.79	0.73	0.79	0.79	0.66
20	0.79	0.71	0.78	0.79	0.66
30	0.78	0.68	0.77	0.79	0.65
40	0.77	0.65	0.76	0.79	0.63
50	0.74	0.59	0.73	0.79	0.59
60	0.70	0.50	0.68	0.79	0.52
70	0.60	0.38	0.58	0.78	0.39
80	0.41	ARR	0.40	0.77	0.21

Data set numbers are indicated (*see* text). U = raw data, F = output after FILCON program operated on the data. ARR means the signal was deemed arrhythmic. For data in SET III, the RI for the circadian component is reported for the raw data, and for the data treated with FILCON in the F group.

3.3. Use of Cross-Correlation Analysis to Estimate Phase and Periodicity in Common

Phase was assessed in data SET IV by running the program CROSSCO. This program uses a reference data set of known phase and amplitude as a base to measure the phase of an experimental vector. The algorithm is identical to standard autocorrelation with the exception that two data sets are being compared, instead of a single data set being compared with itself (7). If the data are in phase, there will be a peak at lag zero, although it will likely not be unity, as is always seen in autocorrelograms, given that the two sets will differ. Displacement of the first peak from lag zero in either direction is a direct measure of the phase difference between the reference and experimental periodicities (*see* **Note 12**).

Figure 9 depicts the output of CROSSCO, comparing a reference data set with phase defined as zero and a data set that is phase-delayed by 6 h. All data had 80% noise added. The peak in correlation occurs at the lag corresponding to a 6-h difference. This analysis also serves to illustrate what two rhythms with a periodicity in common would look like when analyzed in this manner. The robust crosscorrelation, even in the presence of considerable noise, indicates the periods of the two data sets are close. (*See* **Note 12**).

4. Notes

1. MATLAB is a numeric computation software package offered by The Math Works in Natick, MA (www.mathworks.com). It can be configured to compile and run software written in other languages—in this case, FORTRAN. Other

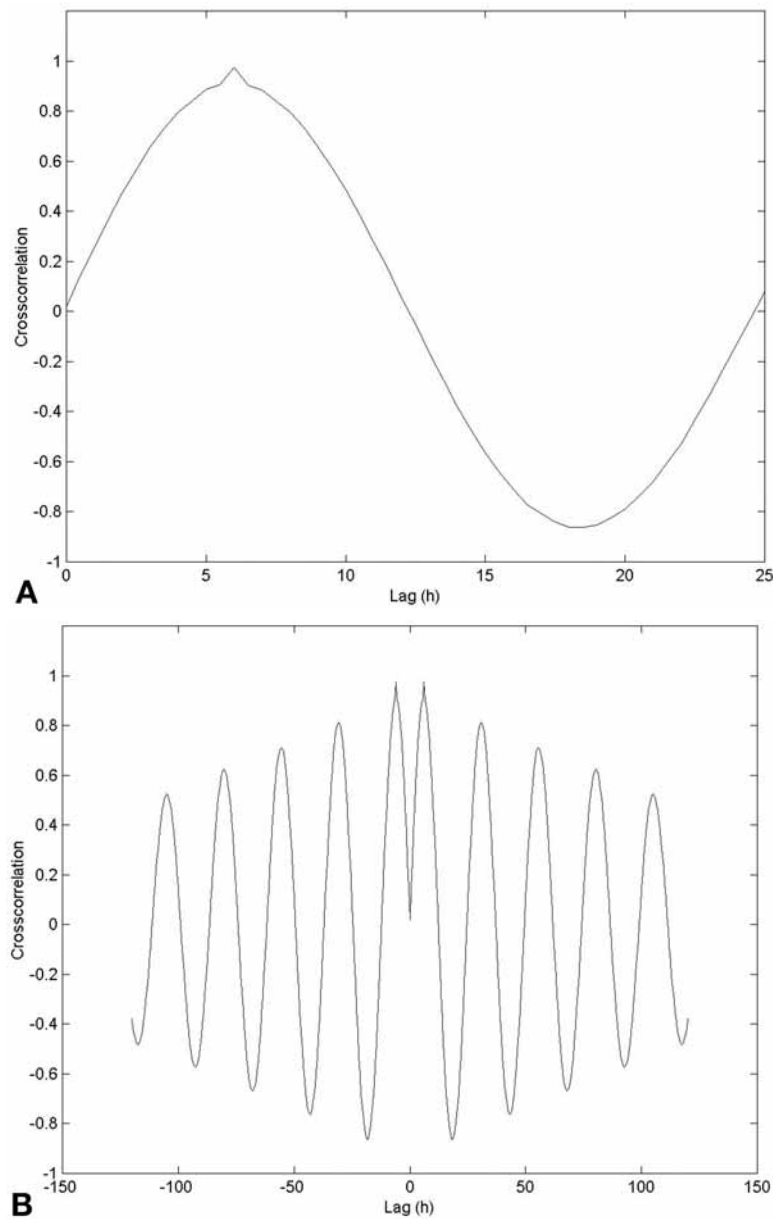


Fig. 9. (A) A short segment of the crosscorrelation of two data vectors differing in phase by 6 h. The periods in the two data sets are the same and both have 80% noise added. (B) The entire crosscorrelation shown to emphasize the periods held in common between the two vectors. If the periods were different, there would be a concomitant decay in the envelope of the function depending on the difference in the periods.

systems have this capability, and the author and others have converted the programs here to run in these systems (8).

2. The programs employed here were run in their DOS window executable file format, as it was desired to maintain the maximum flexibility in producing the examples. In general, this would not be necessary for normal work. Except as noted, all programs were written in FORTRAN.
3. MESA has proven its worth in analysis of biological time series over a period of 20 yr. It was instrumental in uncovering ultradian periodicities in the behavioral rhythms in strains of *D. melanogaster* having no circadian rhythmicity, such as *period⁰¹* (17,18). MESA was set to a very high resolution here, which might not be necessary for normal usage.
4. FILCON: This program takes the discrete Fourier transform of the data and may then be directed to zero out coefficients in a frequency range to be eliminated. Owing to the great sensitivity of MESA, FILCON was not always necessary here to demonstrate the periodicities, but in practice, the author has found it essential as a first step when trends or strong confounding rhythms are present.
5. The programs used to extract the spectral peaks and the RIs were written in Turbo Basic, but could be implemented in several languages. They involve simple bubble sort analysis at the core. In this algorithm, values from a set are ordered in a column by magnitude. The RI program incorporates criteria to ensure that the proper peak is reported. If the autocorrelation function is sufficiently weak, the program reports out arrhythmicity.
6. Heartbeat of *Drosophila* is in the range of 1 to 4 Hz and is monitored in several ways, including optically. The data are best presented as frequency rather than period (9–11).
7. Noise was added by incorporating the output of a white noise generator. The noise file is simply added to the output of the signal generating function in a proportion that reflects the percentage and reflects the signal-to-noise ratio.
8. The determination of significance in rhythmicity may be based on a number of methods. MESA lacks any way of inherently testing for significance of the peaks, as would be possible with the Fourier transform, but the strength of the system described here is that it uses an entirely different algorithm, the autocorrelation function, to assess significance. One may calculate a 95% confidence limit to apply to peaks in the autocorrelation functions, namely $2/\sqrt{N}$; however, it is common simply to look for regularly recurring peaks in the correlogram to determine if a genuine periodicity is present (7).
9. The author uses a batch plotting routine written specifically in MATLAB that accommodates this format for heartbeat (frequency output) data, but currently has none for circadian rhythms (period output).
10. The Butterworth is a recursive filter that can be configured in a high- or low- pass form. The one used here was two-pole low-pass with a 3-db cutoff period of 4 h at a sampling rate of two per hour (16). Recursive filters use a combination of raw and previously filtered data in computing the output. Two pole filters have three coefficients in the formula. A 3-db cutoff means a power reduction in the

signal of 50% at the transition period, here 4 h. None of the output shown in the figures had been filtered first, owing to MESA's power, but normally the author looks at data both with and without filters, and actual rhythm data commonly are improved greatly by the process. This filter will induce an approx 4-h phase shift. If this is a problem, one simply runs the filter twice, sending the filtered data set through in reverse order to cancel out the shift (13).

11. The sensitivity and resolution of MESA, coupled with the ability of FILCON to remove strong circadian rhythmicity, was essential to uncovering a circadian rhythm in human core body temperature. Other methods, including fast Fourier transform, failed to detect it despite the periodicity being clearly visible in the raw data plots (14).
12. When assessing phase in this manner with actual biological data, the test data set must have the same period as the experimental set. The periods of each experimental series are first estimated with MESA, and test sets of the same period are created using SIGGEN. The program automatically adjusts and normalizes amplitude before crosscorrelation is estimated. This method is particularly useful for very noisy and irregular data sets because it estimates phase based on the entire signal rather than just one identifiable phase marker, which by itself may not be reliable from cycle to cycle.
13. All programs written by the author are available free of charge by e-mail or FTP either as FORTRAN source code, executable files, or as files executable from MATLAB.

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II

RHYTHMIC READOUTS

Rhythmic Conidiation in *Neurospora crassa*

Cas Kramer

Summary

In the filamentous fungus *Neurospora crassa* the production of asexual spores (conidia) is regulated by its circadian clock. When the fungus is grown on a thin layer of agar medium in long growth tubes (so-called “race tubes”), restricting its growth to one direction only, bright orange bands are clearly visible. This banding pattern persists with a periodicity of approx 24 h in the absence of any environmental stimuli. The bands are formed by alternating zones of nonsporulating mycelium and mycelium laden with orange conidia. Assaying *Neurospora* conidiation on race tubes is a simple yet powerful and versatile tool for studying the circadian clock of this model organism.

Key Words: Circadian; conidia; asexual sporulation; filamentous fungus; bread mold; race tube; aerial hyphae; mycelium; free-run; entrainment; phase response curve; Chrono.

1. Introduction

In 1953 a growth rhythm in the bread mold *Neurospora* was reported in the scientific literature (1). Six years later Pittendrigh and coworkers (2) showed that this rhythm, the production of asexual spores (conidiation), had the hallmarks of a true circadian oscillation. Many subsequent studies further analyzed and confirmed this (e.g., refs. 3–6). Rhythmic production of spores (both sexual and asexual) is not uncommon among fungi; however, most of these developmental rhythms are not circadian (7,8). In *Neurospora crassa* the developmental switch from vegetatively growing mycelia to the production of asexual spores (macroconidia) is under circadian clock control. Mycelia differentiate to form aerial filaments (aerial hyphae) on which bright orange macroconidia are formed (9,10). This process is activated and deactivated once every circadian day, which results in highly regulated rhythmic production of spores (3,9). Although concentric rings of conidiation can be seen in *Neurospora* cultures

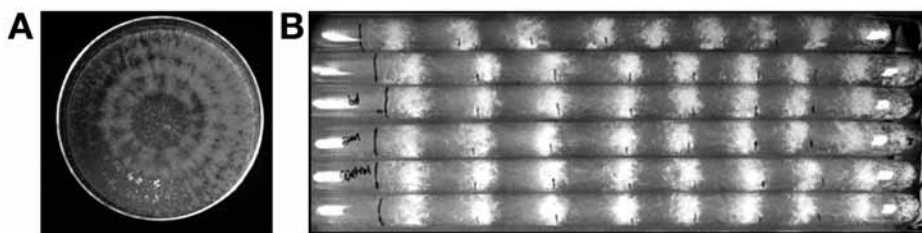


Fig. 1. *Neurospora* conidiation. (A) Concentric rings of *Neurospora* conidiation as observed when *Neurospora* is inoculated in the center of a large Petri dish. Picture by P. Ruoff, Stavanger University College, Stavanger, Norway. Taken from www.ux.his.no/~ruoff/Neurospora_Rhythm.html, reprinted with permission. (B) Bands of *Neurospora* conidiation as observed when *Neurospora* is grown in race tubes (see also Fig. 2).

grown on agar plates (Fig. 1A), the focus of this chapter will be on the assay of “conidial bands” that can be observed when the fungus is grown in long glass growth tubes, known as race tubes (Fig. 1B). The use of race tubes was first reported in 1943 when used by Ryan and coworkers (11) as an accurate tool for growth rate measurements in *Neurospora*. A key event in *Neurospora* circadian biology was the discovery of a mutant strain that showed a distinct banding pattern when grown in race tubes (3). This strain was found to carry a mutation, designated the *band* (*bd*) mutation (3,12), which allowed the clear observation of conidial bands in race tubes because of its reduced sensitivity to CO₂. Conidiation is inhibited by elevated levels of CO₂ (13), which clearly plays an important role when a culture is grown in a long glass tube. The existence of race tubes combined with the discovery of the *bd* mutation has contributed greatly to our current understanding of circadian clock function in *Neurospora*.

2. Materials

1. Race tubes (see Subheading 3.1.1.).
2. Nonabsorbent cotton wool or small foam stoppers.
3. Large (2–5 L) glass beaker or flask.
4. 25-mL Pipet or a Fill-O-Matic dispenser (or any automated peristaltic filler).
5. 50X Vogel’s salts (see Note 1): Per 1 L, 150 g Na₃ citrate·5 H₂O, 250 g KH₂PO₄, 100 g NH₄NO₃, 10 g MgSO₄·7 H₂O, 5 g CaCl₂·2 H₂O (predissolved in 20 mL H₂O; see Note 2), 5 mL trace elements (see item 7), 2–5 mL chloroform (see Note 3). Store at room temperature in the dark.
6. 5X Fries salts solution (see Note 4): Per 1 L, 25 g NH₄ tartrate, 5 g KH₂PO₄, 5 g NH₄NO₃, 2.5 g MgSO₄·7H₂O, 0.5 g NaCl, 0.66 g CaCl₂·2H₂O, 0.5 mL trace elements (see item 7). Store at room temperature.

7. Trace elements: in 100 mL distilled H₂O, 5.0 g citric acid·H₂O, 5.0 g ZnSO₄·7H₂O, 1.0 g Fe(NH₄)₂SO₄·6 H₂O, 250 mg CuSO₄·5 H₂O, 50 mg MnSO₄·H₂O, 50 mg H₃BO₃ (anhydrous), 50 mg Na₂MoO₄·2 H₂O, 1 mL chloroform (see **Note 3**). Store at room temperature.
8. 1000X Biotin stock : 0.5 mg/mL in 50% ethanol. Store at 4°C in foil-covered bottle.
9. Glucose/arginine Vogel's medium (see also **item 5** and **8**): 0.1% glucose, 0.17% arginine, 1X Vogel's salts, 1X biotin, 1.5% agar.
10. Glucose/arginine Fries medium (see also **item 6** and **8**): 0.1% glucose, 0.17% arginine, 1X Fries salts, 1X biotin, 1.5% agar.
11. Sodium acetate race tube medium (see also **item 5** and **8**): 1.2% NaAc (anhydrous), 0.025% casamino acids (made up as 5% stock, stored at 4°C), 1X Vogel's salts, 1X biotin, 2% agar.
12. Minimal sucrose medium (see also **item 5** and **8**): 2% sucrose, 1X Vogel's salts, 1X biotin, 1.5% agar. Boil to dissolve the agar, aliquot into "slants," and autoclave. Slants are cotton wool-plugged 150-mm test tubes containing approx 5 mL medium, slanted at a steep angle when agar is setting after autoclaving. Autoclaved slants can be stored at 4°C for months (in a plastic bag to prevent drying out and contamination).
13. Inoculation loop or large clinical swaps (~150 mm).
14. Large autoclave (minimum internal measurements 500 mm × 400 mm × 300 mm).
15. Temperature- and light-controlled incubators.
16. Darkroom facilities, including standard "safe" red light.
17. CHRONO analysis software ([14](#)).

3. Methods

3.1. Race Tubes

3.1.1. What Is a Race Tube?: Purchasing/Manufacturing

Race tubes are long hollow glass tubes with a thin layer of agar medium, in which growth of *Neurospora* can be assayed accurately for several days (see [Fig. 2](#)). Race tubes are generally approx 400 mm in length, of which 45 to 50 mm on both ends are bent upward at an angle of 30 to 45° (see **Note 5**). Race tubes have an internal diameter of approx 14 mm and a glass thickness of about 1 mm. Uniformity of race tubes is an important parameter in obtaining reproducible results. Race tubes can be purchased "custom made" (see **Note 6**) or brought in as long glass tubes and cut and shaped by an in-house workshop (or even by hand on the bench; see **Note 7**).

3.1.2. Preparation of Race Tube "Six-Packs"

1. Using autoclave tape, tape together clean and dry race tubes, creating "six-packs" (see also **Note 8**; for cleaning of race tubes, refer to **Subheading 3.1.3.**).

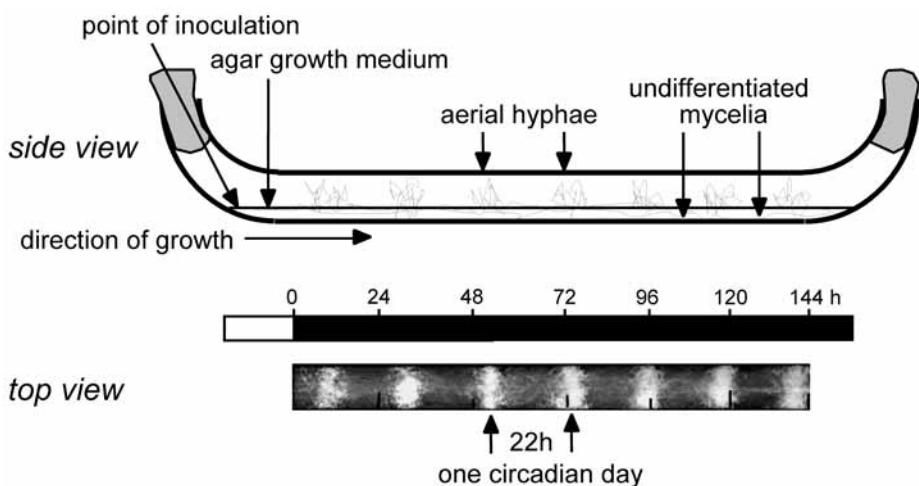


Fig. 2. Graphic representation of a race tube. Race tubes are long, hollow glass tubes with a thin layer of agar growth medium. *Neurospora* is inoculated at one end of the tube, causing its growth to be restricted to one direction only (here from left to right). When grown in constant darkness (black bar), defined areas of the mycelium differentiate and aerial hyphae with bright orange conidia (white “bands” in top view picture) appear once every circadian day (free-running period of 22 h). These conidial bands are separated by areas with undifferentiated mycelia (gray “interbands” in top view picture). Race tubes are marked every 24 h (black marks).

2. Prepare race tube medium in a large glass beaker or flask and bring to a boil (on a heater under constant stirring) to allow the agar to dissolve (*see Note 9*).
3. Fill each race tube with 13 to 18 mL (*see Note 10*) of hot medium using a 25-mL pipet or a Fill-O-Matic dispenser (*see Fig. 3*). When large numbers of race tubes are to be filled, it is recommended to keep the agar medium on a hot plate at low setting to prevent the agar from cooling down too quickly.
4. Prior to autoclaving, plug each race tube on both ends with nonabsorbent cotton or foam stoppers (50 × 25mm, cut in halves; *see Note 11*).
5. Stack race tube six-packs, cover the plugged ends with foil, and autoclave, making sure all tubes stay horizontal at all times (*see also Note 12*).
6. During the autoclaving process, free enough level bench space to lay out all six-packs next to one another.
7. After autoclaving carefully remove six-packs from the autoclave, when still very hot (~80°C; *see Note 13*).
8. Taking a small number of six-packs at a time, make two or three circular swirling movements while keeping the race tubes horizontal (*see Fig. 4* and *Note 14*), before laying the six-packs out flat on a level lab bench. When dealing with large

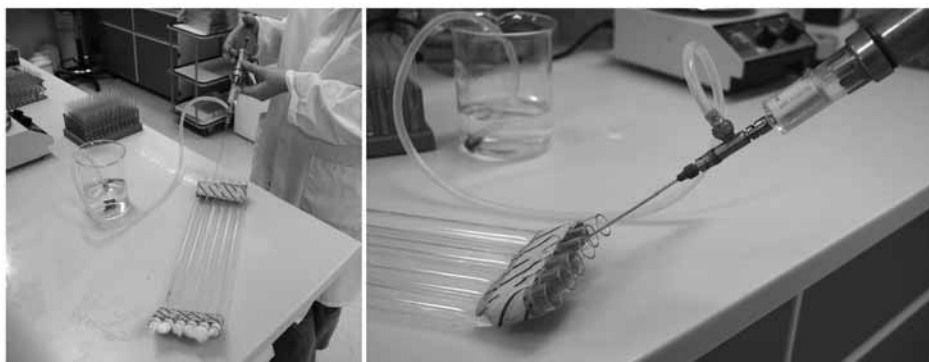


Fig. 3. Filling race tube six-packs with Fill-O-Matic dispenser. (Pictures by S. K. Crosthwaite, University of Manchester, UK.)

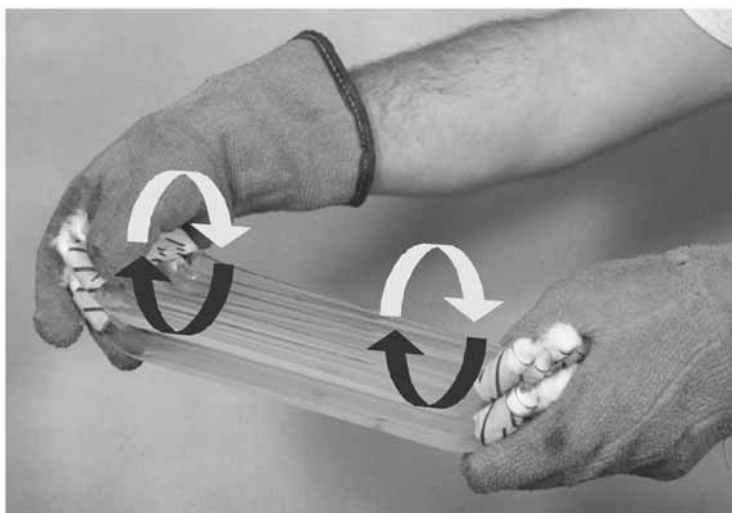


Fig. 4. “Swirling” of race tubes. Two or three circular movements are performed as indicated while holding the race tubes horizontally. The swirling of hot race tubes is an essential step that prevents condensation within the race tubes (*see also* **Note 14**).

numbers of six-packs, work quickly. Make sure each six-pack, as well as each individual race tube, is level.

9. While agar medium is still liquid, remove all air bubbles (*see* **Note 15**). Again, work quickly when dealing with large numbers of six-packs.
10. Replace wet or soiled cotton wool plugs or foam stoppers with fresh, clean ones (there is no need for replacement plugs or stoppers to be sterile). Once the agar medium has solidified, six-packs can be stacked.

11. Leave race tubes to dry at room temperature at least overnight before inoculation (see **Subheading 3.2.1.**). However, drying for 2 to 3 d is normally better. Quick drying in an incubator or drying cabinet is not very effective and therefore is not recommended.

3.1.3. *Cleaning* (See Also **Note 16**)

Once the race tubes have been scanned and analyzed, the six-packs should be cleaned for reuse. It is recommended to clean the race tubes sooner rather than later after the end of the experiment, as drying out of the agar makes the cleaning process more difficult.

1. Reautoclave the six-packs in stacks, placed in a large tray to prevent spillage.
2. Remove six-packs from autoclave when still hot (~80°C) (see also **Note 13**).
3. Take out foam stoppers or cotton wool plugs with forceps (see also **Note 17**). When dealing with large numbers of six-packs, work quickly.
4. Pour out hot molten agar into a container (to set and waste) and immediately run hot tap water under great pressure through each race tube. Run tap water through from both ends of the race tubes. Again, work quickly. Water pressure can be easily increased by squeezing the end of the tube attached to the tap. If possible, leave six-packs intact.
5. Afterward, rinse the race tubes thoroughly with distilled H₂O and dry in a drying cabinet.

3.2. *Rhythmic Conidiation in Circadian Experiments*

In *Neurospora*, the conidial banding pattern is strikingly visual and can be accurately assayed using race tubes, which makes race tubes such a versatile tool to study circadian biology. A wide variety of circadian experiments can be performed in race tubes, as circadian parameters—for instance, the period and phase of the rhythm—are easily examined. For each experiment, of course, the exact protocol will differ. However, some general conditions and considerations regarding the inoculation, incubation, and analysis of race tubes are given under **Subheadings 3.2.1.** through **3.2.3.**, respectively. Subsequently, examples of “classical” circadian experiments—free-run, phase-shift, and entrainment—are given (see **Subheading 3.2.4.**).

3.2.1. *Inoculation of Race Tubes*

1. Make sure to have one or two fresh slants (3–10 d old) for each *Neurospora* strain to be used (see **Note 18**).

For a small number of race tubes:

2. Inoculate each race tube at one end with a small number of conidia using a flamed loop or a large, sterile clinical swab (pre-wetted in sterile H₂O). There is no need to flame the necks of the race tubes; just work cleanly and near a flame.

For a large number of race tubes:

3. Add 1 to 2 mL sterile H₂O to each slant, replace cotton wool plug, and vortex vigorously. Take off the spore suspension and transfer to a 1.5-mL Eppendorf tube.
4. Using a sterile filtered tip add 10 to 20 μ L of spore suspension to one end of each race tube. Again, there is no need to flame the necks of the race tubes; just work cleanly and near a flame. Vortex or shake the spore suspension regularly to maintain even distribution of spores throughout the suspension.
5. To prevent the drop of spore suspension from running toward the middle of the race tube, place a pen under the opposite end of each stack to slightly tilt the race tubes. Keep tilted for the first day of incubation.

3.2.2. Incubation of Race Tubes

1. In general, all race tube experiments are started with a period of 24 h at 25°C under constant light. This allows spores to germinate and form a straight mycelial growth front.
2. After this initial 24 h, mark the growth front with a permanent marker over the full width of each race tube to indicate the start of the experiment and change growth conditions to the experimental conditions—e.g., constant darkness (DD)—for free-run.
3. Incubate race tubes at temperatures between 18°C and 30°C.
4. Make sure that temperature and light conditions are constant within the incubator or be consistent regarding the position of the race tubes within the incubator.
5. Mark growth fronts regularly over half the width of each race tube (*see Note 19*). Marking at least once every 24 to 36 h is recommended (*see Note 20*).

3.2.3. Analysis of Race Tubes

Analysis of race tubes is generally performed after the experiment has finished and the fungus has grown the full length of the race tube. As interbands of undifferentiated mycelia (**Fig. 2**) will not fill in with conidia, the banding pattern is static once formed and can be thus assayed postexperiment. A more advanced and technically challenging analysis of rhythmic conidiation is the use of time-lapse video under constant red light (**15,16**), with very eye-pleasing results. Assuming a linear growth rate for *Neurospora*, the period and phase of the rhythm can be estimated using the 24-h marks. Although this key assumption has recently been proved to be incorrect (**16**), it has been used extensively (and still is used) in the majority of race tube analyses. *Neurospora*'s growth rate can vary by up to twofold with the circadian cycle, causing an estimated maximal error of 1 to 2 h (**16**).

3.2.3.1. USING CHRONO SOFTWARE (**14**) AND SCANNING DENSITOMETRY

1. Create a picture of the race tubes by means of scanning or digital photography.
2. Save each six-pack as a separate PICT file. Use grayscale and a resolution not greater than 150 dpi.

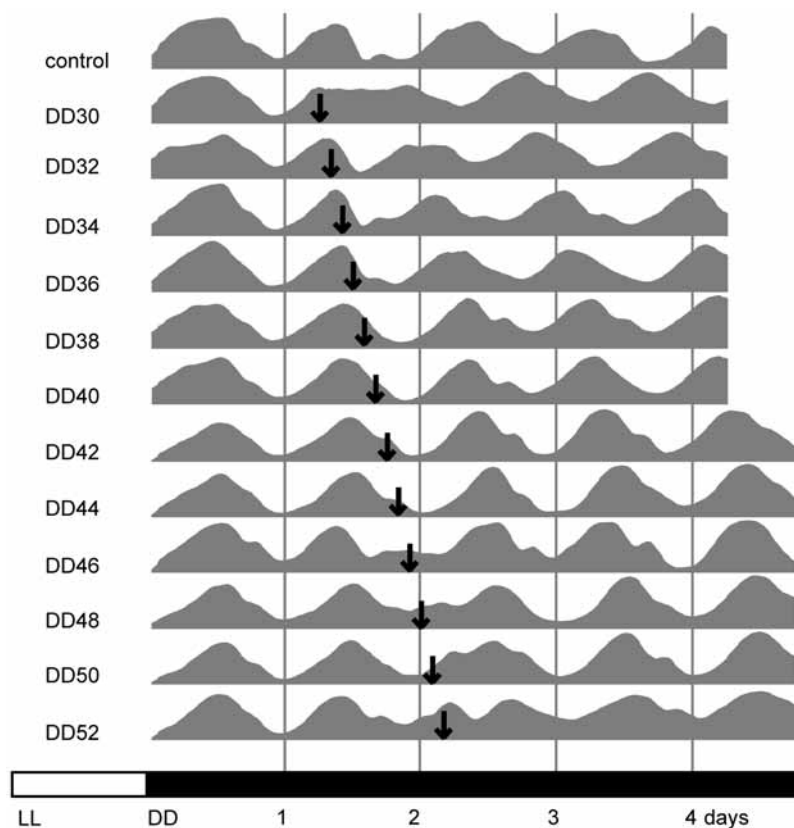


Fig. 5. Densitometry plots generated using the CHRONO software. Example of densitometry plots from race tubes used in a phase response curve experiment (data from [ref. 18](#)). Race tubes were grown at a constant temperature of 25°C for 24 h in constant light (LL) and then transferred to constant darkness (DD). At different times after lights-off (DD times indicated and represented by the black arrows) a 2-min saturating light pulse was given, resulting in strong clock delays and clock advances in the antisense *frq*-defective strain *frq¹⁰frqccg-2* ([18](#)).

3. Import the .PICTfile into CHRONO.
4. Enter the exact time for all time marks (including the start of the experiment) for each individual race tube.
5. Double-check the peaks on the densitometry plot generated by the software for each individual race tube and adjust if necessary (*see Note 21*).
6. Period, phase, advances, and delays can be read or calculated. Densitometry plots can be exported for easy visualization of results ([Fig. 5](#)).

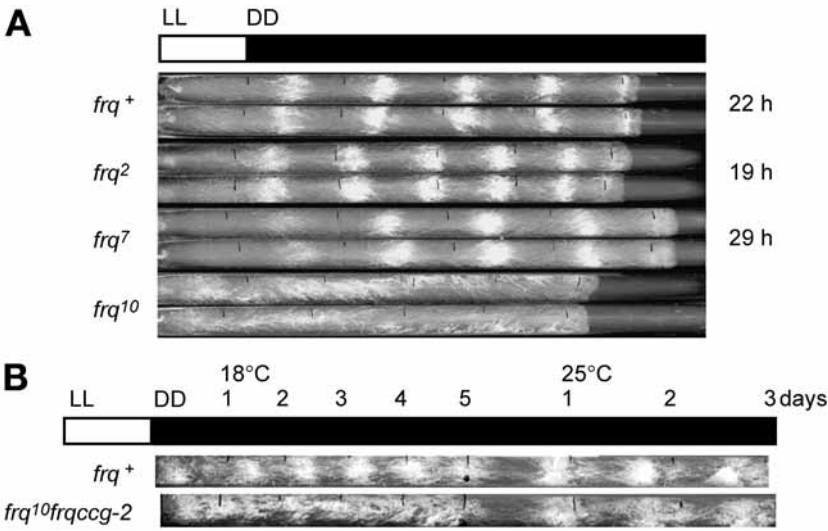


Fig. 6. Visualization of the period of the oscillator. *Neurospora* strains were grown for 24 h in constant light (LL) and then transferred to constant darkness (DD), allowing free-run of the clock. (A) The free-running period of the clock in different clock mutants is easily visualized and assayed when strains are grown in race tubes. Wild-type *bd* strain *frq*⁺ (22 h), short-period mutant *frq*² (19 h), long-period mutant *frq*⁷ (29 h), null mutant *frq*¹⁰ (arrhythmic). Two race tubes per strain are shown. Strains were grown at 25°C. (B) Rhythmic conidiation is temperature-dependent in antisense *frq*-defective strain *frq*¹⁰*frqccg-2* (18). (Pictures by S. K. Crosthwaite and C. Kramer, University of Manchester, UK.)

3.2.4. Examples of Circadian Experiments in Race Tubes

Below, some race tube examples are shown (Figs. 6–8) to emphasize the ease and versatility of race tube assays in general, which have contributed greatly to our current understanding of circadian rhythmicity in *Neurospora*.

3.2.4.1. FREE-RUN

When *Neurospora* strains carrying the *bd* mutation (3,12) are inoculated in race tubes and are left on the lab bench under ambient light/dark conditions, distinct bands of conidia are observed once every sidereal day—every 24 h. For the *Neurospora* strains in these race tubes, as in all circadian systems, the presence of light is the dominant zeitgeber (17). When cultures are transferred into DD (and under constant temperature conditions), conidial bands continue to appear once every circadian day—every 22 h (the free-running period of the *Neurospora* clock; see Fig. 6A; see also Fig. 2). The free-running period of the

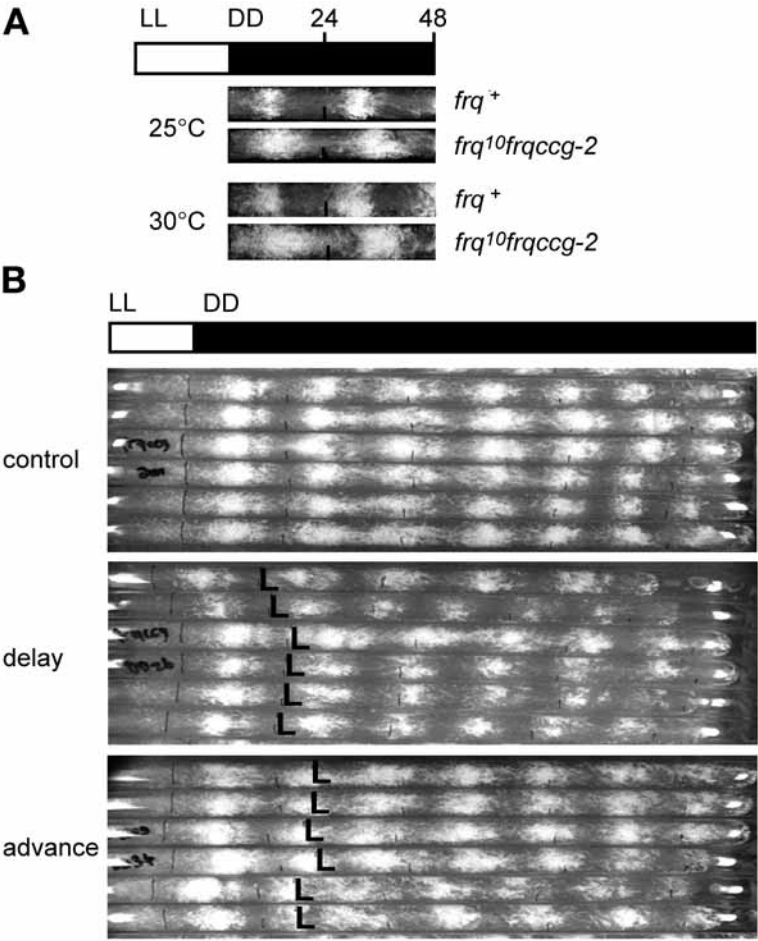


Fig. 7. Visualization of the phase of the oscillator. *Neurospora* strains were grown for 24 h in constant light (LL) and then transferred to constant darkness (DD). (A) Rhythmic conidiation is delayed in antisense *frq*-defective strain *frq*¹⁰*frqccg-2* (18). The light-to-dark transfer sets the clock to an earlier phase in this mutant strain compared with the wild-type *bd* strain *frq*⁺. Strains were grown at 25°C and 30°C, as indicated. (B) Examples of clock delay and clock advance responses of the antisense *frq*-defective strain *frq*¹⁰*frqccg-2* (18), grown in race tube six-packs. At 26 h and 34 h after lights-off (DD26 and DD34) a 2-min saturating light pulse was given (indicated by “L”). Strains were grown at 25°C. The phases of the clock before and after the light pulse (old phase and new phase) are easily visualized and assayed in this way. (Pictures by C. Kramer and S. K. Crosthwaite, University of Manchester, UK. Pictures from Fig. 7A first published in ref. 18 and pictures from Fig. 7B first published online in ref. 26.)

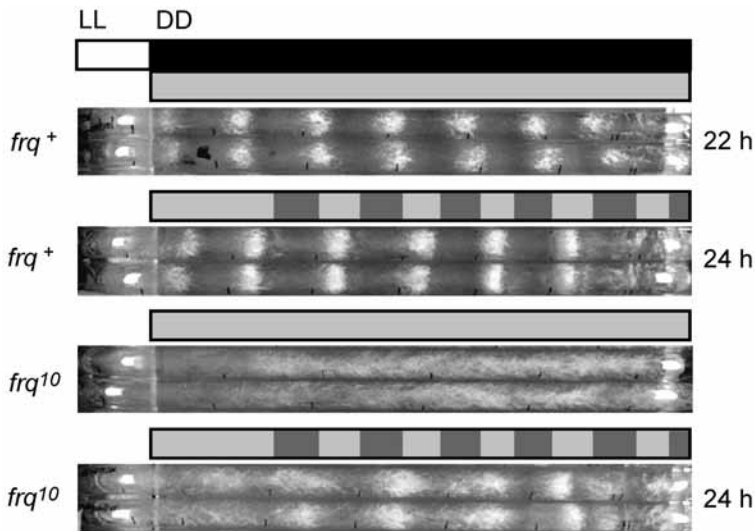


Fig. 8. Entrainment of the oscillator. *Neurospora* strains were grown for 24 h in constant light and then transferred to constant darkness (DD). In DD and at a constant temperature of 25°C the clock is free-running, wild-type *bd* strain *frq*⁺ (22 h) and null mutant *frq*¹⁰ (arrhythmic). In DD and under a temperature regime of 12 h at 25°C/12 h at 27°C, a 24-h rhythm of conidiation is observed in both *frq*⁺ and *frq*¹⁰. (Pictures by P. Gould and S. K. Crosthwaite, University of Manchester, UK.)

clock in long-period, short-period, and arrhythmic clock mutant strains can easily be visualized and assayed when grown in race tubes (Fig. 6A). Whether conidiation appears to be arrhythmic on race tubes can sometimes be temperature-dependent (Fig. 6B).

3.2.4.2. PHASE-SHIFT

Another important feature of the circadian clock, the phase of the oscillator, is also easily assayed when *Neurospora* is grown on race tubes. A light-to-dark transfer sets the clock, and hence the physiological state of the culture, to a defined time (6). In wild-type *frq* strains the peak of conidiation is observed approx 10 h after lights off, whereas in an antisense *frq*-defective strain (18) the appearance of the conidial band is significantly delayed (Fig. 7A), which means that the endogenous clock in this mutant strain is set to an earlier phase by a light-to-dark transfer. Deliberate phase-shifting of the *Neurospora* clock can be established by exposure to light (and also temperature and social cues). In all organisms, light pulses given at different times of the circadian day will

have different effects on the clock (19). Clock delays and clock advances can be easily and accurately measured when *Neurospora* race tube six-packs are exposed to a short light pulse (Fig. 7B).

3.2.4.3. ENTRAINMENT

Apart from light, temperature is the other major zeitgeber for circadian oscillators (17). When *Neurospora* is grown in DD, the conidiation rhythm is in free-run (22 h; see Fig. 6A). However, when in DD the temperature is varied with 12-h alternating periods of cooler and warmer temperature, the conidiation rhythm has a period of 24 h (Fig. 8), the rhythm is entrained. Temperature entrainment of *frq*-null mutant strains has been suggested previously (20) (see also Fig. 8); however, a recent investigation revealed that rhythmic conidiation is characteristic of a driven rather than entrained rhythm (21).

4. Notes

1. In 1956 Vogel (22) described a formula for a 50X-strength salt solution, now commonly known as 50X Vogel's, which is still used today in the majority of *Neurospora* minimal growth media. For 50X Vogel's salts solution, dissolve the chemicals in the given order in 750 mL H₂O with vigorous stirring. It is essential to dissolve each ingredient completely before adding the next chemical. For some chemicals this can take many hours. Failing to do so can create insoluble precipitates. Vigorous stirring using a large stirring bar may speed up the process. Remember, it is better to leave the solution stirring overnight rather than rushing the preparation and allowing precipitates to form. When all chemicals are dissolved, adjust volume to 1 L, pH 5.8 (no adjustment in pH should be necessary). Finally, add the chloroform as preservative (see Note 3).
2. Predissolving the CaCl₂ in water helps to prevent the formation of insoluble precipitates, which will almost inevitably appear when solid CaCl₂ is used. Addition of the CaCl₂ solution to the salt stock solution must be carried out slowly, allowing cloudiness to disappear after every few drops.
3. Addition of chloroform to the 50X Vogel's salts and trace elements is an essential step. Failing this, airborne fungal spores will quickly form myriad fungal colonies on its surface, as these stock solutions are not sterilized.
4. A slightly different salt stock solution may be used. 5X Fries salts (23) is prepared in a similar fashion as described above for 50X Vogel's salts (see Note 1), making sure that the chemicals are added in the given order and dissolved fully before adding the next ingredient.
5. Extra-long race tubes of approx 600 mm in length can be used to allow growth for up 2 wk. Also, 150 mm straight test tubes (capped) can be used as baby race tubes for mutant screening. Both these types of race tubes are not described in further detail in this chapter.

6. Race tubes can be purchased ready-made from, for instance, Lichfield Studio Glass Ltd (www.litchfieldstudioglass.co.uk) or from Chemglass (Vineland, NJ; www.chemglass.com). The use of disposable plastic race tubes has been reported (24), but this interesting concept has strangely never been commercialized.
7. "Do-it-yourself race tubes" is a possibility, but it is a tricky, potentially dangerous, and especially time-consuming process (C. Heintzen, personal communication). To cut glass tubes to the desired length, score the glass with a glass cutter or fine-toothed metal saw, immediately wet the scratch with a little water or saliva (which prevents the glass, which acts in many cases like a liquid, from "healing the cut"), and quickly break by holding the tube on either end, using the thumbs in a smooth but strong outward movement. To bend the tubes, use two Bunsen burners (secured in stands) tilted toward each other, in such a way that the hottest part of the flames meet. This will produce enough heat to bend the glass. Getting a smooth and uniform bend without kinking the glass is the major problem of do-it-yourself race tubes. Finally, flame both ends the race tubes to smooth the ends.
8. A variety of different racks and clips have been used for making race tubes and race tube six-packs. However, the existence of strong autoclavable tape has pushed most of these items into forgotten drawers or display cabinets with old laboratory equipment. The taped six-packs can be reused several times without replacing the autoclave tape, although it should be remembered that repeated autoclaving weakens the tape considerably (*see also* **Note 12**). When preparing six-packs, care should be taken to make sure all six race tubes are level when the six-pack is laid flat. Failing to do so will result in race tubes in which the agar medium is at a slight angle. This will create a gradient in CO₂ levels within the race tube during growth, which will influence conidiation (*see also* **Note 10**).
9. Race tube agar medium should be prepared only at a time when race tubes are clean and ready to be filled, as once aliquoted into race tubes, the medium is autoclaved. The most commonly used race tube media are glucose/arginine and sodium acetate race tube media, the latter causing slightly denser conidial bands. Which race tube medium to use often seems a matter of preference, strain-dependence, and trial and error.
10. Different amounts of agar medium to fill race tubes are used by different experimentors. The exact volume *per se* is not the major parameter that determines the outcome of the experiment. However, it is important to consistently use the same volume of agar medium within an experiment and between representative experiments. The amount of agar used determines the volume of air within the race tube, and thus the CO₂ levels during growth. The inhibitory effect of CO₂ on conidiation is very strong in wild-type *Neurospora* and less pronounced but still detectable in strains carrying the *bd* mutation (13). Therefore, fluctuations in CO₂ levels between race tubes (or within a race tube) may affect conidial banding, which is the cornerstone of all circadian assays performed in race tubes. For this reason it is also advisable to use race tubes identical in internal diameter, length, and shape.

11. Cotton-wool plugs or foam stoppers should be packed tightly, but not too tightly that it would prevent CO_2/O_2 diffusion. As a general rule of thumb, one should be able to just about lift the tube by pulling the cotton wool or foam stopper only.
12. When autoclaving, do not stack six-packs too high or secure the stacks with extra autoclave tape to the sides of the tray. Remember, seemingly rigid stacks of race tubes will reshape (or can become a loose pile of race tubes) once autoclave tape weakens during the autoclaving process. Repeatedly reused tape around six-packs is likely to tear when wet. When stacks of race tubes are to be autoclaved in a large tray, place several supports (for instance, some small tube racks) under the race tube stacks, as water may collect in the tray during autoclaving, flooding the bottom six-packs.
13. Take race tubes out of the autoclave when still very hot, as swirling ([Fig. 4](#)), laying out, and removing air bubbles can take a surprisingly long time when dealing with large numbers of race tubes. When the temperature of the race tubes is too low, the agar medium will set too quickly. However, take great care because when the temperature of the race tubes is too high, the agar medium may still boil and cotton wool or foam stoppers and hot medium will often shoot out of the tubes.
14. The swirling of hot race tubes ([Fig. 4](#)) must be done before the agar growth medium has set. It is an essential step in the process of making good race tubes. The swirling prevents condensation on the “roof” (inner top) of the race tubes ([11](#)). If condensation occurs, dispersed conidia are often “carried by the water droplets” over the full length of the race tubes. The mycelial growth front will “jump” or reach the end of the tube in a couple of days, making the assay of that race tube impossible.
15. Removing air bubbles from race tubes can be tricky. One effective method is to lay one hand flat on the six-pack and make sharp, jerky movements in the direction parallel to the race tubes. Another method is to use a Bunsen burner to “burn away” the bubbles, in a similar fashion as removing air bubbles from poured agar plates. Take care not to set fire to the autoclave tape. A third method is to heat up a spatula (red-hot) and tap the surface of the glass with it where an air bubble sits, until the bubble bursts.
16. Never soak or rinse race tubes in the disinfectant TriGene (MediChem International, UK), as this will ruin the race tubes. *Neurospora* is very sensitive to TriGene—even after an acid wash and repeated rinsing and soaking in H_2O , fungal growth is affected (C. Kramer and S. K. Crosthwaite, unpublished results).
17. Foam stoppers can be reused several times. Wash foam stoppers in distilled H_2O , squeeze out the liquid, and dry in a drying cabinet.
18. To prepare fresh *Neurospora* slants, inoculate minimal sucrose medium slants from frozen stock slants ([25](#)). Incubate for 2 to 3 d at 30°C until a large amount of light orange spores have developed. Slants can then be stored on the lab bench at room temperature until use. Exposure to the light will intensify the color of the spores to bright orange, will also color the aerial hyphae, and will increase the conidial yield in young cultures ([10](#)). Spores should be collected from fresh slants

to obtain consistent results. Spores may be taken from frozen stock slants, but the germination and the initial growth may be inconsistent, and is therefore not recommended. Spores should not be used when slants are older than 10 d because *Neurospora* conidia lose viability quickly after 10 d and the chance of picking up mutants increases significantly.

19. The race tubes should be marked with tiny marks, less than half the width of the race tube (instead of across the full width). This allows densitometric analysis of the race tubes without interfering black marks.
20. Marking race tubes once a day is highly recommended for consistent analyses. Although the CHRONO software (see **Subheading 3.2.3.**) can easily deal with missing marks and marks at varying time-points, it is much easier for the experimenter to remember to mark each day at a set time. When marking at varying times, write it down. For entrainment experiments it is also essential to mark each transition, as the mycelial extension rates under the different entrainment conditions can vary considerably. If marked only once a day, the phase of the conidial banding pattern cannot be reliably analyzed (21).
21. When two peaks in the densitometry plot are very close, the CHRONO software often fails to detect either. Also, when one peak is “dented at the top,” the software will recognize two peaks. Ignore shoulder peaks; however, remember that in phase response curve experiments an advanced peak sometimes appears to be a shoulder peak. In most cases, the last peak at the end of a race tube should be ignored, as growth rate and conidiation are affected by the bend in the glass tube (this is often very clear when regression analysis is applied).

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Monitoring and Analyzing *Drosophila* Circadian Locomotor Activity

Mauro A. Zordan, Clara Benna, and Gabriella Mazzotta

Summary

In the 1970s, the intriguing discovery of autonomous circadian rhythmicity at the behavioral level in *Drosophila* set the starting point for one of the most remarkably rapid advancements in the understanding of the genetic and molecular bases of a complex behavioral trait. To this end, the design of appropriate electronic devices, apt to continuously monitor behavioral activity, has proven to be fundamental to such progress. In particular, most of the mutational screens performed to date in the search for genes involved in circadian rhythmicity were based on monitoring *Drosophila* mutants for alterations in the circadian pattern of locomotor activity. Many different experimental paradigms, based on the use of circadian locomotor activity monitors, have been developed. Experiments can be designed to determine (1) the natural period, (2) the capacity to adapt to day–night cycles with photoperiods of differing length, and (3) the phase of the circadian activity cycles with respect to the entraining stimulus. Here we describe some of the rationale and the steps required to set up experiments to monitor circadian locomotor activity in *Drosophila*. Suggestions for the statistical analysis of the data obtained in such experiments are also provided.

Key Words: *Drosophila*; locomotor activity; circadian rhythms; spectral analysis; CLEAN; Python; open source; actograms; period; phase; infrared emitter.

1. Introduction

In insects, such as *Drosophila melanogaster*, locomotor activity is implicated either directly or indirectly in activities such as courtship behavior, foraging for food, and exploration of the environment. It is important to realize that in all these cases, locomotor activity as such is the overt manifestation of a chain of events that are coordinated by the central nervous system of the fly (2). Indeed it is known that after decapitation, *Drosophila* maintains a normal

standing posture, shows spontaneous grooming, and can also display locomotor activity (3). The fact that a fly can walk at all without a head raises the obvious question of what role the head (and of course the brain within) plays in this phenomenon. The answer to this fascinating question is beyond the scope of this chapter, the point being, however, that locomotor activity constitutes one of the most important behavioral readouts in the study of the genetics and molecular biology of circadian rhythms in *D. melanogaster*. What matters in this specific instance is that wild-type flies show regular circadian (daily) “sleep–wake” cycles (4) that are paralleled, in particular, by overt cycles of locomotor activity and inactivity.

On average, fruit flies “wake up” just before sunrise, at which time they begin to move about, foraging for food, exploring their environs, interacting with other individuals, and perhaps also engaging in courtship behavior with the other sex. This state of affairs usually continues till about midday, when the flies reduce their activity and take an early afternoon nap. In the late afternoon, before sunset, they once again animate themselves until just before nightfall, at which time they reduce their activity in preparation for the night’s rest. In this simplified description of a typical day in the life of a fruit fly, the central issue is that the distinction between moments of activity and those of inactivity can be further simplified by stating whether the fly is “moving about” at a certain moment in time or not. In this case, the circadian patterns of locomotor activity are taken as representing the circadian patterns of the fly’s overall activity. Researchers in circadian biology have put this behavioral paradigm to good use as a handy means to screen for *Drosophila* circadian clock gene mutants (e.g., refs. 5–9). To this end the imperative becomes devising an approach that would allow automatic monitoring of the locomotor activity of single flies “24–7.” To anyone with an electronic twist, this definitely sounds like a problem requiring a “triggerable event counter.” This sort of device is normally employed in industrial production lines, where large numbers of small parts often need to be counted automatically. This can be done by conveying the objects into a narrow passage that will allow only one object at a time to pass through. The passage is equipped with an infrared light-emitting diode (LED) and a phototransistor receiver, facing each other on opposing sides of the passage. Interruption of the infrared beam by a passing object can thus be detected and the event scored as a count by appropriate circuitry, which is interfaced to a data-collecting device, such as a computer.

The same principle, applied to the “*Drosophila* circadian locomotor activity” problem, led to the conception of a glass tube container of a diameter just sufficient to house a single fly and long enough to allow adequate space for the fly to “walk” back and forth freely. The container is sealed at both ends; one

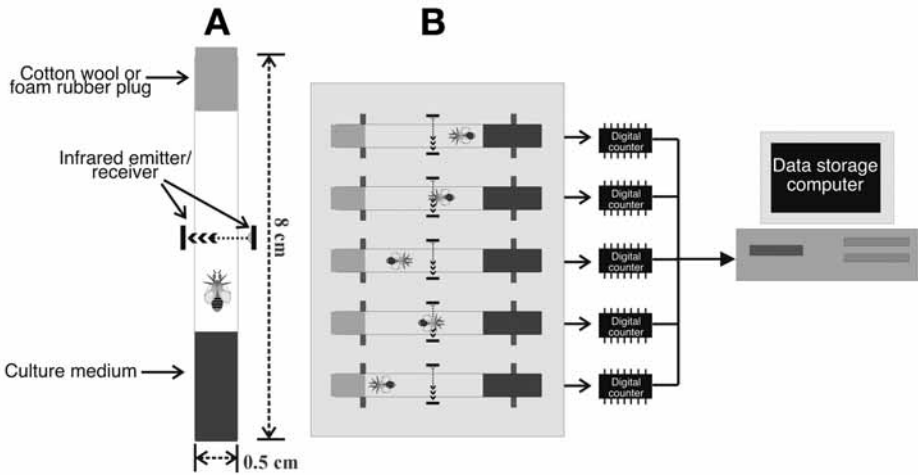


Fig. 1. General structure of a locomotor activity monitoring device. (A) A glass tube (length and diameter, not to scale), containing a single fly, ready to be loaded into the activity monitor. The infrared emitter/detector is also shown. (B) Typical organization of the glass tubes on a circuit board and the schematic connection through a digital counter.

harbors a source of food and water and the other is plugged with foam rubber or cotton wool. Each unit so prepared can be mounted on a circuit board between a pair of infrared emitter/detectors (Fig. 1). Every time the fly passes in front of the emitter/detector pair during its meanderings, the infrared beam will be interrupted; this will be scored as a single event by an electronic counter. A computer is programmed to “interrogate” the event counter at a set frequency (usually once every 5–30 min), after which the counter is reset. It is apparent that what we are scoring in this way is a further simplification of what we had set out to score in the first place because we are not scoring locomotor activity *per se*, which would entail also gathering information concerning, for example, the speed and direction of the fly’s motion during its bouts of activity. However, apart from the consideration that this would entail the use of an altogether different activity monitoring technology (see ref. 2), one also needs to keep in mind that the more information one wishes to gather, the more one is then confronted by the problem of data storage: typical circadian rhythm experiments consist of up to a few hundred flies monitored individually “around the clock” for several days at a time (typically, at least 7 d). Commercial versions of devices based on the above logic are currently available (for example, Trikinetics, Waltham, MA; www.trikinetics.com).

2. Materials

1. Agar.
2. Sugar.
3. Dried yeast.
4. Nipagin (*p*-hydroxy-4-methylbenzoate).
5. Absolute alcohol.
6. Depilatory wax.
7. Paper-backed adhesive tape.
8. Glass tubes (length = 80 mm, diameter = 5 mm).
9. Locomotor activity monitoring equipment.
10. Controlled-environment incubator(s).
11. Data analysis computer and software.

3. Methods

This section describes the preparation of experiments to monitor circadian locomotor activity in *D. melanogaster*. The following subsections will deal with the preparation of the glass tubes for the locomotor activity monitor; the characteristics of light, temperature and humidity required during the monitoring of circadian locomotor activity; the principal experimental paradigms used to assess the main characterizing aspects of the circadian rhythms of locomotor activity; and the software used for the statistical analysis of data obtained from the various experimental paradigms.

3.1. Preparing Glass Tubes for Locomotor Activity Monitor

In order to prepare the glass tubes to be placed in the locomotor activity monitor, it is necessary to provide each tube with a source of food and moisture, usually in the form of an agar-based *Drosophila* culture medium.

3.1.1. Preparing *Drosophila* Culture Medium

Our laboratory currently employs the following formulation, but there are many variations on the theme, and each laboratory has its own favorite recipe (see, for example, refs. [10](#) and [11](#)).

1. Weigh 44 g dried yeast, 44 g sugar, 12 g agar, and place in a 2-L conical flask.
2. Add 1 L tap water, plug the flask with a cotton and gauze plug, wrap the top of the flask with aluminium foil, and place the flask into an autoclave.
3. Cook the medium in an autoclave for 15 min and allow to cool down enough (to about 80°C) to allow extraction of the flask from the autoclave.
4. Place a Teflon magnetic bar into the hot medium and allow to cool down to about 50°C, while standing the flask on a magnetic stirrer.
5. At this point, add 4 g of nipagin (previously dissolved in 40 mL of 95% ethanol) and allow to stir for 2 to 3 min.

3.1.2. Loading Glass Tubes With *Drosophila* Medium

1. Pour the medium into a Pyrex container (~20 cm diameter) to a depth of approx 2 to 2.5 cm.
2. Place clean glass tubes standing up into the hot liquid medium.
3. Let the medium solidify with the glass tubes standing in it. During this time, cover the tubes with a fine mesh net to avoid contamination by flies that might be free in the laboratory.
4. Once the medium has cooled down, extract the tubes with a slight rotation and gentle side-to-side movement. This overcomes the suction that may prevent the food from remaining in the tube.
5. Wipe the outer surface of each tube well with a piece of paper towel.
6. Seal the “medium end” of the tubes.
 - a. Melt depilatory wax on a heating plate, held at the wax’s melting temperature.
 - b. Briefly dip a tube into the melted wax to a depth of about 0.5 to 1 cm.
 - c. Quickly solidify the wax by immersing the waxed end of the tube into a beaker containing cold water.

This type of treatment ensures a watertight seal, guaranteeing maximum protection of the medium inside the tube from drying out. Once the tubes have all been prepared in this way, they are ready to be loaded with their solitary fruit fly occupants.

3.1.3. Placing Flies Into Glass Tubes

Sex-specific characteristics in circadian locomotor activity patterns have been reported; thus, it is a good idea to keep note of the sex (and age) of the flies tested (**refs. 12,13**; see **Note 1**).

1. Place CO₂-anesthetized flies, of appropriate age and sex, into a Petri dish lined with a piece of paper towel and placed onto a bed of crushed ice (see **Note 2**).
2. Put the cold-anesthetized flies individually into the glass tubes with the help of a soft, fine-tipped paintbrush.
3. Plug the open end of each tube with a small piece of cotton wool or rubber foam and leave in a horizontal position. Make sure that the flies remain away from the medium until they wake up.
4. Once all the tubes have been loaded, check that all the flies have awoken and that they are in a good state; replace the dead or unfit individuals.

3.1.4. Washing Glass Tubes

Glass tubes should be perfectly clean at the start of each experiment, as any residual dirt on the glass surface may interfere, among other things, with the infrared detector system. Consequently, the glass tubes need to be freed from

the depilatory wax plug and from traces of medium and fly droppings left from the previous experiment.

1. Bunch together with an elastic band up to 50 to 60 tubes and wrap them in aluminium foil (it is not necessary to remove the flies from the preceding experiment).
2. Place the bunches into a freezer (-20°C) for a few hours. This is done because the wax becomes brittle with the cold treatment and does not cling to the glass very well.
3. Take the bunches out from the freezer a few at a time (as the effect of cold on the wax is reversible) and gently scrape off the wax from the tubes with a scalpel blade.
4. Remove the cotton or rubber foam plugs.
5. Bunch all the tubes together and place them vertically in a beaker of slightly larger diameter.
6. Fill the beaker with a solution of dishwashing detergent in warm to hot tap water.
7. Let stand for a couple of hours, then rinse the tubes by repeatedly flushing the beaker vigorously under running cold water and by raising and lowering the bunch of glass tubes, while letting the tap water run through the top open end and into the beaker. Repeat this operation until there are no traces of impurities left on the glass tubes.
8. Rinse the tubes two or three times with distilled (or deionized) water.
9. Shake off the excess of water by holding the bunch of tubes (kept together with an elastic band) in one hand and hitting the open ends against a wad of absorbent paper held in the palm of the other hand.
10. Place the semidry tubes in a standing position, on a shelf of a dry-heat incubator at a temperature of 60 to 70°C .
11. Once the tubes are perfectly dry, wrap them in aluminium foil and store until further use.

3.2. Ambient Conditions

Experiments that aim at acquiring information regarding the patterns of circadian activity shown by the organism of interest should be conducted under highly controlled environmental conditions. Such experiments should be conducted in strictly light-tight, possibly also soundproof, housings that should also allow the full control of temperature, humidity, and internal lighting conditions. Ideally, both temperature and light should be independently programmable. Better still would be to have light, temperature, and locomotor activity monitors controlled by the same computer so that, particularly in the case of light cycles, these will always be perfectly in synch with the locomotor activity readings, as both will be under the control of the same timing apparatus. What often happens, instead, is that lighting control is assigned to a dedicated timer, which must be synchronized by hand to the clock of the data-collection computer.

3.2.1. Temperature and Humidity

Temperature is an important variable to control during circadian locomotor activity monitoring of *Drosophila*. Thus, although it is well-known that true circadian clocks are temperature-compensated ([14,15](#)), temperature nonetheless does have an effect on basal metabolism; this can be reflected, for example, on the amplitude of the locomotor activity patterns (*see* **Note 3**).

Humidity should be controlled to ensure a level typical of normal *Drosophila* culture conditions, which is usually in the range of 60 to 70% relative humidity.

3.2.2. Lighting

Typically, appropriate lighting conditions are ensured by fluorescent lighting tubes. The type to be preferred is the normal “daylight” tube, rather than the “cool white” or “warm white” kind (*see* **Note 4**). An important point also regards the positioning of the lights: these should possibly irradiate light perpendicularly onto the glass tubes containing the flies in the activity monitors (*see* **Note 5**).

3.2.2.1. MEASURING LIGHT INTENSITY

Light intensities are often measured using easily available and relatively low-cost luxmeters (such as the ones normally employed in photography). However, this is not necessarily the best approach, as luxmeters provide a measure that is referred to the spectral sensitivity of the human eye (which, for daylight vision, is centered at 555 nm; *see also* [refs. 16–18](#)). A more unbiased approach would be to measure the actual flux of photons reaching the flies by using a (rather more expensive) photometer. Photometers provide a measure of the flux of photons per unit surface and time, irrespective of the wavelength of the light source used (*see* **Note 6**).

3.3. Experimental Paradigms

Experiments can be planned with different goals in mind. In particular, considering a *Drosophila* mutant or wild-type strain, one may be interested in determining, for example, (1) the natural free-running period of the strain, (2) the capacity for such individuals to adapt to different light–dark (LD) cycles, and (3) the phase of the circadian locomotor activity cycles with respect to the zeitgeber (*see* **Note 10**) used. In each of the above cases, the experimental design will differ accordingly. For an excellent recent review on various aspects of entrainment *see*, for example, [ref. 19](#). It is also important to note that the following subheadings contain information that applies equally well to nonphotic entraining stimuli (such as temperature, for example).

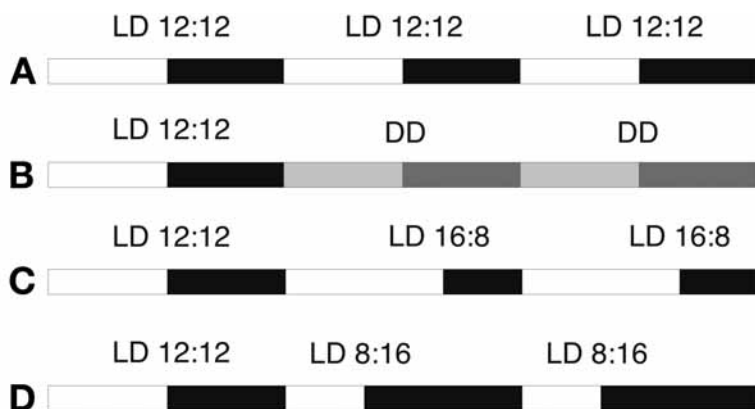


Fig. 2. Schematic representation of different types of light–dark (LD) cycles showing, in each case, examples of how it is possible to change from 12 h:12 h LD to the other regimes. As explained in the text, a typical experiment requiring switching from one LD regime to another, would entail at least 3 d of 12 h:12 h LD, before switching to the new cycle. For brevity, **B–D** show only the last of 3 d of 12 h:12 h LD. **(A)** 3 d of 12 h:12 h LD cycles. **(B)** 1 d of 12 h:12 h LD, followed by 2 d of constant darkness; here the light gray bars represent the subjective daylight and the dark gray bars the subjective night. **(C)** 1 d of 12 h:12 h LD, followed by 2 d of 16 h:8 h LD; **(D)** 1 d of 12 h:12 h LD, followed by 2 d of 8 h:16 h LD.

3.3.1. Determining Natural Free-Running Period (*t*) of a *Drosophila* Strain

1. Initially entrain, for instance, with 12 h light:12 h dark (12:12 LD) cycles for at least 3 to 4 d.
2. Continue the experiment for at least another 5 to 7 d, keeping the flies in free-running conditions—constant temperature and constant darkness (DD; see **Notes 7–9**).

3.3.2. Determining Capacity to Adapt to Different LD Cycles

In order to assay the capacity of flies to adapt to different LD cycles, experiments can be conducted in many different ways. The important variable to constantly bear in mind is that whatever changes to the lighting regime are adopted, the new condition should be monitored for at least 3 to 4 d (if not longer) in order to make sure that transients from the previous condition have disappeared, or at least become attenuated. For example, one may be interested in assaying the circadian locomotor activity response of flies to LD cycles consisting of a long day (16 h:8 h LD) or, conversely, a short day (8 h:16 h LD; **Fig. 2**). In each case, as flies are normally raised in incubators with 12 h:12 h

LD cycles, it is a good idea to start off the experiment by monitoring 3 to 4 d of 12 h:12 h LD cycles, followed by 5 to 7 d with the new LD condition.

3.3.3. Determining Phase–Response Characteristics

This type of experiment aims at obtaining information on the way flies respond to flashes of saturating (25–40 $\mu\text{mol photons/m}^2/\text{s}$) light given at different times of the night or the following subjective day.

1. Treat the flies with a pulse (usually lasting 10–20 min) of saturating white light (25–40 $\mu\text{mol photons/m}^2/\text{s}$). Start from the last night of the LD period and continue on the following first day of DD, at equally spaced intervals, each to be assayed in a separate experiment.
2. Monitor the activity of the flies in DD for at least 3 to 4 d.
3. Analyze the actograms of individual flies to establish whether the morning and evening locomotor activity peaks have remained in phase with the original zeitgeber time (ZT), or whether they have been influenced (phase-shifted) by the flash of light (*see Note 11*), and if so, by how many minutes or hours.
4. Plot the average (of all flies) of each phase delay/advance produced at each ZT and circadian time (CT) to obtain a phase–response curve. This provides important dynamic information on the state of the circadian clock at different times during the day.

3.4. Data Analysis

The many issues concerning the analysis of data collected during the monitoring of *Drosophila* circadian locomotor activity have received a great deal of attention from the very outset of circadian rhythms biology. In particular the major issue of concern has nearly always been the choice of the statistical approach used to extract information on the periodicities that would best describe the nature of the experimental data. An in-depth treatment of such issues can be found in Chapter 2. However, in this chapter it may be of some use to consider our personal experience in this respect. In particular, one of us (M. A. Z.) became involved in a project for the implementation of time series analysis software, possibly using platform-independent programming tools. We then chose programming tools that were not only platform-independent, but also open source (for a complete definition of the open source concept, see http://www.opensource.org/docs/definition_plain.php). In particular, all the routines so far developed have been coded using the Python (www.python.org) scripting environment, enhanced by the Numeric library (Numerical Computing for Python library; (www.numpy.sourceforge.net) and the Tcl/Tk cross-platform graphics library (www.tcl.tk). Currently, routines for producing double-plotted actograms and for performing autocorrelation plots and CLEAN (21,22) spectral analysis have been implemented. CLEAN is further enhanced by us-

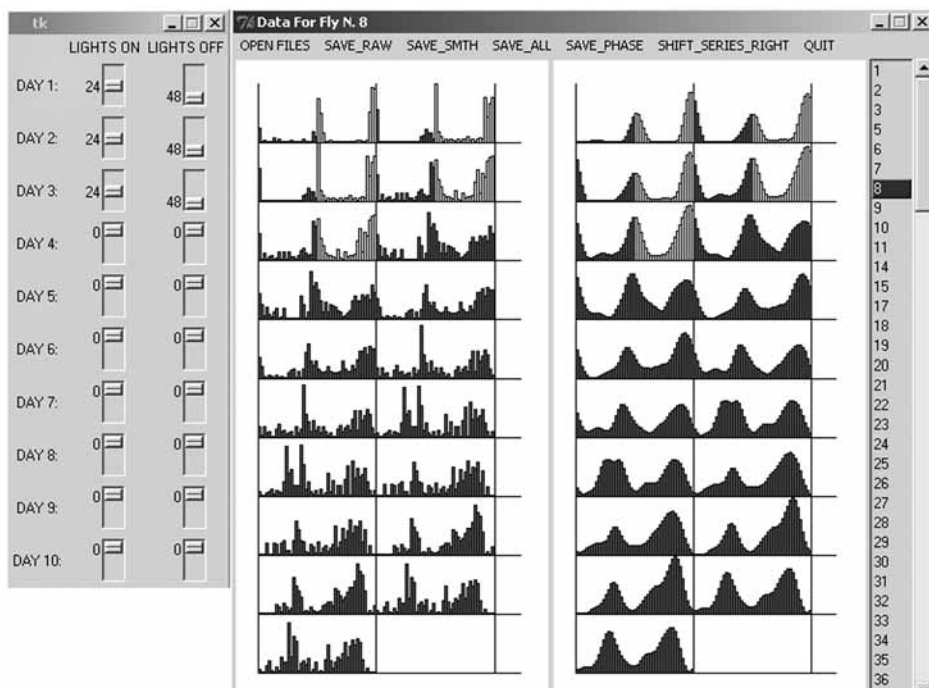


Fig. 3. The figure shows the graphical user interface of the actogram plotting program used in our laboratory. The left-hand part of the figure shows the slide controls that allow the assignment of the time of lights on and lights off for each single day, “on the fly.” This leads to the coloring of the bars in the two double-plotted actograms (left-hand panel = raw data; right-hand panel = smoothed data) accordingly (i.e., white bars = lights on; dark gray bars = lights off). The smoothed actogram was obtained by applying a two-pole Butterworth filter to the raw data, in order to filter out high-frequency (i.e., <4-h periodicity) “noise.”

ing a Monte Carlo approach to generate 95 and 99% confidence limits, to be used as an objective criterion for the assessment of the significance of the peaks present in the CLEAN spectrum (*see Note 12*).

In addition, the actogram plotting routines generate two side-by-side plots consisting of (1) the double plot of the original (raw) locomotor activity data and (2) the double plot of the same data after smoothing (following the application of a two-pole Butterworth filter designed to filter out data with a periodicity below 4 h [24]). The latter plot usually facilitates the observation of the main peaks present in the behavioral data even when these are relatively “noisy” (Fig. 3). The actogram-plotting program provides a graphical user interface with slide controls (*see* left-hand part of figure) that allow the assign-

ment of the time of lights on and lights off for each single day, in a dynamic fashion. This leads to the coloring of the bars in the two actograms (raw and smoothed) accordingly (i.e., white bars = lights on; dark gray bars = lights off). An additional tool allows the user to select particular bins in the smoothed actogram. These choices can be saved in a text file, which will then contain information regarding the main phase reference points (i.e., time of lights on and lights off, mean daily activity, as well as the position of the bins selected graphically by the user; see **Note 13**).

4. Notes

1. Most published reports relate to data obtained only from male flies, the main reason being one of convenience, which stems from the complicating necessity that female flies must be virgin in order to avoid egg deposition in the glass tube in which the fly is housed during the data acquisition period. The eggs and the ensuing larvae can interfere seriously with the infrared locomotor activity detector.
2. The paper towel will absorb atmospheric humidity (which may condense in the Petri dish); this guarantees that the flies will stay asleep for all the time necessary to load them into the tubes, but without risking the irreversible damage (often leading to death) following prolonged periods (>20 min) of CO₂ anesthesia.
3. This means that, within a physiologically well-tolerated range of temperatures (i.e., 18–28°C), the general circadian characteristics of the activity patterns will in fact be conserved, although the daily mean of locomotor activity may show subtle changes, and the relative heights and position of the morning and evening activity peaks may also be influenced (**16**).
4. The “daylight” type tube has a definitely more intense spectral emission in the 400- to 570-nm range, and has a relatively lower emission at wavelengths greater than 570 nm (**20**). Fluorescent tubes in general show relatively little changes in emission during their normally fairly long lifetime (several months of continuous usage); furthermore, they emit little far red/infrared radiation, which entails the generation of relatively little heat.
5. Recently it has become possible to consider employing high-efficiency LEDs, as these are available in types that can provide full visible spectrum emission (i.e., 400–600 nm) as well as fairly narrow bandwidth monochromatic light (usually available in the colors of blue, green, yellow, orange, and red). The main advantage of such illumination devices is their low energy consumption, limited generation of heat, and, owing to their small size, the ease of placement in the experimental setup (which can even envisage having an array of LEDs so that each fly is directly illuminated by its own personal “sun”).
6. It is, however, important to point out that, should one wish to simply monitor the lighting conditions occurring in an incubator during an experiment in which *Drosophila* circadian locomotor activity is being monitored, it is sufficient to employ a luxmeter (or even a simple phototransistor) to provide information on whether the lighting source is turned on (with an intensity reading as well) or off at a given moment in time.

7. The data corresponding only to the time spent by the flies in DD will then be used for the appropriate statistical analyses (see **Subheading 3.4.**) in order to obtain an estimate of the desired τ value. As an extra precaution, the first day of DD data should be excluded from the statistical analysis to avoid, at least in part, the effects of transients (i.e., aftereffects reflecting the fly's previous LD experience). Such transients may take (on average) a day or two to completely disappear (for example, see **ref. 17**).
8. Because one has gone to the trouble of organizing the experiment, it is not a bad idea to continue collecting data even after the end of the DD period, by providing the flies again with 3 to 4 d of 12 h:12 h LD cycles with the same phase as the LD cycles used at the beginning of the experiment. Inspection of actograms obtained from the data collected during the whole experiment (i.e., 3–4 d LD + 5–7 d DD + 3–4 d LD) can provide useful information on the capacity of the flies to readapt to a zeitgeber once they have been left in free running conditions for some days.
9. As stated in the introduction, the collection of locomotor activity data, among other things, entails the choice of an appropriate sampling frequency. The answer to this question is strictly related to how this choice will affect the type of inferences we wish to be able to make from our data. In particular, as circadian rhythms biologists, our major goal (normally) is the identification of patterns of events occurring with circadian or close to circadian regularity. In general, information and signals analysis theory state that the sampling frequency should be twice that of the highest frequency to be analyzed (in other terms, the interval between samples should be half that of the shortest periodicity of interest), a concept that is also known as the “Nyquist limit” (**23**). This implies that with a sampling frequency of 0.02 Hz (i.e., one sample every 10 min), we would be limited to the analysis of periodicities no shorter than 20 min. For anyone interested in circadian rhythms this would already be much more than actually required. In fact, most circadian experimental setups use sampling rates included between 0.04 and approx 0.06 Hz (5 and 30 min, respectively). There is also an upper limit to the determination of periodicities in time-series analysis (i.e., the longest periodicity that can be determined can be no longer than half of the length of the series itself), which means that, let us suppose, if we are interested in periodicities in the circadian range (i.e., 24 h) then our data should consist of at least 48 1-h samples (**23**).
10. By convention, times of the day during 12 h:12 h LD cycles are referred to as ZTs, so that the time at which lights come on is referred to as ZT 0, and lights off is referred to as ZT 12. Thus, daylight hours are included between ZT 0 and ZT 12, whereas hours of darkness are included between ZT 12 and ZT 24 (or ZT 0). During free-running conditions (DD), it is customary to refer to the original ZTs as reference points during the circadian cycle, but in this case the times are referred to as CTs, with the same numeration as above (i.e., CT 0 is the time when lights would have come on if the flies were in an LD cycle; similarly CT 12 is the time when lights would have been turned off in an LD cycle).
11. Typically, in *D. melanogaster*, flashes of light given between ZT 12 and ZT 18 will tend to produce a phase delay in the flies (i.e., the morning peak of activity, which is normally due at around CT 0, will tend to occur later); flashes of light

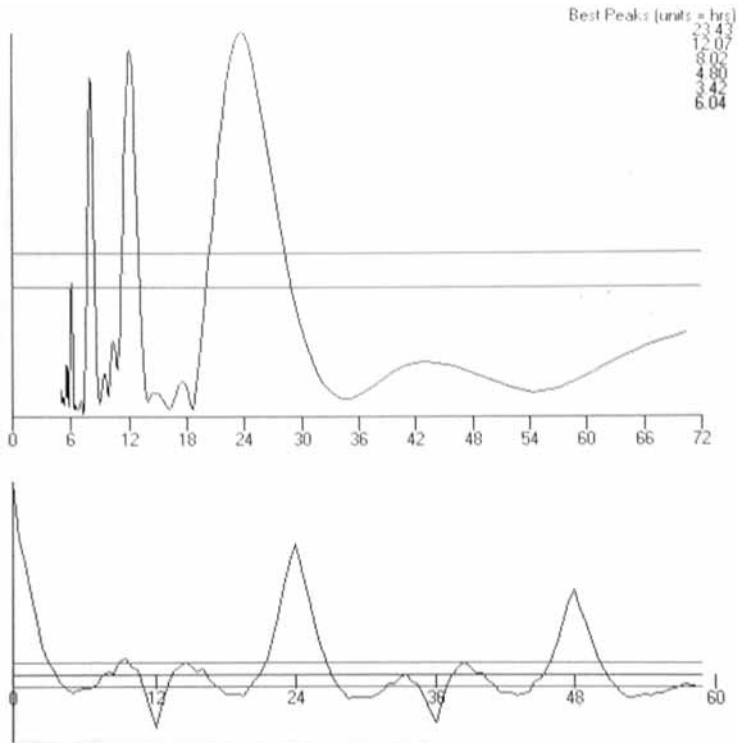


Fig. 4. Our data analysis software package currently contains routines that allow on-screen viewing, in a single screen shot, of: (1) **upper panel:** the CLEAN spectral plot along with the 95 and 99% Monte Carlo confidence limits, while also providing the periods corresponding to the first 10 most significant peaks; (2) **lower panel:** the autocorrelogram, along with the canonical 95% confidence limits.

given after ZT 18 will tend to produce a phase advance in the flies (i.e., the morning peak of activity, which is normally due at around CT 0, will tend to occur earlier).

12. The algorithms employed for the CLEAN spectral analysis software were ported from original FORTRAN listings by Dr. J. Lehar (MIT, Cambridge, MA). We are also planning to implement the algorithms necessary to perform maximum entropy spectral analysis (25) in the same package. The software package currently contains routines that allow the on-screen viewing of the CLEAN spectral plot along with the 95 and 99% confidence limits (which also provides the periods corresponding to the first 10 most significant peaks) and the autocorrelogram in a single screen shot (Fig. 4).
13. These data form the basis of phase analysis of the locomotor activity data under study. In this respect, we adopted an external statistical package, which we found to be very useful in performing circular statistics as well as cross-correlation

analysis (which are both related to phase analysis [25])—the open source R package (the R Foundation for Statistical Computing; see ref. 25), integrated by the CircSats package from the Comprehensive R Archive Network (CRAN, www.cran.r-project.org).

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Automated Video Image Analysis of Larval Zebrafish Locomotor Rhythms

Gregory M. Cahill

Summary

A method is described for measurement of the circadian activity rhythms of up to 150 larval zebrafish simultaneously with a single video image analysis system. Most of the required equipment and software are commercially available, although some components are custom-built.

Key Words: Zebrafish; circadian; locomotor activity; automated activity monitoring; image analysis.

1. Introduction

Our goal in development of this automated, video-based method for monitoring zebrafish locomotor activity was to develop an efficient screen for circadian clock mutants with defects in the timing (period or phase) of free-running behavioral rhythms. The widespread use of the zebrafish in genetic analysis of vertebrate embryonic development has driven rapid development of genetic and genomic techniques and resources that can be exploited in genetic analyses of other complex phenomena, such as circadian rhythmicity ([1](#)). A mutant screen requires maximization of the number of animals that can be tested and minimization of the effort devoted to raising and testing each animal, because most will not carry a detectable mutation. The method described here can be used to record behavioral rhythmicity from up to 150 larval zebrafish simultaneously with a single video camera ([2,3](#)). In the age range that we have used (5–20 d of age), up to 100 larvae can be raised in a 1-L beaker with minimal effort. The recording apparatus is completely automatic, so no intervention is required during behavioral testing.

During activity monitoring, larval zebrafish are maintained individually in 0.5-mL wells drilled in a translucent, white polyethylene specimen plate. The plate is illuminated continually with infrared light, and a charge-coupled device video camera is focused on the plate from above. Automated acquisition and analysis of images from the camera are controlled by customized software running on a desktop computer. For each activity sample, a series of 30 to 60 images (1/s) is captured, digitized, and stored in RAM (Fig. 1). The image analysis software then determines the $x - y$ coordinates of each fish (recognized as an object with an optical density below a threshold) in each image. The total path length for each fish is determined from the series of coordinates and stored in a text file. The images are then erased from memory, and a new cycle of image capture and analysis is initiated. Typically, we record an activity sample every 4 min for 1 wk.

Optimization of the procedure for maximal numbers of animals required balancing several parameters. One set of parameters relates to maintaining the fish in a state of health that supports behavioral rhythmicity. The minimal volume that reliably keeps 10- to 20-d-old larvae healthy for 1 wk is 0.5 mL. Excreted ammonia and decaying food are toxic, so if the fish were to be fed during the recording period, the water in the wells would have to be changed regularly. Continuous food delivery and water exchange would be difficult to accomplish with the imaging configuration that we use, and food and water exchanges at daily intervals could influence circadian periodicity. Therefore, we chose to starve the fish during recording, which eliminates the need for water changes but limits the duration of experiments. We record at a relatively low temperature of 24°C to reduce metabolic rates. We find that in most experiments all larval zebrafish survive 7 d in the system and can be raised to adulthood afterward.

Another important parameter was the age of the fish. Zebrafish raised at 28.5°C begin actively swimming and feeding at about 5 d of age, when their yolk is depleted. We can record swimming rhythms at this stage (3), but their small size and a lack of body pigmentation make them difficult to detect consistently in the 150-well format. They are also less likely to survive than older larvae. We find that 10-d-old zebrafish fed live *Paramecium* from 5 to 9 d of age produce the best results.

A third issue was balancing digital image resolution and sampling parameters against available RAM, microprocessor speed, and storage capacity. These algorithms were developed several years ago, when digital imaging was less advanced, microprocessors were slower, and RAM and disk storage capacity were much more expensive. The procedure described here will certainly work at least as well or better with today's increased computing power, but it is also possible that different algorithms could make better use of these advances.

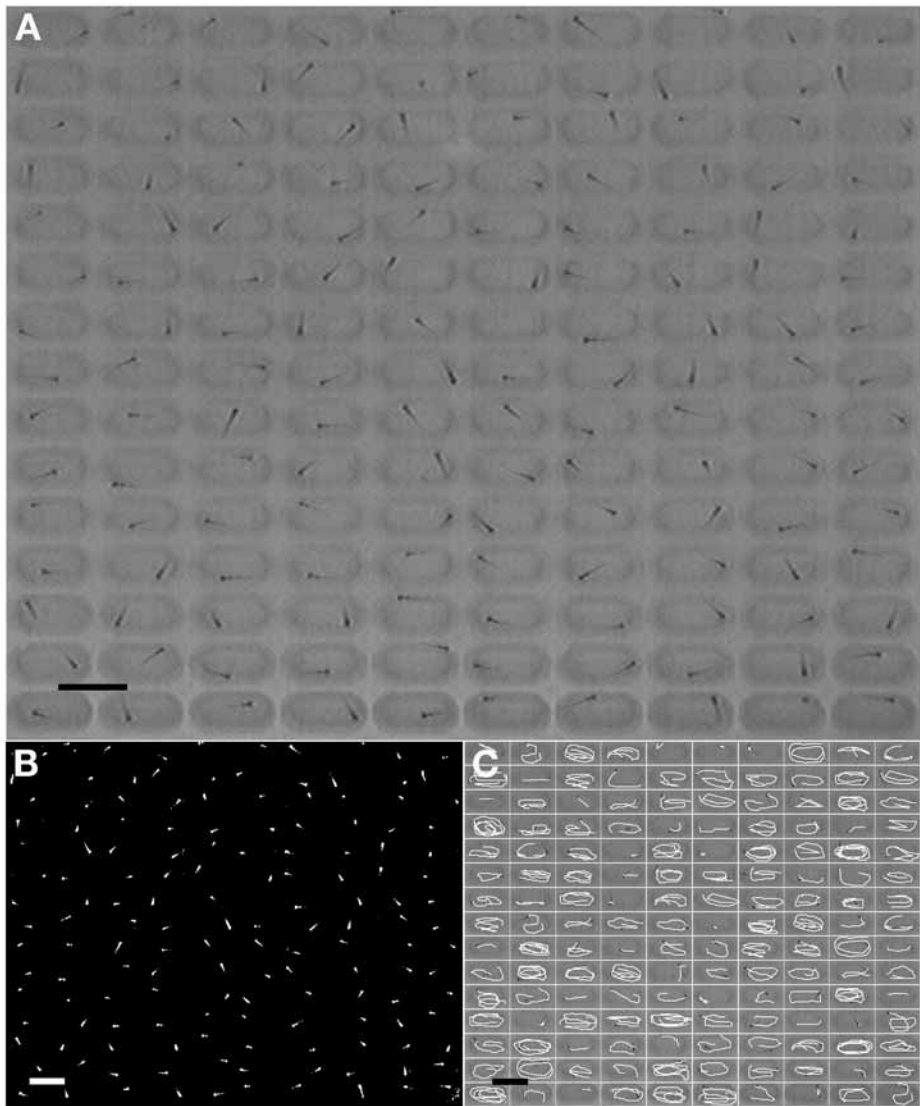


Fig. 1. Measurement of larval zebrafish activity by video image analysis. (A) digitized image of 150 10-d-old zebrafish, each in an oval, 0.5 mL well. (B) Binary image at 50% scale of the image in A, produced by setting a pixel value threshold that distinguishes fish (white) from background. For each image, the software determines the coordinates of each object that is below threshold. (C) Swimming paths typical of those recorded from a series of 38 images (1 captured/s) during the subjective day. Scale bars = 1 cm.

Finally, as the number of animals monitored simultaneously (and thus the area imaged) increases, the number of pixels representing each fish and their differences from background decrease. This makes a uniform background crucial, so that every fish is darker than any part of the background. This is addressed primarily by even illumination, and secondarily by image preprocessing.

We have also reported circadian locomotor rhythmicity in adult zebrafish, recorded with an infrared beam detector system (4). Although activity rhythms were detectable in approx 70% of fish tested, they were variable and noisy, so that approach cannot be recommended. Limited preliminary experiments suggest that adult rhythms could be measured with video image analysis, but we have not optimized conditions for adults.

Detailed methods for raising, maintaining, and breeding zebrafish have been described previously (5). Here, the only issues in those categories that are addressed are those that seem to affect success in locomotor rhythm recording.

2. Materials

2.1. Breeding and Raising Zebrafish

1. Fish strains: AB (Zebrafish International Resource Center, Eugene, OR) and SJD (Steven Johnson Laboratory, Washington University; *see Note 1*).
2. Egg water: Reverse osmosis, deionized water (conductivity $<1 \mu\text{S}/\text{cm}$), with Instant Ocean sea salt ($\sim 60 \text{ mg}/\text{L}$) and NaHCO_3 added to bring the final conductivity up to $100 \mu\text{S}/\text{cm}$ and the pH to 7.0, aerated for at least 12 h before use (*see Note 2*).
3. Zebrafish breeding: We maintain adult zebrafish in holding systems produced by Marine Biotech (Beverly, MA) or Aquatic Ecosystems (Apopka, FL). These systems circulate outflow from multiple 1- to 2-L polycarbonate aquaria through biological, particulate, and carbon filters, a UV sterilizer, and heater before returning it to the aquaria (*see Note 3*). The system water is maintained at a conductivity of $200 \mu\text{S}/\text{cm}$ by daily replacement of 10% of the water with a combination of deionized water and egg water.

Breeding traps are rigid plastic mesh aquarium inserts that allow eggs to fall through to the bottom of the tank and prevent the adults from eating them.

Baby tubes are polycarbonate, 10 cm D $\times$ 15 cm L; one end is covered with 0.5-mm nylon mesh.

2.2. Paramecia Culture and Filtration

1. *Paramecia multimicronucleatum*.
2. Open plastic tubs (45 cm L $\times$ 38 cm W $\times$ 12 cm D).
3. Brewer's yeast tablets (500 mg).
4. Whole-grain wheat, boiled slowly (150 mL wheat/700 mL water) for 2 h or until all kernels split open. Store at 4°C for up to 1 mo.
5. Cloth prefilters.
6. 24-cm Fluted paper filters, grade P8.

2.3. Activity Recording

1. Specimen plate: translucent white polyethylene, 18.5 cm × 13.5 cm × 8 mm thick. Wells are oblong, 12 mm L × 6 mm W × 7 mm D, arranged in 10 × 15 array with 1-mm-thick walls.
2. Illumination: Fiberoptic light source, 150 W tungsten halogen, with heat filter removed and long-pass filter (700 nm cutoff) added. Diffuse axial illuminator (see **Note 4**). Mirror, 18.5 × 13.5 cm.
3. Video camera: Hamamatsu model 2400-77E. The relevant features are a remote head 1.7-cm charge-coupled device sensor, a control unit with automatic gain control and shading correction, and horizontal center resolution greater than 750 TVL. The standard infrared-blocking filter is removed, and a 50-mm macro lens is used. The camera is mounted on an adjustable copy stand.
4. Computer hardware: desktop computer with a 333 MHz microprocessor, 128 MB RAM, a 4-GB hard drive and a Flashpoint 128 frame capture card (640 × 480 pixels, 8-bit gray scale).
5. Computer software: Windows NT operating system and Optimate 6.2 (MediaCybernetics, Silver Spring, MD) image processing software with the Swimming1.1 macro (Meyer Instruments, Houston, TX) for image analysis; Microsoft Excel and CHRONO, a Macintosh program for rhythm analysis created by Till Roenneberg, University of Munich (6).

3. Methods

3.1. Production of Larval Zebrafish

1. Maintain breeding zebrafish, 3 to 12 mo old at 28.5°C, under a 14:10 light–dark (LD) cycle. Feed lightly three times daily with commercial flake food in the morning, newly hatched brine shrimp (*Artemia* sp.) nauplii at midday, and adult brine shrimp in the afternoon, providing enough food so that all fish get some, but all is eaten within 5 min (see **Note 5**).
2. Select breeder aquaria containing either pairs or groups of fish in a 1:2 male–female ratio. Nine hours or more after lights-on, feed with adult brine shrimp, clean the aquaria, and insert breeding traps to protect the eggs, which usually are produced around the time of lights-on the next day.
3. Day 1: Collect eggs and rinse to remove all feces, debris, and dead (opaque white) eggs. Transfer up to 100 eggs to each 1-L beaker along with 200 mL egg water containing 0.01% methylene blue (see **Note 6**). Keep beakers in a water bath at 28.5°C throughout embryonic and larval stages.
4. Days 2–4: In the mornings, remove any dead embryos and shed chorions, and replace the water with 200 mL of fresh egg water. In the afternoon on day 4, increase the water in each beaker to 500 mL, and add the *Paramecia* filtered from 500 mL of a recently cleared culture to each beaker (see **Subheading 3.2.**).
5. Days 5–6: Do not change water. *Paramecia* can be added on day 6 if they are depleted (see **Note 7**).

6. Day 7: Transfer larvae into baby tubes in 1-L beakers filled with fresh egg water. Add the *Paramecia* filtered from 1 L of a recently cleared culture to each beaker (see **Note 8**).
7. Days 8–10: Each morning, replace 95% of the water with fresh egg water, pouring the old water gently from the outside of the baby tube to strain out the larvae. Add the *Paramecia* filtered from 1 L of a recently cleared culture.
8. Day 11: In the morning, transfer larvae to clean baby tubes and beakers with fresh egg water and no food. They will be transferred to recording wells in the afternoon.

3.2. *Paramecia Culture and Harvest (see Note 9)*

3.2.1. *Culture*

1. Culture *Paramecia* in eight tubs in the fish colony room. Discard the oldest culture and start a new one every Monday, Wednesday, and Friday, or whenever a culture is used up.
2. Clean the culture tub with hot tap water, but no detergent. Wipe with 95% ethanol and rinse with deionized water.
3. Fill the tub three-fourths full with egg water. Add 2 L of a culture that is growing at a high rate (7–10 d old, cloudy, yellow to pink, with many very active *Paramecia*). Fill the tub to within 1 cm of the top with egg water. Scatter 15 brewer's yeast tablets around the bottom of the tub. Scatter 30 mL of drained boiled wheat (about 250 kernels) around the bottom of the tub.
4. Stir each culture every day to break up the film that forms on the top (see **Note 10**).

3.2.2. *Harvest Paramecia From Recently Cleared Culture to Feed Larval Zebrafish*

1. Mix up culture, and then allow the debris to settle out.
2. Scoop up culture supernatant with a 1-L beaker, avoiding debris.
3. Filter through four layers of prefilter to remove large particles.
4. Transfer to fluted paper filter and wash with egg water to completely replace culture fluid and concentrate the *Paramecia*. Do not let the filter drain completely.
5. Wash clean *Paramecia* into a beaker and dilute with egg water, then feed to larvae.

3.3. *Activity Recording Setup*

1. Soak specimen plate in 1% bleach for 1 h, then rinse repeatedly with hot tap water, then deionized water, then egg water.
2. In the afternoon or evening of the first recording day, transfer larvae to specimen plate wells. Use the baby tube to gently strain out larvae, then invert the tube and wash the larvae into clean egg water in a 100-mm Petri dish.
3. Fill the specimen plate wells with egg water. Use a large-bore pipet to transfer larvae to individual wells. Avoid transferring water with the fish. Touch the tip of the pipet to the water surface and encourage the fish to swim down with gentle tapping (see **Note 11**).

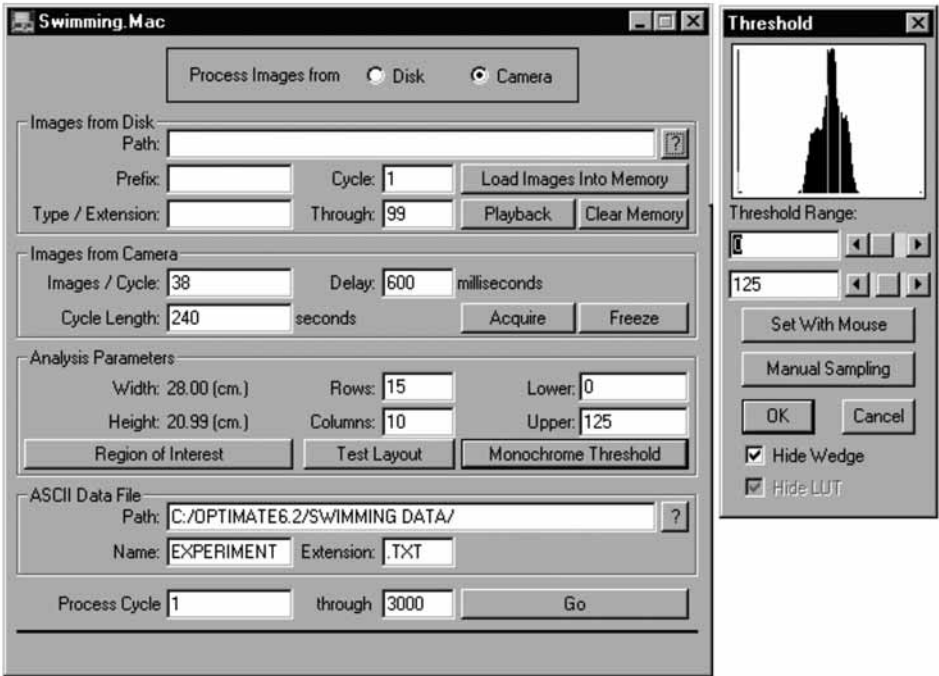


Fig. 2. Control panels for the image analysis software.

4. Set up the camera and illumination system in a light-tight, refrigerated incubator maintained at a constant 24°C. The light source is located outside the incubator, with fiberoptic leads passing in through a light baffle. Set the illuminator at 50% power. Humidify the incubator by bubbling air through a large open reservoir of water.
5. Place the specimen plate directly on the reflecting surface of the mirror, and position the diffuse axial illuminator over the specimen plate.
6. Launch Optimate and the “Swimming” macro (Fig. 2, left), and click on “Acquire” to view the digitized video image (Fig. 1A). Focus the camera so that the array of occupied wells fills the image field.
7. Set the camera controller: “Automatic gain control” on; “Autoenhance” off; “Boost” off; “Detail” at minimum; “Shading correction” normal.
8. With the “Swimming” macro in “Acquire” mode, use the four “Shading Correction” dials to even out any detectable variations in background shading from center to periphery, side to side or up and down. To fine-tune the image shading, click on “Freeze” to capture a still image, then on “Monochrome Threshold” to bring up the “Threshold” dialog window (Fig. 2, right). Set the lower threshold to 0, and then scan the upper threshold up and down to visualize any shading variation in the image. If shading variations exist, close the “Threshold” window,

click on “Acquire,” and repeat these steps until variations are minimized (*see Note 12*).

9. Set a threshold range that, as much as possible, includes all the fish and excludes all the background (*see Note 13*).
10. Set sampling parameters in the “Images from Camera” and “Process Cycle” sections of the “Swimming” macro window (**Fig. 2**, left). “Images/Cycle,” 38; “Delay,” 600 ms; “Cycle Length,” 240 s (*see Note 14*).
11. Freeze the image and click on “Region of Interest,” then use the cursor to draw a rectangle that encompasses all the wells. Set the number of “Rows” and “Columns” of wells. Click on “Test Layout” to superimpose a grid produced by dividing the region of interest into the specified rows and columns onto the image. Repeat these steps if every cell in the grid does not encompass one and only one well.
12. Create an ASCII data file by clicking on the “?” button, selecting a folder, and entering a file name with a .txt extension. If a file with the same name and path already exists, a dialog that asks whether to “Replace the existing file,” “Append the new data,” or “Cancel” pops up.
13. Click on “Go” to start automated image capture and analysis. A window with a “Stop” button will pop up.
14. Once each day, check the swimming paths that are superimposed on the image at the end of a cycle (**Fig. 1C**). If any problems appear (*see Note 15*), click on the “Stop” button, which works only during the image capture phase of the cycle, close and reopen the “Swimming” macro, optimize the image, and repeat **steps 9** through **13**.

3.4. Data Analysis (*see Note 16*)

1. At the end of the experiment, stop the “Swimming” macro and check the threshold to see if there are any problems. Exit the macro and Optimate.
2. Import the data file and preprocess it in Excel. The first column contains time stamps, and each succeeding column contains the measurements for one well.
3. Scan the data for blank rows that are inserted whenever the macro is restarted and delete them.
4. In new columns, calculate the averages for each treatment group or genotype.
5. Note the starting date and time, ending date and time, total number of time points, and number of columns; these are required to create CHRONO files.
6. Create a new worksheet and transform all the values in the raw file worksheet with the formula: = Round(100*value,0) + 10. This generates integers between 10 and 1000 for import into CHRONO.
7. Save the integer worksheet as a text file.
8. Import the text file into CHRONO for plotting and analysis (**Fig. 3**).
9. During import, fuse six time points to produce 24-min bins; enter other experimental information as directed in dialog windows.
10. Smooth the data with a 4-h centered running average (**Fig. 3B**).

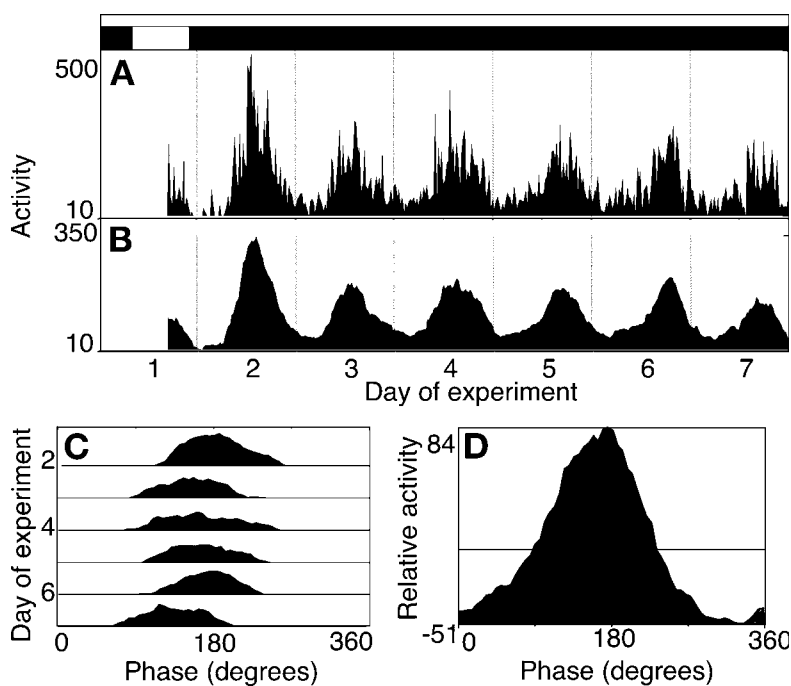


Fig. 3. Display and analysis of zebrafish activity records. All records are from the same wild-type individual. (A) Raw activity reduced to 24-min bins. (B) Activity record smoothed with a 4-h running average. (C) Actogram plot of activity peaks, plotted modulo 25.3 h. (D) Composite peak for days 5 through 7.

11. To produce an actogram of activity peaks (Fig. 3C), subtract the trend calculated from a 25.6-h centered running average; make a signal ratio of the trend; set modulo τ to 25.3 h or to the average free-running period of the control group.
12. Set the time limits to days 5 to 7, and plot the composite curve (Fig. 3D).
13. Measure the phase of the composite peak's center; this is reliable only if the composite curve has a single symmetrical peak with a peak–trough amplitude greater than 40 relative activity units.

4. Notes

1. The AB strain is a closed, outbred line that breeds well in the laboratory. The SJD strain is an inbred line, genetically distinct from AB. This strain does not breed naturally, so in vitro fertilization is required to propagate it. AB, SJD, and AB/SJD hybrid larvae have the strongest behavioral rhythms of the strains we have tested. Strains that express weaker rhythms in our hands include C32 (an inbred derivative of AB), Tubingen, and some strains derived from pet store fish.

2. Although adult zebrafish can thrive in dechlorinated, oxygenated tap water in many areas, we use only deionized water with salts and pH buffer added to avoid contaminants and seasonal variation in the water supply. Embryos and larvae require egg water.
3. Several companies market similar recirculating systems that enable maintenance of adult zebrafish at high densities. Zebrafish can also be maintained in good breeding condition in plain aquaria without recirculation or filtering if the density is kept lower than 1 fish/2 L and the water is changed daily.
4. A diffuse axial illuminator produces even illumination across the specimen plate and eliminates uneven reflection from the water surface. This is critical for producing a uniform background. The principle is that incoming horizontal light is reflected down onto the specimen plate by a mirror-type beam splitter, then light reflected from the specimen plate passes vertically up through the beam splitter to the camera. Only axial (vertical) light reaches the camera, eliminating glare. The largest commercially available diffuse axial illuminators (Edmund Industrial Optics, Barrington, NJ; RVSI/NER, Weare, NH; Advanced Illumination, Rochester, VT) illuminate a field of 10×10 cm, which would illuminate 100 wells. We constructed our own to illuminate a 17×14 cm field. The mirror, placed under specimen plate to reflect diffuse light up through wells, enhances contrast.
5. Critical environmental considerations for breeder maintenance are water quality (particularly zero ammonia and nitrite, nitrate <10 ppm, pH 7.0), long photoperiod (but constant light inhibits breeding), and water temperature ($25\text{--}30^\circ\text{C}$). Growth rate is inversely proportional to fish density.
6. It is important to get rid of all debris. Methylene blue inhibits fungal growth, which starts on dead eggs and can spread to kill healthy eggs; it is required only on the first day.
7. At this stage, larvae maintain an upright swimming posture, are rhythmically active, and begin to feed. Rhythms can be recorded reliably from as many as 63 fish (in a 7×9 array of wells) of this size.
8. From this stage on, the larvae should always have an excess of *Paramecia*. They tend to swim near the surface, and a visible cloud of them should fall from the surface when the beaker is swirled. It is important that all of the culture medium is filtered away from the *Paramecia* and replaced with egg water to prevent growth of bacteria in the baby tubes.
9. This low-tech method is adapted from a published method (5). As described here, it produces enough *Paramecia* to raise 10 beakers of larvae each week. Cultures that grow slower and last longer can be made by omitting the brewer's yeast.
10. The *Paramecia* feed on bacteria. The bacteria form a smelly white scum on the surface of the culture that can suffocate the *Paramecia* if it is not broken up regularly. If the scum turns black, discard the culture. When the culture supernatant clears, most of the bacteria and nutrients are gone, and the cultures begin to decline.

11. The key to the survival and strong rhythmicity in this system is clean water. This is the reason for starving the fish, changing the water, and transferring as little water as possible from one vessel to the next.
12. It can take several rounds of adjustment to optimize background shading initially, because adjusting in one dimension can affect shading in the others. Although we use the camera controller to make this adjustment, digital methods could be used instead.
13. With 150 fish, there is a very narrow range of upper thresholds (1–3 pixel values out of 256 total) that discriminate all the fish from all of the background. The system recognizes an object as a fish only if four or more contiguous pixels are in the designated threshold range, so single background pixels that sometimes fall into the range are not a problem. Two types of recognition errors unavoidably occur in some wells in some images. The first is that the fish is positioned in such a way that it is not detected. If this happens the software inserts the last known coordinates of the fish. The second is that two or more objects are recognized in one well, either because two separate parts of the fish cross the threshold or because a section of background fluctuates into the threshold range. When this happens, the software computes the average of the objects' coordinates.
14. These values were developed under constraints of available image capture and processing speed. A 600-ms delay results in image capture at a rate of one per second. We arbitrarily chose a 4-min cycle length, and maximized the number of images that can be processed within that time. Our computer takes about 5 s to extract coordinates for 150 fish from each image, so 38 images is the maximum possible within a cycle.
15. The most common problem is an unexpected change in illumination intensity that requires a change in the threshold range. Dimming that brings background into the range produces paths that jump back and forth, rather than the circles typical of swimming fish. Brightening (more rare) that puts the fish out of the threshold range appears as no paths at all.
16. A variety of statistical time series analyses are used to extract rhythm parameters of period, phase, and amplitude. Any of these would work well with the type of record shown in [Fig. 3](#). However, with these relatively short data sets, all the methods that we have tried are very sensitive to noise, particularly missed peaks or the timing of the first and last peaks. They often return period estimates that are not believable. We have found that measuring the phase of the composite peak produced by averaging the last three cycles of data, as described here, gives timing estimates that are less variable.

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Locomotor Activity in Rodents

Gianluca Tosini

Summary

Many of the behavioral parameters exhibited by an organism show daily fluctuations. These may persist under constant environmental conditions, demonstrating that they are governed by an endogenous (circadian) clock. The monitoring of locomotor activity in rodents is probably one of the most common methods to track this endogenous timing system. The analysis of locomotor activity rhythms can provide several parameters that may be used to describe the status of this endogenous clock. In the past few years several companies have developed hardware and software systems that allow the collection and the analysis of activity data using a personal computer.

Key Words: Locomotor activity; free-running period; phase shift; amplitude; rodents.

1. Introduction

Many biochemical, physiological, and behavioral parameters exhibited by the majority of organisms on the planet show daily fluctuations. Importantly, these rhythms persist under constant conditions with periods close to 24 h, demonstrating the presence of an endogenous circadian clock.

The description of a rhythm can be obtained by regular recording of events in a numeric time series, thus providing information on three fundamental characteristics: period, amplitude, and phase. The period is the length of time necessary to complete one full cycle; the amplitude is, roughly, the difference between the peak and the trough; and the phase refers to the temporal relationship between a specific identifiable point on the cycle and a point on a reference cycle (e.g., the relationship between onset of locomotor activity and the beginning of the light cycle).

To study the clock it is useful to have an indirect measure of its activity that reflects its performance as accurately as possible. During the past few years

several studies have identified many physiological events (body temperature, heart rate, etc.) or behaviors (locomotor activity, drinking, etc.) that can be used as an accurate expression of clock functioning. However, the most widely used, and probably the most reliable, expression of the clock in rodents is the rhythm of locomotor activity. Such a rhythm can persist under constant conditions for many months or even years ([1–3](#)).

2. Materials

1. Cages.
2. Running wheels.
3. Implantable telemeters and receivers.
4. Infrared sensors.
5. Infrared camera.
6. Computer and data acquisition hardware.
7. Data acquisition software.
8. Data analysis software.

3. Methods

3.1. Care of Animals

1. House animals in temperature-controlled rooms with light-tight doors.
2. Place the light–dark cycle of the room under control of a timer and choose the cycle appropriately.
3. Change bedding and/or cage without interfering with the light conditions (i.e., intensity and/or wavelength). Experimental evidence indicates that even a brief exposure to a different light condition can affect the circadian clock ([2](#)) and therefore the rhythms of locomotor activity (see **Note 1**).

3.2. Locomotor Activity

3.2.1. Running Wheel

This method takes advantage of the propensity of nocturnal rodents to actively seek out wheels and turn them for many hours each day. In nocturnal rodents this rhythm persists under constant conditions for months or even years. The number of turns of a running wheel are recorded using a magnetic switch (see **Note 2**).

1. Mount the switch on the support frame of the wheel.
2. Attach the magnet to the wheel itself. As the wheel turns, the switch counts the number of times it is passed by the magnet.
3. Connect the system to a computer. The counts for a defined period of time (usually 5 or 10 min) are transmitted from the switch to the computer, where they will be stored for later analysis.

3.2.2. Telemetry

The motor activity measured by telemetry provides a basic index of movement for the subject implanted with the transmitter. As the animal moves, movements of the implanted transmitter result in changes in the transmitted signals. These small changes are detected by the receiver via telemetry and noted as activity counts. Such information is then transmitted to a computer to be stored and then analyzed (*see* **Notes 3** and **4**).

1. Choose a transmitter (from 1.2 to 4.5 g) appropriate for the size of the experimental animal.
2. Implant the transmitter in the abdominal cavity of the animal.
3. Position a receiver within the transmitter range and connect to a computer.

3.2.3. Infrared Sensors

The principle of this method is that when an animal crosses an infrared beam between an emitter and a receiver, such an event is counted and then transmitted to a computer, where it is stored for later use (*see* **Note 5**).

1. Arrange a series of infrared sensors at a fixed distance within the animal cage, covering every possible path.
2. Connect the sensors to a computer.

3.2.4. Video Tracking

This system can track the distance traveled by each of several monitored animals within a fixed period of time (usually 1–5 min).

1. Select an appropriate infrared-sensitive video camera and connect to a recording device.
2. Position the camera in order to have a full view of the experimental field.

3.3. Data Analysis

1. Plot the activity data as a vertical stack of 24-h traces. This is called an actogram or actograph (*see* **Fig. 1**). Alternately, produce double- or triple-plotted actographs (**Fig. 1**) by stacking the data in overlapping 48- or 72-h traces.
2. Analyze the actogram by visual inspection in order to estimate the following parameters:
 - *Amplitude*: The difference between peak and trough of activity.
 - *Circadian time* (CT): Subjective time of the organism in which one circadian period is divided into 24 equal parts (circadian hour). By convention CT 0 corresponds to subjective dawn and CT 12 corresponds to subjective dusk.
 - *Activity onset and offset*: The times of day when the animal starts and ends its activity, respectively (**Fig. 2**; *see* **Note 6**). Calculation of these parameters can be easily obtained by eye-fitting connecting with a line the start or the end

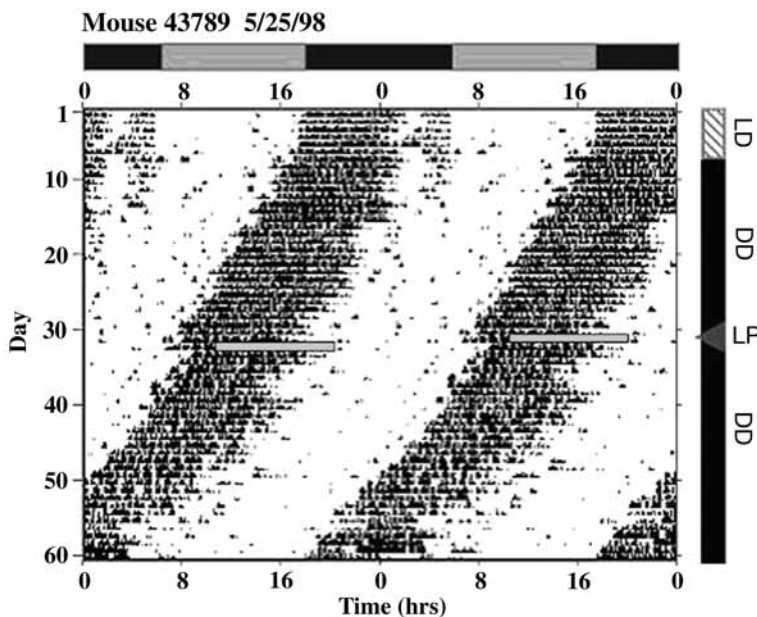


Fig. 1. Double plotted actogram of locomotor activity over a 60-d period. Changes in the light–dark cycle are shown on the right. Note the effect of a light pulse (LP) on the circadian rhythms (courtesy of Actimetrics and Dr. J. S. Takahashi, Northwestern University).

of locomotor activity (represented by the dots on the left and right side of Fig. 2, respectively) for each subsequent day.

- *Alpha* (α): The portion of the daily rest-activity cycle corresponding to the activity period (Fig. 2). Alpha can be easily calculated by measuring the interval (in units of time) between activity onset and offset.
- *Rho* (ρ): The portion of the daily rest-activity cycle corresponding to the rest period (Fig. 2). Rho corresponds to the interval (in units of time) between activity offset and onset.
- *Free-running period (FRP) or tau* (τ): The periodicity of the rhythm in the absence of any external cue. It can be estimated by calculating the linear regression of the activity onset.
- *Total activity*: the total number of running-wheel turns (or movements) recorded during 24 h.
- *Phase shift*: The shift in locomotor activity with respect to a reference (typically the pattern of activity under light–dark conditions) after a disturbance of the circadian clock (Fig. 3). It is determined by measuring magnitude and direction (i.e., advance or delay) of the difference in the onset of activity before and after the phase shifting stimulus was presented (see Note 7).

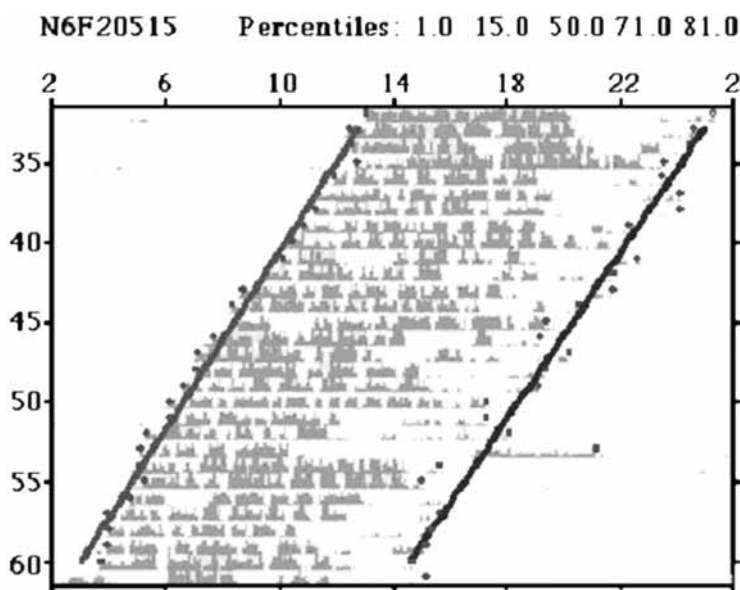


Fig. 2. Calculation of α and ρ using the ClockLab data analysis kit. The dots on the left and right sides indicate the onsets and offset of the daily activity, respectively. The interval between the left and right lines represents α , whereas ρ is represented by the opposite. Usually, as shown by this example, the activity onset show a more consistent pattern than the activity offset (courtesy of Actimetrics and Dr. J. S. Takahashi, Northwestern University).

- *Transients*: the variability in the onset of locomotor activity observed in the 2 to 3 d that follow a phase shift (see **Note 7**).
- *Splitting*: this term is used to describe when the FRP separates into two different components.

Although the visual inspection of actograms can provide useful information about the periodicity of the rhythm, it cannot provide an accurate estimate of the FRP. The most commonly used statistical method to estimate the FRP is the periodogram analysis developed by Enright (4) and then refined by Sokolove and Bushnell (5). This method utilizes the chi-square (χ^2) distribution to determine the statistical significance of the calculated FRP. The χ^2 periodogram analysis presents the best combination of accuracy, tolerance to waveform irregularity, and tolerance to noise. However, it is important to note that the χ^2 periodogram analysis cannot be applied to time-series data that have been collected at irregular intervals or with many missing points. Other methods currently used to calculate the FRP of locomotor activity include Fourier analysis (6), autocorrelation (7), and linear regression of the onset (8).

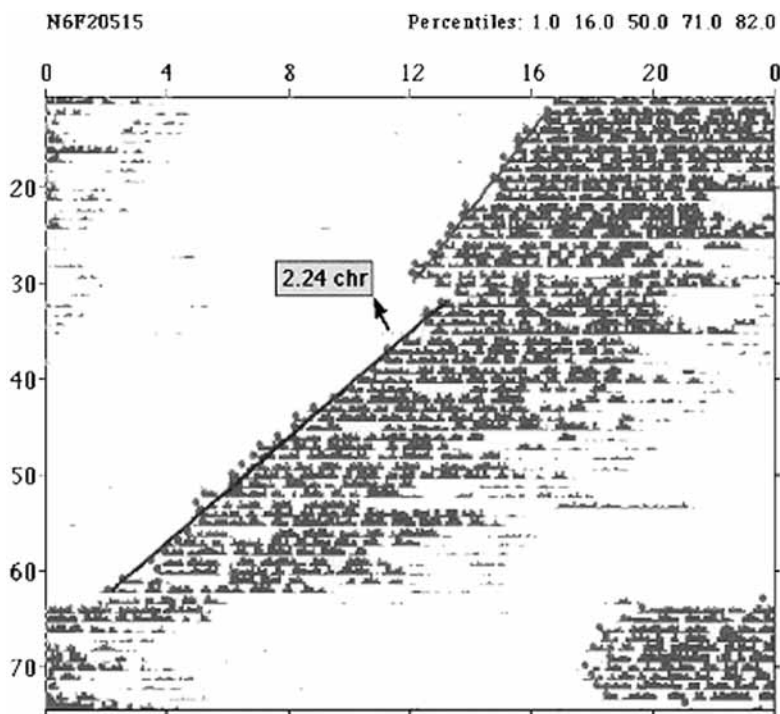


Fig. 3. Phase shift of locomotor activity calculated with ClockLab data analysis kit. Note the instability of the activity onset immediately after the phase shift (courtesy of Actimetrics and Dr. J. S. Takahashi, Northwestern University).

Several companies have now developed software packages that allow automated analysis of circadian parameters. The following list provides a brief description (and the company website, where more detailed information is available) of the most commonly used programs:

- *Actiwatch*. This software has been developed by Minimitter (www.minimitter.com) and allows the analysis of most circadian parameters.
- *ClockLab*. This program has been developed by Actimetrics (www.actimetrics.com) and allows the calculation of all circadian parameters previously mentioned.
- *The Chronobiology Kit*. This software (www.query.com) includes the capability to produce actogram, periodogram, and rhythm profiles.

4. Notes

1. Because working in complete darkness is very difficult, many laboratories are now using red dim light (<1 lux) instead of complete darkness.

2. The monitoring of locomotor activity by running wheel is relatively inexpensive, as running wheels can be easily built in the laboratory. It is also very reliable and convenient, as it can be used for several different species (i.e., rat, mouse, and hamster).
3. Because the transmitter needs a battery, the major limitation of this method is that the transmitter must be replaced every 2 to 3 mo, making it unsuitable for long-term studies. However, it must be mentioned that a new technology (transponder) that does not require batteries has been recently developed.
4. The major advantage of telemetry is that the same transmitter can monitor other parameters, such as body temperature and heart rate, in addition to locomotor activity. However, telemetry monitoring can be expensive and the need for surgery to implant the transmitter can be seen as a further complication.
5. As for the running-wheel method, the infrared sensor system is relatively inexpensive and can be easily assembled in the laboratory.
6. The FRP varies slightly among individuals and different animals may be at different circadian times at the same local time on the same day. Therefore, establishing the activity onset is very important to calculate the subsequent CT when the experiment requires the sampling of fluids or tissues of free-running animals at different CTs.
7. Because the onset of activity may show some variability for 2 to 3 d immediately after the phase shift, it is preferable to measure the phase after this interval and when the FRP has reached the steady state (see [Fig. 3](#)).

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Analysis of Circadian Leaf Movement Rhythms in *Arabidopsis thaliana*

Kieron D. Edwards and Andrew J. Millar

Summary

Arabidopsis thaliana is the model organism for the study of the higher plant circadian clock. The physiological change in position of young leaves and cotyledons in *Arabidopsis* seedlings reveals an overt circadian rhythm. Measuring these leaf movements provides a simple and reliable assay of the plant circadian clock and, unlike systems based on the firefly luciferase reporter gene, requires no prior genetic manipulation of the plant. As such, leaf movement can be used to measure circadian rhythms in plants lacking luciferase reporter genes, or as an independent measure of the clock in plants that do possess the transgene. The imaging system described in this chapter can also be adapted to measure circadian rhythms in other plant species displaying rhythmic leaf movements.

Key Words: *Arabidopsis thaliana*; biological clock; leaf movement; cotyledon movement; physiological rhythm; plant.

1. Introduction

Arabidopsis thaliana has become established as the model system for higher plant genetics (1,2) and subsequently as the model for plant circadian biology (3). Estimates suggest that between 6 and 16% of the *Arabidopsis* genome is regulated by the circadian clock (4,5) and the expression of such genes is often used to assay the clock. This can be achieved using traditional molecular approaches, such as Northern blotting, but more recently use of the firefly luciferase (*Luc*) reporter gene has come to the fore (6). The coupling of *Luc* to clock-controlled promoters provides a reliable, real-time measure of the plant circadian system, and is described elsewhere in this book (see Chapter 10). However, this method requires plants to be transformed with *Luc* constructs prior to assaying, which can be a time-consuming process.

An alternative to measuring the expression of clock-controlled genes lies in measuring rhythmic changes in plant physiology. Leaf position (7), hypocotyl elongation (8), elongation of the inflorescence stem (9), and stomatal opening (10) all display circadian rhythms and have been measured as markers of the clock in *Arabidopsis*. Such overt rhythms allow direct measurement of the circadian clock without the need for prior genetic manipulation of the plants. Indeed, leaf movement in the plant *Mimosa pudica* provided the first recorded example of a circadian rhythm (11).

The leaves of *Arabidopsis* seedlings are supported on organs called *petioles*. Antiphased oscillations in elongation of the abaxial and adaxial cells of these petioles causes the position of young leaves to oscillate (12) so that they are lowered during the day and raised during the night. The physiological importance of this rhythm is unproven, but higher leaf angles may enhance light harvesting in the diffuse light at the beginning and end of the day. This change in leaf position is demonstrated in Fig. 1, which shows two images of a seedling under constant conditions, one taken during the subjective day and one during the subjective night. The clear difference in position of leaves between the images serves to demonstrate the principle behind assaying leaf movement rhythms, which is based on collecting a series of images of a seedling under constant conditions and measuring the vertical position of its primary leaves in each image. By plotting the position of a leaf against time the rhythm is revealed, as shown in Fig. 2.

Manual collection and measuring of a series of images would prove a very time-consuming process (13); thus, automated systems were developed. The first of these for *Arabidopsis* was reported by Engelmann et al. (7). This system used a set of charge-coupled device (CCD) video cameras to capture images of seedlings at regular intervals and image analysis software to measure the vertical position of the seedlings' primary leaves in each image. Several other systems for measuring leaf or cotyledon movements have since been developed based on these same principles (8,14,15), with multiple cameras taking images of multiple *Arabidopsis* seedlings, allowing a relatively high throughput.

2. Materials

2.1. Leaf Movement System

1. Desktop PC (see Note 1).
2. MetaMorph software (Molecular Devices Corp., Downingtown, PA).
3. 16 Monochromatic KC6400E CCD video cameras (Ultrak, Carrollton, TX; see Note 2).
4. VXS-416K 16-channel camera switcher (Videoswitch, Church Crookham, Hants, UK; see Note 3).

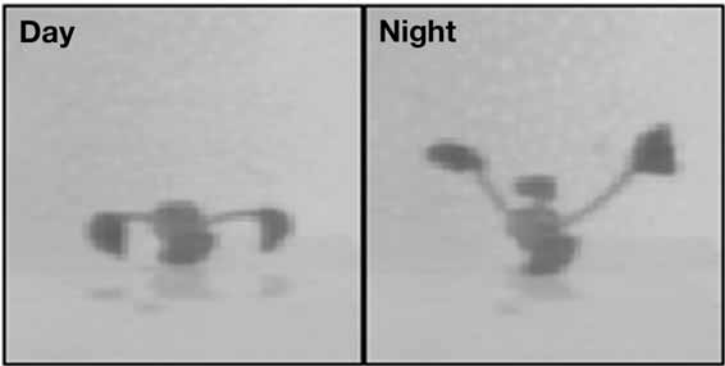


Fig. 1. Circadian leaf movement in an *Arabidopsis thaliana* seedling. The 14-d-old seedling was entrained under light–dark cycles and then placed into constant white light. The “day” image was taken during the subjective day (approx circadian time [CT] 6) and the “night” image during the subjective night (approx CT 18).

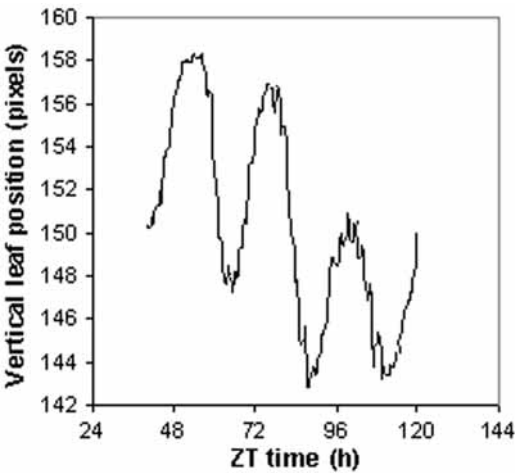


Fig. 2. Vertical position of an *Arabidopsis* seedling’s primary leaf plotted against time. The seedling was entrained with light–dark cycles and then released into constant light and temperature conditions at dawn. The vertical position of a primary leaf was measured at 20-min intervals over the course of several days, revealing a circadian rhythm.

5. Parallel port controller unit (Roper Scientific, Marlow, Bucks, UK; *see Note 4*).
6. Flashbus MV card (Integral Technologies Inc., Indianapolis, IN).
7. MLR-350 Versatile Environment test chamber (Sanyo Gallenkamp PLC, Loughborough, Leicester, UK; *see Note 5*).

2.2. Growth and Preparation of Plants

1. 70% Ethanol: 70% (v/v) ethanol.
2. Bleach: 50% (v/v) bleach, 0.01% (v/v) Tween-20.
3. Top agar: 0.01% agar.
4. MS 3% sucrose: Murashige & Skoog Basal salt mixture (MS), 3% (w/v) sucrose, 2% (w/v) agar.
5. 25-Well tissue culture plates (Bibby Sterilin Ltd, Stone, Staffs, UK).
6. Parafilm (Pechiney Plastic Packaging, Menasha, WI).

3. Methods

3.1. Leaf Movement System

As indicated in the introduction, the principle of assaying leaf movement rhythms lies in collecting a series of images of seedlings at regular intervals. We achieved this by connecting standard CCD video camera equipment to a computer, and controlling the hardware with the image analysis software package MetaMorph. **Figure 3** summarizes the “KujaMorph” leaf movement system that we developed.

In outline, 16 monochromatic CCD video cameras were connected to a 16-channel camera switcher. Output from the camera switcher was connected to a computer via a flashbus, allowing the computer to acquire live images from the cameras. A parallel port controller unit was custom-built, to enable MetaMorph to select the active channel from the 16-channel camera switcher unit via its alarm circuitry. MetaMorph was configured to capture and save images from each of the cameras at 20-min intervals over the course of a week, saving approx 3 GB of image data per experiment (*see Note 6*).

We placed the cameras in a growth chamber to allow careful control of the light and temperature environment. We have assayed seedlings at a range of temperatures between 12 and 30°C, and use 22°C under approx 25 $\mu\text{mol}/\text{m}^2/\text{s}$ cool white light as a standard (*see Note 7*). It is also important that the cameras are faced toward a light background (*see Note 8*).

3.2. Growth and Preparation of Plants

The stages below deal specifically with preparing *A. thaliana* seedlings for leaf movement. Other species of plant show rhythms in leaf movement, which can be assayed using the system outlined above, but these may require different preparation procedures and growth conditions (*see Note 9*).

1. Surface-sterilize *Arabidopsis* seed by soaking in 70% ethanol for 1 to 2 min, followed by soaking in 50% bleach for 8 min.
2. Remove the bleach and rinse two to three times in sterile distilled water, before placing the seed in top agar.

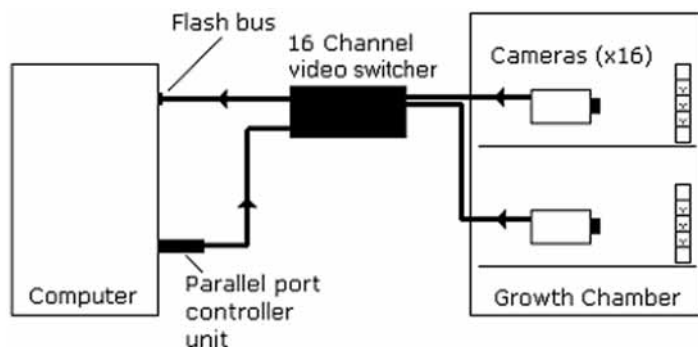


Fig. 3. Figure summarizing the components of the KujaMorph leaf movement system. Arrows represent the direction of information flow. Square 25-well tissue culture dishes containing 12 10-d-old *Arabidopsis* seedlings are placed vertically within growth chambers. Sixteen charge-coupled device cameras controlled by a 16-channel switcher are set up facing the plates inside the chambers. Images are transferred from the video switch to the computer via a flash bus. A parallel port controller unit enables MetaMorph to select the channel on the video switcher and capture and store images from every camera at 20-min intervals.

3. Store the seed in top agar at 4°C in a refrigerator for 4 d to stratify (see **Note 10**).
4. Following stratification, plate the seed onto MS 3% sucrose plates and grow at 22°C under constant white light for 6 d (see **Note 11**).
5. After 6 d of growth, transfer the seedlings to 12 h:12 h light–dark (LD) cycles for 3 d (see **Notes 12** and **13**).
6. Using aseptic technique, transfer the seedlings into 25-well tissue culture plates as outlined here (see **Note 14**):
 - a. Using a sterile scalpel or forceps, manipulate the seedlings so that they stand up straight in the media. This can best be achieved by gently pressing the hypocotyl–root junction into the medium.
 - b. Cut blocks of approx 1 to 2 cm² from the media surrounding individual seedlings and transfer them into vertically placed 25-well tissue culture plates (see **Fig. 4** and **Note 15**).
 - c. Place 12 seedlings into each plate, leaving the bottom and top rows and the left-hand column empty (see **Fig. 4** and **Note 16**).
 - d. Once all 12 seedlings are placed in the plate, add a drop (50–100 mL) of sterile distilled water to each of the four corner wells.
 - e. Place the lid on the plate and secure it with Parafilm to prevent infection and loss of moisture.
7. Once all plates are set up, they can be placed in front of the cameras.

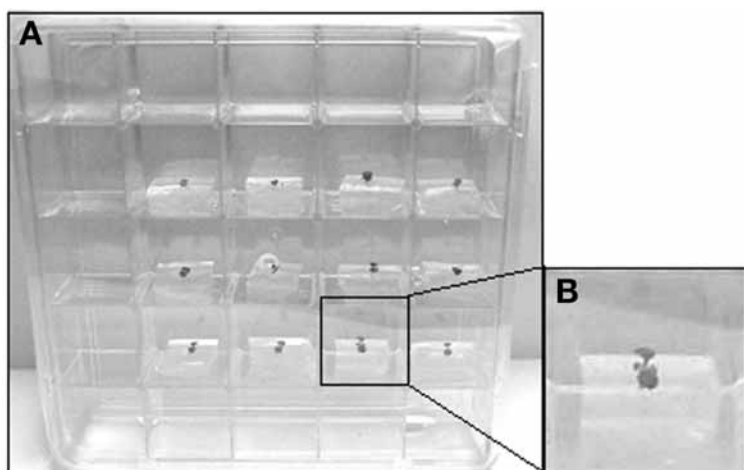


Fig. 4. *Arabidopsis* seedlings prepared for leaf movement imaging. (A) 25-Well tissue culture plate placed vertically, containing 12 *Arabidopsis* seedlings. (B) Exploded view of an individual seedling within the plate. Note that the cotyledons face toward the front and back of the plate, whereas the emerging primary leaves face toward the sides.

3.3. Data Acquisition

Each camera should be focused directly onto the 12 seedlings, and the lens aperture should be adjusted to give the image with the highest contrast possible (see **Notes 17** and **18**).

If phase information is required, data collection must be initiated immediately upon transfer of the seedlings to constant light. If only period information is required, the first 24 h of data are excluded from analysis to avoid any transient cycles following the LD entrainment; data collection can then be initiated at any point in the first 24 h.

At each timepoint, MetaMorph journals direct the video switcher to:

- Move to the next channel (i.e., camera).
- Wait for several seconds to allow synchronization of the flashbulbs to the video signal (see **Note 19**).
- Acquire five frames.
- Store the average of all frames as an 8-bit grayscale image (see **Note 20**).

3.4. Data Analysis

3.4.1. Measuring Leaf Position by Image Analysis

As with the collection of data, the software package MetaMorph is used to analyze the image data. Leaves can be identified in MetaMorph by applying a

threshold to the images. The threshold effectively reduces the image from an 8-bit grayscale image to a 1-bit image. Each pixel that was darker than the threshold level in the 8-bit image is set to black; all other pixels are white. As the seedlings are relatively darker than the light background that they are imaged in front of, it is possible to adjust the threshold to pick out only the seedlings. The user can then define regions of the image (“regions of interest”) where leaves are moving and configure MetaMorph to measure the position of thresholded leaf signals within each region. The journals required to carry this out are available at www.amillar.org.

This process is outlined below and should be carried out for one camera at a time.

1. Build a stack of the images from a camera (*see Note 21*).
2. Apply a threshold to dark objects so that the seedlings are picked out against their light background.
3. Set regions around each of the primary leaves of each seedling (*see Note 22*). These should be set to encompass the entire range of movement for at least three circadian cycles of a leaf. Do not set overlapping regions and, where possible, avoid other leaves or cotyledons entering a region, as this may cause problems later on in analysis (*see Note 23*).
4. Configure MetaMorph to log the Image Name, Image Plane, Image Date and Time, Centroid X, and Centroid Y for each region and then measure and log data for each image throughout the stack (*see Note 24*).
5. This will produce a log file of raw data in spreadsheet format for further analysis.

Figure 5 shows a sample of a log file, with all the data collected. The most important data for circadian rhythm analysis are the image acquisition time (referred to as “Date” in **Fig. 5**), region number, and the leaf centroid positions.

3.4.2. Analysis of Leaf Movement Rhythms

Data logged and saved in the format described above are suitable for period analysis by several methods, including the Biological Rhythm Analysis Software System (BRASS) package, which is available from www.amillar.org. BRASS is a user-friendly interface in MS Excel, for fast-Fourier transform–nonlinear least squares analysis of the period, phase, and amplitude of circadian rhythms (**16**). BRASS imports log files of leaf movement data, reformats them, exports them to fast-Fourier transform–nonlinear least squares for analysis, and produces various summaries of the results, in numeric and graphic format.

It is characteristic of *Arabidopsis* leaves and cotyledons that they move rhythmically only during active growth of the petiole. Elongation of each petiole ceases after about 5 d, before and after which the data are unreliable for circadian rhythm analysis. In order to avoid such data, BRASS allows one to analyze a time window of the data from each trace. The window is automati-

	A	B	C	D	E	F	G
1	CAM01-	1	13:43.6	10	154	331.964	
2	CAM01-	1	13:43.6	11	299.429	335.286	
3	CAM01-	1	13:43.6	18	153.429	476.286	
4	CAM01-	1	13:43.6	24	632.667	483.333	
5	CAM01-	1	13:43.6	21	483.629	496.886	
6	CAM01-	2	33:43.4	10	154	331.964	
7	CAM01-	2	33:43.4	9	145.333	332	
8	CAM01-	2	33:43.4	11	299.429	335.286	
9	CAM01-	2	33:43.4	18	153.5	476	
10	CAM01-	2	33:43.4	24	632.25	483.25	

Fig. 5. Sample of data logged from a leaf movement experiment. The data are logged into an MS Excel spreadsheet, with columns A through G representing image name, image plane, image date and time, region number, Centroid X, and Centroid Y, respectively. Logs in this format can be imported into Biological Rhythm Analysis Software System for further circadian analysis.

cally moved along the trace in order to identify the most reliable data. Clearly, potential changes in the rhythm over time are a concern. Many other types of analysis are possible.

4. Notes

1. The PC should meet the minimum system requirements of MetaMorph and have at least two spare PCI expansion slots. Also, it is best to have a reasonably sized hard disk (more than 40 GB) to allow storage space for more than one experiment at any one time.
2. We used Ultrak KC6400E cameras, but any standard CCD cameras should suffice. It may be preferable to select cameras that can be synchronized with one another to improve the stability of the system.
3. As with cameras, any standard video channel switcher unit should suffice. The switcher should have an alarm circuit that can be addressed using a parallel port,

to follow our design exactly. Switchers that change video channel using a serial signal can also be used, but would require further programming of the serial codes into MetaMorph.

4. This parallel port controller unit was custom built for us by Roper Scientific, which has since become part of the UK representative of Universal Imaging Corp. (West Chester, PA). All the hardware (aside from the cameras) can be supplied by UIC or its local representative. It may be preferable to purchase a system in this way to remove compatibility issues.
5. We required careful control of the light and temperature environments for our experiments and so elected to house the cameras in a growth chamber.
6. MetaMorph journals written to carry out the collection are available at www.amillar.org. Please note that the multijournal time-lapse function of MetaMorph is required for them to work.
7. Light intensity and temperature affects the seedlings' morphology, which can alter the ease of assaying leaf movement rhythms. We empirically identified 25 $\mu\text{mol}/\text{m}^2/\text{s}$ cool white light at 22°C as standard conditions, although we have had success measuring leaf movement rhythms at different temperatures and light intensities.
8. The importance of the light background will be made clear in **Subheading 3.3.1**.
9. We have used KujaMorph to measure leaf movement rhythms in *Brassica oleracea* seedlings.
10. Stratification induces and synchronizes the germination of seedlings.
11. The seeds should be plated in a grid pattern, at least 1 cm away from one another for ease of manipulation at later stages. Similarly, it is best to pour thick plates and use medium containing 2% agar.
12. The seedlings should be entrained to synchronize their rhythms prior to experiments by growth in LD cycles. They can be grown under entrainment conditions for the entire time. We grow them only under constant light initially to save on incubator space.
13. We tend to transfer the seedlings to the multiwell plates the day before we wish to start the experiment and return them for a final night of LD entrainment. They can then be transferred to the experimental conditions at dawn on the day of the experiment to minimize any perturbation of the circadian system.
14. We randomize the position of different genotypes between plates to reduce experimental error caused by positional effects.
15. At this age you should be able to see the primary leaves, which should be placed into the tissue culture wells facing sideways. One cotyledon should face toward you and the other away from you (as in [Fig. 4B](#))
16. Should higher resolution cameras be used, it may be possible to fill the entire plate.
17. Focusing at short working distances may require a spacer ring to increase the distance from the lens to the CCD. Sets of rings can be obtained from many video supply catalogs (e.g., Edmund Optics Ltd., York, UK).

18. Focus, aperture, and other camera features should be fixed. Mechanisms that automatically correct the focus and/or image brightness (including electronic and optical adjustments) are unlikely to be sufficiently stable to allow good image analysis over time courses.
19. If synchronization is poor, “flagging” may result, causing lateral displacement of parts of the image.
20. The averaging greatly reduces the noise that is common in low-cost video cameras. The later image processing steps can be sensitive to such noise.
21. Building a “stack” effectively turns the series of images into a movie.
22. Clearly, it is crucial to record which seedling is analyzed by each region. Labeling seedlings in a way that is visible in the images can be helpful, particularly when seedling positions are randomized.
23. Note that each distinct thresholded object within each region will be recorded individually, so it is possible to get more than one measurement per region. Later analysis of the data attempts to remove unwanted objects, but where possible, try to set regions to minimize crossover with other leaves.
24. Centroid X and Y refer to the x and y coordinates of the central point of a thresholded object within a region.

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Detection of Rhythmic Bioluminescence From Luciferase Reporters in Cyanobacteria

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Summary

The unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 is the model organism for studying prokaryotic circadian rhythms. Although *S. elongatus* does not display an easily measurable overt circadian behavior, its gene expression is under circadian control; hence, a “behavior” is created by linking a cyanobacterial promoter to either the bacterial *luxAB* or firefly *luc* luciferase genes to create reporter fusions whose activity can be easily monitored by bioluminescence. Numerous vectors have been created in our lab for introducing luciferase reporter genes into the *S. elongatus* chromosome. A choice of methods and equipment to detect light production from the luciferase fusions provides a means for high-throughput, automated mutant screens as well as testing rhythms from two promoter fusions within the same cell culture.

Key Words: *Synechococcus elongatus* PCC 7942; cyanobacteria; luciferase; bioluminescence; circadian rhythm; neutral site.

1. Introduction

Cyanobacteria are among the growing group of organisms that have been shown to exhibit 24-h rhythms under the control of a central biological clock. *Synechococcus elongatus* PCC 7942 is the model organism for studying prokaryotic circadian rhythms because of its small genome size (2.7 Mb), the ease with which it can be manipulated genetically by transformation (1) or conjugation (2) from *Escherichia coli*, and the availability of vectors for genetic and molecular manipulations (3). *S. elongatus* does not display an overt circadian rhythm of behavior that can be easily monitored; however, it exhibits circadian control of expression of its genome. The organism has been genetically engineered to produce light, using genes that encode a luciferase enzyme, as a readily measurable reporter of circadian gene expression. Luciferase is ideal

for this purpose because it does not affect cellular growth and its short half-life accurately reflects real-time expression from promoter fusions because its protein product does not accumulate and obscure troughs of expression. In addition, luciferase reporting provides an opportunity to measure the circadian rhythm of *S. elongatus* gene expression in a high-throughput and automated manner. We are able to utilize luciferases from both *Vibrio harveyi* and the firefly to study circadian rhythmicity through mutant hunts and specific gene inactivations (see Chapter 11). This chapter describes the luciferase vectors and monitoring devices that we use to identify and characterize elements of the cyanobacterial circadian system.

2. Materials

1. Neutral site vector that harbors a promoterless luciferase reporter gene.
2. Modified BG-11 (BG-11M) liquid medium (4): 1.5 g/L NaNO₃, 0.039 g/L K₂HPO₄, 0.075 g/L MgSO₄·7H₂O, 0.02 g/L Na₂CO₃, 0.027 g/L CaCl₂, 0.001 g/L EDTA, 0.012 g/L FeNH₄ citrate, and 1 mL of the following microelement solution: 2.86 g/L H₃BO₃, 1.81 g/L MnCl₂·4H₂O, 0.222 g/L ZnSO₄·7H₂O, 0.391 g/L Na₂MoO₄, 0.079 g/L CuSO₄·5H₂O, and 0.0494 g/L Co(NO₃)₂·6H₂O.
3. BG-11M solid medium (5): equal volumes of twice-concentrated (2X) BG-11M liquid medium and Difco agar solution (3% in sterile water), mixed together with filter-sterilized Na₂SO₃ added last (final concentration of 1 mM).
4. 10 mM NaCl.
5. Antibiotics (spectinomycin, streptomycin, kanamycin, gentamycin, and/or chloramphenicol).
6. Laminar flow hood with ultraviolet (UV) light.
7. Packard TopCount Microplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences, Boston, MA).
8. Black 96-well microtiter plates and clear plastic lids (ThermoLabsystems, Franklin, MA).
9. Clear 96-well plates (ThermoLabsystems).
10. Packard Topseal (Perkin Elmer Life Sciences).
11. D-Luciferin sodium salt monohydrate (Biosynth, Naperville, IL).
12. Decanal (decyl aldehyde; Sigma, St. Louis, MO).
13. Canola oil.
14. Fluorescent light bulbs (25 W), compact fluorescent light bulbs (40 W), and corresponding fixtures.
15. Cooled charge-coupled device (CCD) camera equipped with a liquid nitrogen/CCD Detector (Roper Scientific, Trenton, NJ) and liquid nitrogen cooling system, Liquid Level Instrument (American Magnetics, Oak Ridge, TN).
16. Programmable timer and controller to regulate lighting for the CCD camera system (VWR, West Chester, PA)
17. Liquid scintillation counter.
18. 6-mL Scintillation vials.

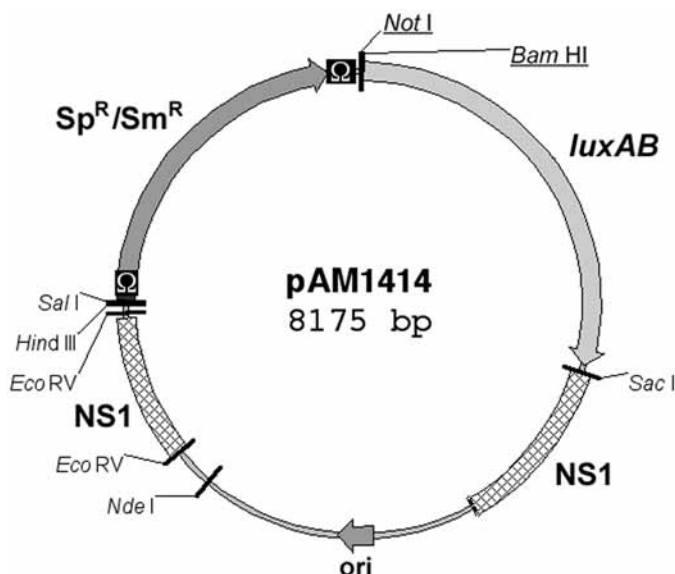


Fig. 1. Schematic drawing of promoterless *luxAB* NS1 vector, pAM1414. This vector contains restriction sites (*NotI*, *BamHI*) for cloning a promoter of interest upstream of *V. harveyi luxAB* genes. The *luxAB* genes and selectable Sp^R/Sm^R Ω cassette are bordered by *Synechococcus elongatus* NS1 sequence to allow for homologous recombination, which transfers the reporter cassette into neutral site I of the *S. elongatus* chromosome.

3. Methods

3.1. Description of *luxAB*, *luxCDE*, and *luc* Vectors

We have constructed a number of vectors that contain the promoterless *V. harveyi luxAB* genes, which encode the luciferase enzyme, and an antibiotic-resistance marker, flanked by the sequences from one of two identified “neutral sites” of the cyanobacterial genome (Fig. 1). These neutral sites are regions of the *S. elongatus* chromosome that can be disrupted without any discernable phenotype (neutral site I, NS1, GenBank accession number U30252; neutral site II, NS2, GenBank accession number U44761). Vectors for NS2 target two adjacent regions of the chromosome (NS2.1 and NS2.2) based on the location of the antibiotic resistance cassette and cloning site for insertions within neutral site DNA on the plasmid. Because these vectors can replicate in *E. coli* but not in *S. elongatus*, a homologous recombination event (an apparent double cross-over in which the *trans*-gene and selective marker are inserted into the neutral site and the vector sequence is lost) must occur between the neutral site vector

and the chromosome in order for genes to be expressed in the cyanobacterium (5,6). A number of unique and compatible restriction sites are available in each vector for ease of cloning *luxAB* transcriptional promoter fusions or translational fusions by standard recombinant DNA methods (7). Upstream of the multiple cloning site is a transcriptional terminator to eliminate expression of *luxAB* from transcriptional elements other than the promoter being tested.

The product of a *luxAB* transcriptional or translational fusion is bioluminescent in the presence of its long-chain aldehyde substrate (see **Note 1**). Because it is difficult in some monitoring environments to add exogenous substrate to cell cultures (see **Subheading 3.4.**), other neutral site vectors have been designed to express the *luxCDE* genes from *Photorhabdus luminescens*, which encode the enzymes required for the synthesis of the long-chain aldehyde. We typically use the strong promoter of the *psbAI* gene (*PpsbAI*) to maintain a high level of expression from the *luxCDE* genes. Strains that express both *luxAB* and *luxCDE* are autonomously bioluminescent. The rhythm of light production reflects the transcriptional patterns of the promoter driving *luxAB*, even when levels of *luxAB* peak 12 h out of phase with the peak level of *luxCDE* expression, which suggests that the necessary reaction substrates for the luciferase reaction (FMNH₂, O₂, and long-chain aldehyde) are present in saturating levels throughout the circadian cycle (8). Features of the available neutral site vectors and those vectors that harbor *luxAB* and *luxCDE* are described in **Table 1**.

In most of the autonomously bioluminescent strains, both NS1 and NS2 are occupied by the *luxAB* and *luxCDE* constructs, respectively, making it difficult to introduce other *trans*-genes into the chromosome. We have developed two methods that leave one neutral site unoccupied for complementing a mutation or overexpressing a gene. In pAM2195, the *PpsbAI::luxAB* and *PpsbAI::luxCDE* constructs are both cloned into NS2.1, leaving NS1 (and NS2.2) available for genetic manipulation. We also developed the use of an engineered allele of firefly luciferase, *luc*. Here, as in the *luxAB* system, a promoter of interest can be cloned upstream of the promoterless *luc* gene (Promega, Madison, WI; **Table 1**), subcloned to a neutral-site vector, and crossed to the chromosome of the cyanobacterium. The resulting strain will produce light in the presence of exogenous 5 mM D-luciferin substrate, and has one neutral site available for introduction of other genes.

3.2. Transformation of *S. elongatus* PCC 7942

The following section describes the conditions typically used to transform *S. elongatus* with the bioluminescence vectors and the propagation of positive clones (adapted from **ref. 5**).

Table 1
Plasmids Engineered for Expression of Bioluminescent Reporters
in *S. elongatus* PCC 7942

Plasmid ^a	Promoter	<i>lux/luc</i> genes	Marker ^b	NS ^c	Description
pAM1303	None	None	Sp ^r /Sm ^r	1	Cloning sites <i>NotI</i> , <i>Bam</i> HI, and <i>Sma</i> I
pAM1411	None	None	Cm ^r (Ap ^r) ^d	2	Cloning site <i>Sma</i> I
pAM1414	None	<i>luxAB</i>	Sp ^r /Sm ^r	1	Derivative of pAM1303; cloning sites <i>NotI</i> and <i>Bam</i> HI
pAM1504	None	<i>luxCDE</i>	Sp ^r /Sm ^r	1	Derivative of pAM1303; cloning sites <i>NotI</i> and <i>Bam</i> HI
pAM1518	<i>PpsbAI</i>	<i>luxCDE</i>	Sp ^r /Sm ^r	1	Derivative of pAM1504; synthesizes luciferase substrate in <i>S. elongatus</i>
pAM1573	None	None	Cm ^r (Ap ^r)	2	Cloning sites <i>Nhe</i> I, <i>Xho</i> I, <i>Sma</i> I, <i>Xba</i> I, <i>Stu</i> I, <i>Sal</i> I, and <i>Eco</i> RV
pAM1579	None	None	Km ^r (Ap ^r)	2	Cloning sites <i>Nhe</i> I, <i>Xba</i> I, <i>Stu</i> I, <i>Sal</i> I, and <i>Eco</i> RV
pAM1580	None	<i>luxAB</i>	Cm ^r (Ap ^r)	2	Derivative of pAM1573; cloning sites <i>Nhe</i> I, <i>Xho</i> I, <i>Sma</i> I, <i>Xba</i> I, <i>Stu</i> I, and <i>Sal</i> I
pAM1607	None	<i>luxCDE</i>	Km ^r (Ap ^r)	2	Derivative of pAM1579; cloning sites <i>Nhe</i> I, <i>Stu</i> I, and <i>Sal</i> I
pAM1619	<i>PpsbAI</i>	<i>luxCDE</i>	Km ^r (Ap ^r)	2	Derivative of pAM1607; synthesizes luciferase substrate in <i>S. elongatus</i>
pAM1667	None	<i>luxCDE</i>	Cm ^r (Ap ^r)	2.1	Derivative of pAM1573; cloning sites <i>Nhe</i> I, <i>Xho</i> I, <i>Sma</i> I, <i>Stu</i> I, and <i>Sal</i> I
pAM1706	<i>PpsbAI</i>	<i>luxCDE</i>	Cm ^r (Ap ^r)	2.1	Derivative of pAM1667; synthesizes luciferase substrate in <i>S. elongatus</i>
pAM1850	<i>PpsbAI</i>	<i>luxCDE</i>	Cm ^r (Ap ^r)	2.2	Derivative of pAM1411; synthesizes luciferase substrate in <i>S. elongatus</i>
pAM2314	None	None	Sp ^r /Sm ^r	1	Derivative of pAM1303; cloning sites <i>NotI</i> , <i>Sac</i> II, <i>Bgl</i> II, <i>Sna</i> BI, <i>Bsi</i> WI, <i>Mlu</i> I, <i>Eco</i> RI, <i>Xho</i> I, <i>Stu</i> I, <i>Spe</i> I, and <i>Bam</i> HI
pAM2195	<i>PpsbAI</i> <i>PpsbAI</i>	<i>luxAB</i> <i>luxCDE</i>	Cm ^r (Ap ^r)	2.1	Derivative of pAM1580; synthesizes luciferase and substrate in <i>S. elongatus</i>
pSP- <i>luc</i> +NF	None	<i>luc</i>	(Ap ^r)	None	pSP- <i>luc</i> +NF Fusion Vector from Promega; cloning sites <i>Kpn</i> I, <i>Nhe</i> I, <i>Bgl</i> II, <i>Avr</i> II, <i>Hind</i> III, <i>Nco</i> I, and <i>Bst</i> EII

^aAll neutral site vectors are based on a pBR322 replication origin.

^bAntibiotic markers are used for the selection of cyanobacterial transformants after homologous recombination event at either NS1 or NS2.

^cNeutral site.

^dMarkers shown in parentheses are additional markers used for selection of plasmids in *E. coli*.

Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin; Sm, streptomycin.

pAM1303 and its derivatives contain an Ω cassette that is Sp^r/Sm^r and carries transcription and translation terminators at either end (16).

3.2.1. *S. elongatus* Transformation

1. Grow 100 mL of *S. elongatus* PCC 7942 at 30°C in BG-11M liquid medium (4), shaking at 250 rpm with constant light of about 300 $\mu\text{E}/\text{m}^2/\text{s}$ (see **Note 2**). Cells will reach an optical density at 750 nm (OD_{750}) of 0.7 in 4 to 7 d depending on the inoculum used (typically 5–10 mL of a fully grown culture into fresh medium). Cells used for transformation are typically used in log or early stationary phase.
2. Collect cells from 15 mL culture by centrifugation at 6000g for 10 min. Discard medium.
3. Resuspend collected cells in 10 mL of 10 mM NaCl. Repeat centrifugation at 6000g for 10 min.
4. Resuspend pellet in 0.3 mL of BG-11M liquid medium. We usually concentrate cells to between 5×10^8 to 1×10^9 cells/mL as the transformation efficiency (per input DNA) increases linearly with increasing cell concentration (5).
5. Add plasmid DNA (50 ng to 2 μg) to cells (see **Note 3**). Wrap tubes in aluminum foil to keep out light and incubate at 30°C for 15 to 20 h with gentle agitation.
6. Plate the entire 0.3-mL cell suspension on BG-11M agar that contains the appropriate selective antibiotic. The concentrations of antibiotics typically used are as follows: 2 $\mu\text{g}/\text{mL}$ gentamycin, 5 $\mu\text{g}/\text{mL}$ kanamycin, 2 $\mu\text{g}/\text{mL}$ spectinomycin + 2 $\mu\text{g}/\text{mL}$ streptomycin, and 7.5 $\mu\text{g}/\text{mL}$ chloramphenicol (see **Note 4**). After 7 to 10 d of incubation under standard light conditions, transformed colonies will appear.

3.2.2. Chromosome Segregation and Clonal Propagation

1. Pick single transformants using a sterile toothpick and patch onto a fresh BG-11M agar plate that contains the selective antibiotic to ensure that all chromosomes have incorporated the *trans*-gene (*S. elongatus* carries multiple copies of its chromosome). Many single colonies can be streaked onto one plate if patches are kept to areas less than 1 cm^2 .
2. After 5 to 7 d of growth, the cyanobacteria can be used to inoculate a BG-11M liquid culture that contains the selective antibiotic(s), or used directly for the measurement of bioluminescence by any of the methods described in **Subheadings 3.3.** through **3.5.**

3.3. TopCount Measurement

The Packard TopCount Microplate Scintillation and Luminescence Counter utilizes single photomultiplier tubes to measure the light produced from recombinant *luxAB* or *luc* reporter cyanobacteria in counts per second (cps). The advantage of using the TopCount to measure circadian bioluminescence is multifold: one can screen hundreds of strains at a time, and the automated counting protocol allows for rhythms to be monitored 24 h a day, for weeks at a time, with little attention from an otherwise sleep-deprived worker.

3.3.1. Plate Preparation

1. Sterilize a black 96-well microtiter plate (ThermoLabsystems; *see Subheading 3.3.3.*) and clear plastic lid with 70% ethanol (EtOH; *see Note 5*). Evaporate the EtOH in a laminar flow hood under UV light for at least 30 min before use.
2. Prepare 50 mL of BG-11M-2X agar. Place in 65°C water bath to bring to temperature and add 50 mL of BG-11M-2X salts that have been warmed to 65°C. Add antibiotics directly to melted mixture and mix well.
3. In a laminar flow hood, use a multichannel micropipet to add 300 μ L of agar medium to each well of a 96-well black plate. Cover with lid and let solidify at room temperature for at least 30 min.

3.3.2. Sample Preparation

1. Inoculate each well with test strains. If using liquid culture, add 20 to 40 μ L of cell suspension (recommended $OD_{750} = 0.7$) to desired wells. Alternatively, cultures growing on solid medium may be streaked onto the agar pad of each well using a sterile toothpick. If using *luc* strains, we have found that inoculation of TopCount sample plates with liquid cultures provides superior traces.
2. Lay a flat toothpick on either side of the black plate immediately outside the outer wells and place the clear lid on top of the toothpicks to prevent the lid from directly contacting the samples. This will prevent mixing of cell cultures from adjacent wells.
3. Incubate in constant light at 30°C overnight. To synchronize the cells' clocks, a 12-h dark treatment is typically used (*see Note 6*).
4. After dark treatment, in a laminar flow hood, replace the clear lid with a plastic Packard TopSeal (PerkinElmer Life Sciences), being careful not to have any tape hang over the edges of the plate (*see Note 7*). If using *luc* reporter strains, add 10 μ L of a 5 mM D-luciferin solution to each well before applying the TopSeal.
5. Using a 16-gage sterile needle, poke a hole in the plastic seal above each well that contains a sample to allow gas exchange throughout the TopCount run, being careful not to touch the samples with the needle.

3.3.3. TopCount Protocol and Interpretation of Results

S. elongatus is an obligate photoautotroph, so constant light conditions are used for circadian monitoring. A frame that surrounds the TopCount stackers was designed by D. Denke (*see Note 8*) to provide light to the samples. We use a light source consisting of eight 40 W compact fluorescent bulbs (four bulbs on each side) that create a light intensity of about 1300 μ E/m²s at the outer edge of the stacker and about 1000 μ E/m²s in the middle of the stacker, which maintains a light gradient within the wells of the sample plate that ranges from about 230 μ E/m²s in the outer wells that are closest to the light source to about

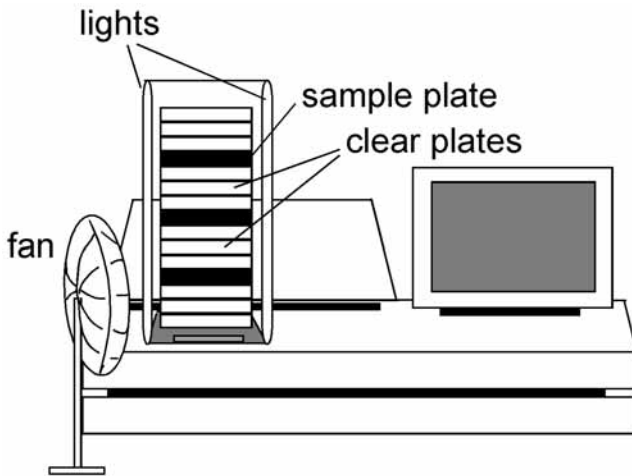


Fig. 2. Schematic drawing of the Packard TopCount Microplate Scintillation and Luminescence Counter. A set of four compact fluorescent light bulbs lines each side of the TopCount stackers. Stackers hold black sample plates separated by three clear plates to allow sufficient light penetration into sample wells. To reduce the heat supplied by the lights, a fan is placed in front of the stackers to maintain a temperature of 30°C. Each black sample plate is taken into the machine approximately every 2 h and bioluminescence is measured for each well. Data are stored on the TopCount computer until retrieved; rhythmic data can be interpreted using the Import and Analysis program.

50 $\mu\text{E}/\text{m}^2\text{s}$ in the inner wells (9). Because the high-intensity lamps cause an increase in temperature, we place a fan in front of the stackers, set at its lowest speed, to maintain a temperature of 30°C across the stackers. The temperature within the measuring chamber is controlled automatically.

Black sample plates are necessary so that luminescence from neighboring wells does not interfere with measurements. We place 6 to 8 sample plates in the TopCount stackers and separate each black sample plate with three clear 96-well plates to allow sufficient light to reach the cells (Fig. 2). Each plate is read every 1.5 to 2 h depending on the number of plates used. The plates enter the machine and are kept in darkness for 3 min at 30°C to allow fluorescence from the photosynthetic apparatus to dissipate before measuring bioluminescence. It takes approx 10 min for the TopCount to count and record bioluminescence from each 96-well plate, thus placing the cells in the dark for a total of 13 min every 2 h, if using eight sample plates. This brief introduction to darkness does not have an effect on the rhythms of gene expression (as far as synchronization, entrainment, or resetting of the cultures) as displayed by the

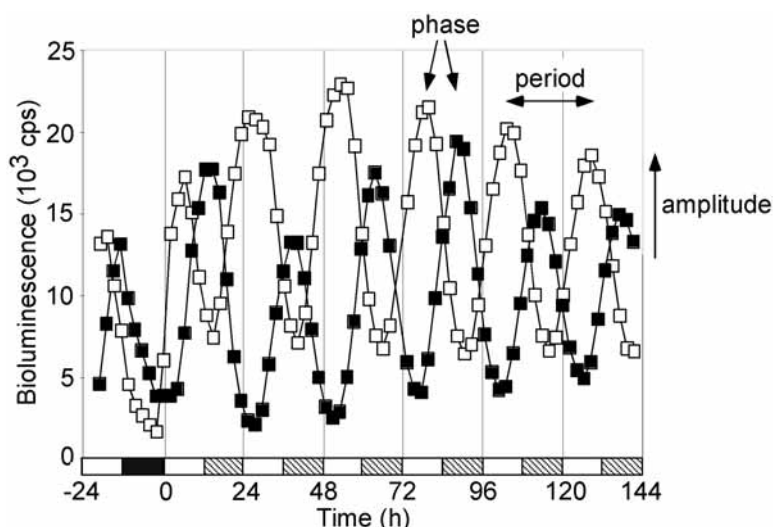


Fig. 3. Characteristics of the *Synechococcus elongatus* PCC 7942 circadian rhythm. Negative time denotes time during light–dark (LD) cycles that synchronize the cells. The black bar indicates time in darkness during the LD cycle; hatched bars indicate “subjective” dark during constant light (LL) conditions. Properties of the curve that are typically measured are the period (the time between occurrence of peaks or troughs), phase (the relative positioning of the curve with respect to time entering LL), and amplitude (the expression level measured from the midline of the curve to either the peak or trough) of the rhythm. Time points from a *PkaiBC::luxAB* reporter (AMC462; closed squares) show a class 1 phase rhythm, peaking at the light to dark transition, or subjective dusk. *PpurF::luxAB* (AMC408; open squares) represent a class 2 phase reporter that peaks at subjective dawn, 12 h out of phase from class 1 rhythms in constant conditions.

bioluminescent reporters. Very clear rhythms have been recorded from both *lux* and *luc* reporters by the TopCount for more than 2 wk (10) with no detectable change in rhythm characteristics.

Measurements recorded by the TopCount can be downloaded and interpreted using the Import and Analysis (I&A) program designed by the S. A. Kay laboratory (available at www.scripps.edu/cb/kay/shareware/) (11). The I&A program creates a Microsoft Excel worksheet that displays the bioluminescence cps for each timepoint collected for each sample in the 96-well plate. From this worksheet, the bioluminescence emitted from the culture in each microtiter well can be graphed. Each graph displays circadian properties that can be analyzed to determine if a particular mutation or environmental cue has caused an alteration of the rhythm (Fig. 3). The period of the circadian rhythm is defined

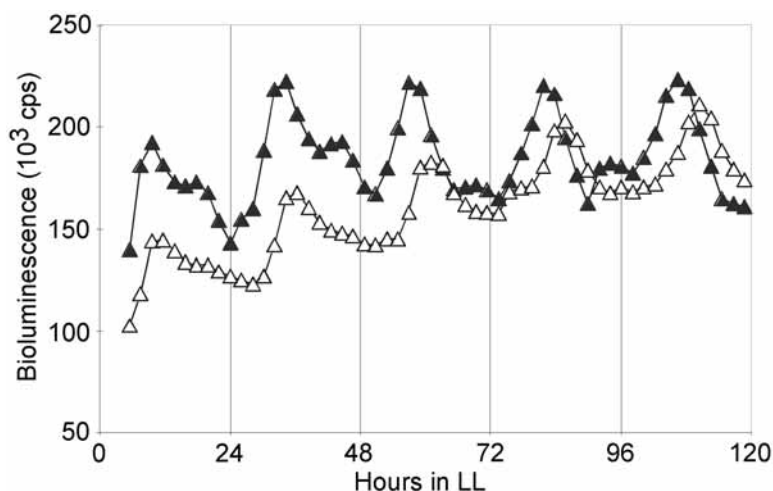


Fig. 4. *Synechococcus elongatus* circadian behavior obeys Aschoff's rule. Bioluminescence traces from a *PpsbAI::luxAB* reporter (AMC393) display a shorter period under high light (closed triangles) than under low light (open triangles).

as the amount of time between two adjacent peaks or troughs; the wild-type period for *S. elongatus* is between 23.5 and 25 h, depending on light intensity. This difference in periodicity occurs because the cells obey Aschoff's rule, a phenomenon of the circadian clock wherein the period of the circadian rhythm of diurnal organisms shortens with increasing light intensity (12,13). Following Aschoff's rule, the cyanobacterial cultures in the outer wells of the 96-well plate, that are closer to the light source, show consistently shorter periods than cultures in the inner wells (Fig. 4). Another noticeable element of the rhythm is relative phase, which is the positioning of the rhythm peak with respect to a reference point, such as when the culture was placed into constant light. A third characteristic is amplitude, the distance from the midline of the curve to either the peak or the trough of the rhythm. This property, though important, is the most variable of the three even among wild-type samples. These characteristics can be analyzed using the I&A computational interface fast Fourier transform–nonlinear least squares, which provides statistical period, phase, and amplitude information.

3.4. Cooled CCD Camera Measurement

Even though the TopCount is an excellent system for measuring bioluminescence, it does have its drawbacks. The TopCount system can be very labor-intensive, as each sample must be transferred to the well of a 96-well plate for analysis. And although the TopCount is a valuable asset for measuring *luxAB*

strains that are autoluminescent, it cannot be used to measure *luxAB* strains that lack *luxCDE*, as it is difficult to add exogenous decanal to the TopCount system. The decanal substrate is extremely volatile and, therefore, must be administered in the vapor phase; the vapor affects the ability of the TopSeal to adhere to the 96-well plate and causes plates to become stuck inside the machine. The use of a cooled CCD camera is ideal in these situations. Because rhythms can be measured directly from an agar plate in this system, mutant bioluminescence rhythms can be detected from a culture that has been mutagenized and plated on BG-11M medium. Either type of luciferase reporter can be used because the cooled CCD camera system is not limited by TopSeal issues, and can therefore measure bioluminescence from *luxAB* with the addition of the decanal substrate. This capability has led our lab to develop the detection of two different rhythms from the same strain by utilizing both the *lux* and *luc* systems for bioluminescence reporting. By creating strains that contain both *lux* and *luc* reporters, and providing these recombinant strains with either decanal substrate or luciferin, two different promoters can be analyzed from the same strain.

To examine existing strains, as opposed to colonies that have arisen following a mutagenesis protocol, patch transformants in 1-cm² sectors on BG-11M plates that contain the appropriate selective antibiotics for 4 to 7 d until cells have formed a thick mat. For *luc* strains, add 10 μ L 5 mM D-luciferin directly to patches and seal the Petri plate with Parafilm. For *lux* strains, add 200 μ L of a solution of decanal dissolved in canola oil (3% v/v) to a small receptacle (the cutoff cap of a microcentrifuge tube works well) (14). Lay the cap, open side, up on the BG-11M agar plate, being careful not to cover any of the cell patches. Seal the plate with Parafilm to prevent the gaseous decanal from escaping.

Synchronize the cells' clocks with 12 h of darkness and then place up to four plates, lid side up, in a dark box with lights on. We use two work lamps, each having a 25 W fluorescent bulb, that provide an intensity of about 300 μ E/m²s under the control of a programmable timer (VWR) that regulates turning the lights off for 5 min every hour for the duration of screening. Within every 5-min period of darkness, a 2-min period of darkness allows any autofluorescence to dissipate before the cooled CCD camera takes a picture with a 1-min exposure (Fig. 5). The cells then remain in darkness for an additional 2 min; this extra period of darkness is precautionary in the event that the camera and light timer become slightly out of phase with one another, as it can be harmful to the camera if too much light enters during exposures.

The MetaMorph v4.6 program (Universal Imaging Corporation, Downingtown, PA) is used to set up the parameters for camera exposures, as well as to save and process the data. Within MetaMorph, the area of each microbial patch is designated, and bioluminescence in counts per minute is

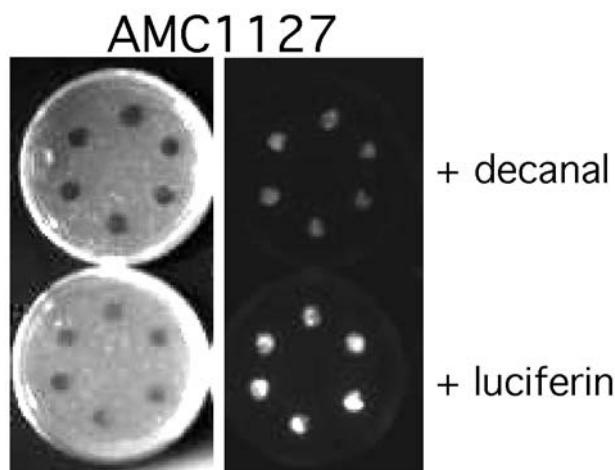


Fig. 5. Bioluminescence from the dual-reporter stain, AMC1127, was measured using a cooled charge-coupled device camera. AMC1127 carries two promoter fusions in its chromosome: *PpsbAI::luxAB* in NS1 and *PkaiBC::luc* in NS2. Cells were patched onto duplicate BG-11M plates. The upper plate was provided with gaseous *n*-decanal to monitor transcription from the *psbAI* promoter, whereas the lower plate received 5 mM luciferin to examine the bioluminescence from the *kaiBC* promoter. Light produced from the luciferase reaction was detected by the camera during a 1-min exposure when the lights were turned off (right). Data were saved and processed by the MetaMorph program. The intensity of light produced over time can be plotted to display the circadian rhythm of each patch of cells in counts per minute.

calculated for each exposure throughout the experiment. These data can then be exported to Microsoft Excel and analyzed by graphic representation.

3.5. Scintillation Counter Measurement

A third method for detecting bioluminescence is the use of a liquid scintillation counter. This method is the most labor-intensive of all bioluminescence measurement techniques used in our laboratory, but allows monitoring of circadian rhythmicity in growing liquid cultures of cyanobacteria that are being sampled for molecular rhythms in cyanobacterial extracts by RNA blots or immunoblot analysis, respectively (*see* Chapter 26).

1. Record the OD₇₅₀ of a 3-mL liquid cell culture.
2. For *luc* strains, add 400 μ L water, 20 μ L 5 mM D-luciferin, and 10 μ L liquid cell culture to a 6-mL scintillation vial. Let the reaction sit for 3 min. Use 410 μ L water with 20 μ L luciferin as the negative control sample for background emission. For *luxAB* strains, use 420 μ L water and 10 μ L liquid cell culture for

samples, and 430 μL water for the control. This volume of liquid is just enough to form a confluent layer along the bottom of the vial.

3. Measure the light production for the control and each sample. Subtract the background value from the sample and then divide that value by the OD_{750} to obtain counts/OD unit value (see **Note 9**).

4. Notes

1. We use a commercially available 10-carbon aldehyde, decanal, to provide exogenous substrate to *S. elongatus luxAB* reporter strains for bioluminescence assays.
2. 1 Einstein = 1 mol of photons.
3. The efficiency of transformation depends on the properties of the donor DNA, including the conformation of the DNA (linear vs circular). The efficiency of chromosomal recombination depends on the extent (number of uninterrupted base pairs) of identity between the *trans*-gene (or neutral site arms in the vector) and the chromosome, and the distance between the selectable marker and the end of the donor DNA fragment (1,15).
4. The concentrations of individual antibiotics may need to be reduced when two or more are used in combination to select for multiple recombination events to the cyanobacterial chromosome. The usable ranges of antibiotics for transformed cells that carry resistance cassettes are: 5–20 $\mu\text{g/mL}$ kanamycin, 1–2 $\mu\text{g/mL}$ gentamycin, 7.5–10 $\mu\text{g/mL}$ chloramphenicol, and 5–20 $\mu\text{g/mL}$ spectinomycin. To avoid spontaneous spectinomycin resistance, we use equal amounts of spectinomycin and streptomycin, e.g., 2 $\mu\text{g/mL}$ spectinomycin + 2 $\mu\text{g/mL}$ streptomycin; the Ω cassette confers resistance to both (16).
5. Black 96-well plates may be reused to cut costs. Remove agar pads from each well with a spatula. Soak plates overnight in 50% bleach and wash thoroughly with water to remove residual bleach. Soak plates in 70% EtOH overnight. Evaporate the EtOH in a laminar flow hood under UV light for 30 min before use.
6. The synchronization of cells may be conducted on or off of the TopCount machine. Off of the TopCount, simply place your prepared sample plates in any dark, 30°C, temperature-controlled chamber for 12 h. If you want to synchronize cells on the TopCount, or want to examine rhythms of bioluminescence during light–dark cycles, a dark box can be constructed to fit over the TopCount stackers. The box should fit snugly but not disrupt the movement of the plates within the stacker or into and out of the machine. It is not necessary to diligently obscure small light leaks; a cardboard box is sufficient.
7. Excess adhesive from the plastic TopSeal can cause black sample plates to become stuck in the TopCount machine. Ensure that no TopSeal is hanging over the edge of the 96-well black microtiter plate by removing it with scissors or a razor blade. To safely remove excess adhesive stuck to the plate, use 100% EtOH on a paper towel to gently remove the adhesive without damaging the adhered TopSeal. If the plate continues to stick in the machine, or a clear plate is sticking, use a paper towel dampened with a very small amount of WD-40 to rub the edges of the plates to prevent further complications.

8. Designed by D. Denke, Instrumentation Shop, Department of Biology, Texas A&M University, College Station, TX.
9. A scintillation counter can be used to determine saturating amounts of luciferin required for an individual reporter fusion. Follow the procedure in **Subheading 3.5.**, except use 20 μ L of a static cyanobacterial culture and add decreasing volumes of luciferin to each of several test vials. Measure the amount of bioluminescence. A noticeable adjustment in the counts of detection shows that the amount of luciferin is not saturating, and therefore not sufficient, for accurate measurements. The smallest volume of luciferin that gives consistent counts can be used for your TopCount and cooled CCD camera measurements.

Acknowledgments

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Analysis of Rhythmic Gene Expression in Adult *Drosophila* Using the Firefly Luciferase Reporter Gene

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Summary

The study of circadian clock function in *Drosophila* relies heavily on the analysis of rhythmic gene expression. Typically, individuals or groups of flies collected during a specific time of the circadian day need to be sacrificed, followed by the extraction of clock gene products. This procedure makes it impossible to analyze molecular rhythms in an individual over time. To measure clock gene expression within the living animal, firefly luciferase can be used as real-time reporter gene. This chapter describes how rhythmic expression of clock or clock-controlled genes can be measured in living adult *Drosophila*. A survey of all existing clock-related luciferase transgenics is given.

Key Words: Luciferase; *period*; *timeless*; *takeout*; transgenics; reporter gene; bioluminescence; circadian rhythms.

1. Introduction

The firefly luciferase reporter gene was first applied in *Arabidopsis* to study circadian clock gene regulation in plants (*see* Chapter 10). In flies, the first description of this reporter gene was by Lockett et al. (1), who used the luciferase reporter to demonstrate the suitability of a new marker gene for identifying transgenic flies with stably integrated transposable elements. In fact, it was those flies that prompted Hall and Kay to see if luciferase could be used as a circadian reporter gene (2). From these initial studies it became clear that luciferase transgenics can easily be sprayed or fed with the substrate luciferin, after which they start to emit green light (visualized by charge-coupled device [CCD] cameras) (2). Motivated by these experiments, the first clock-relevant luciferase transgene was generated by Christian Brandes in the Hall lab (3). In this construct, 4 kb of 5'-flanking regulatory DNA sequences cloned from the *period* (*per*) locus were fused to a cDNA encoding firefly luciferase.

The transgenic flies were analyzed *in vivo* for spatial reporter gene expression using a CCD camera and for overall rhythmic light output using a luminescence counter. Because of technical difficulties the spatial information obtained by inspecting the luciferase CCD camera images was not very informative: the fly had to be kept alive and tethered under a microscope for many minutes in order to obtain a decent image, which—even if it worked—was not of high resolution (*see* examples in **refs. 3** and **4**). In contrast, the quantitative light measures, obtained through an automated luminescence counter, proved to be excellent, and allowed a detailed analysis of rhythmic *per* gene expression in a heretofore-unknown fashion (**3,4**). These recordings are relatively simple to obtain: the flies are placed individually, or in small groups, into a given well of a microtiter plate. These wells contain fly food mixed with the luciferin, so the flies are continuously supplied with substrate throughout the experiment, which usually lasts for 5 to 7 d. Light output from each well containing the fly (or flies), food, and substrate is then automatically measured by the luminescence counter, usually once every hour for the duration of a given experiment.

This chapter describes how quantitative measurements of *per*, *timeless* (*tim*), and *takeout* (*to*) luciferase transgenics are obtained using a PerkinElmer TopCount bioluminescence/scintillation counter. This machine is used by several fly labs to measure luciferase reported rhythms (e.g., J. C. Hall, P. E. Hardin, S. A. Kay, M. Rosbash, A. Sehgal, and R. Stanewsky). It allows one to keep the flies in a temperature- and light-controlled environment while they are not in the dark counting chamber (usually once per hour for ~5 min). The ability to control and manipulate the environmental conditions is important to perform experiments under different entrainment conditions and to study circadian clock function.

2. Materials

2.1. Preparing Sample Plates

1. Fly food with luciferin: 1% Bacto agar (w/v), 5% sucrose (w/v), 15 mM luciferin (D-luciferin firefly potassium salt). Luciferin is light-sensitive and stable at -20°C for at least 6 mo (as powder). Before usage, prepare a 100 mM luciferin stock solution in water. Stock solution is stable at -80°C for at least 6 mo.
2. Microtiter plates (96-well). Use either white (Optiplate, PerkinElmer) or black (Micro-FLUOR, Dynex Technologies) plates (*see Note 1*).
3. Eppendorf repeater pipet (cat. no. 4780).
4. Tips for repeater pipet (e.g., 2.5 mL nonsterile PD Tips, USA Scientific, cat. no. 474-0250).

2.2. Loading the Flies

1. CO₂ station or ether.
2. Transgenic luciferase flies (*see Table 1*).
3. Forceps.
4. Plastic domes: These are modified caps of PCR tubes (MicroAmp Caps; PCR covers, 8 caps per strip; PerkinElmer). Each cap must be poked twice with a needle to allow sufficient airflow to the fly. It is best to do this while the caps are still on the strip. Then caps are separated with a razor blade and further trimmed to remove all excess plastic. This facilitates placing the dome over the fly and into the well. If you are lucky, your machine shop will do the separation and trimming for you.
5. Microtiter press-on adhesive sealing film (TopSeal A for 96-well plates, PerkinElmer).

2.3. Loading Plates Into Bioluminescence Counter

1. Bioluminescence counter; typically a PerkinElmer TopCount bioluminescence/scintillation counter.
2. Clear, 96-well microtiter plates to separate sample plates (*see Subheading 3.3.*).
3. Stop plate (*see Note 2*).

2.4. Running the Experiment

1. Light- and temperature-controlled room or incubator (*see Note 3*).

2.5. Data Handling and Analysis

1. Windows-based PC with Pentium or higher processor.
2. Import and Analysis (I&A) software: available at <http://www.scripps.edu/cb/kay>.
3. An alternative software package was developed in the Hall lab (*see Note 4*). Please contact Jeffrey Hall (hall@brandeis.edu) or Joel Levine (jlevine@utm.utoronto.ca).

3. Methods

3.1. Preparation of Medium and Sample Plates

1. Mix agar and sucrose in water and microwave until solution becomes clear. Let the solution cool down to approx 50°C (until you can touch it with your bare hands) and add the luciferin stock solution.
2. With the Eppendorf repeater pipet load 100 μ L of the luciferin fly food solution (*see Note 5*) in every other well (*see Note 6*). Cover each plate with tissue paper and store in the dark until usage. The medium should be allowed to solidify and dry for at least 1 h. Use plates within the day of preparation.

3.2. Loading of Flies

1. Anesthetize flies with ether or CO₂ on a clean tile or porous CO₂ pad, respectively.
2. Load flies individually (*see Note 5*) in the center of the food-containing wells of the microtiter plate using clean forceps (*see Note 7*). For best results try to lay down the fly on its side and not on its back in order to prevent its wings from becoming stuck to the medium.
3. Carefully cover each fly with a plastic dome. After finishing one plate cover it with an adhesive plastic seal and punch two air holes in the seal above each fly-containing well using a sterile needle (*see Note 8*).
4. Trim off excess adhesive seal that extends over the side of the plate.

3.3. Loading Plates Into Bioluminescence Counter

Plates are loaded in the front stacker of the machine. Start with a clear spacer plate at the bottom, followed by a sample plate. The sample plate is then topped by the next clear spacer plate, which is topped by the next sample plate, and so forth. The spacer plates allow light to reach each sample plate during the time it is not in the counting chamber (*see Note 9*). The last sample plate is covered again by a clear plate, followed by the stop plate (*see Notes 2 and 10*).

3.4. Running the Experiment

Using the software provided by PerkinElmer, you must enter certain counting parameters before starting the experiment:

1. The count delay (time each plate sits in the dark before being counted; *see Note 10*).
2. Count time (time each well is counted; *see Note 10*).
3. Specify which plates should be counted (i.e., the sample plates, and not the clear plates).
4. Specify which wells should be counted (*see Note 1*).
5. Start the assay. The duration of the experiment need not be entered at the beginning of the experiment, as it can be stopped at any time and runs for an infinite number cycles if not programmed otherwise.

3.5. Data Handling and Analysis

3.5.1. Raw Data Plotting

Upon completion of the analysis, the data (usually one data file per hour) are compressed and copied to a floppy disk. After extracting the data on a PC, it is loaded into I&A software (Microsoft Excel-based) developed in the laboratory of S. A. Kay (5). This software allows plotting the raw data of individual wells, generating group averages, and plotting data of several genotypes on a single graph (*see Note 4*).

3.5.2. Data Analysis

The software also allows exporting the data for quantitative analysis. For this a fast Fourier transform–nonlinear least square (FFT-NLLS) analysis is performed (see **Note 4**). It generates estimates for period length and phase, and it helps to judge whether the rhythmic component found in a particular record is significant or not (**4,5**).

3.6. Problems and Troubleshooting

3.6.1. Background Signals

A particular problem arises when experiments are performed in light–dark (LD) cycles. During the light portion of an experiment, background counts reach values between 50 and 150 counts per second (cps), whereas during the dark portion, background is 10 to 40 cps. This means, if you plot an empty well (control well without fly) after an experiment, you see a beautiful 24-h pseudorhythm, caused by the light-induced background. Also, FFT-NLLS or any other method will tell you that this is a highly significant 24-h rhythm. This light-induced rhythm is probably caused by a minimal light leakage into the machine because it cannot be decreased by extended count delays. Although this sounds like a major drawback of the technique, it is not a problem for most applications. This is because the expression levels of most clock-gene–luciferase transgenics are orders of magnitudes higher compared with the background signal. Nevertheless, this “background rhythm” problem can be severe in certain instances. For example, when promoterless *per-luc* fusions are analyzed (*8.0-luc:9*; **Table 1**), expression levels are extremely low, because the transgene is expressed only in approx 100 neurons (**ref. 6**; in contrast, *per-luc* and *tim-luc* transgenes containing regulatory upstream sequences are broadly expressed in the fly, leading to expression levels of 5000 to 80,000 cps, **ref. 8**). This makes analysis of such a “low-*luc* expressor” dubious in LD cycles, but expression rhythms can still be reliably recorded in constant darkness (DD) (**6**). The same low-signal connected problems may arise when body parts or internal organs are recorded (see Chapter 38).

3.6.2. Amplitude Reduction Over Time

A general problem associated with long-term bioluminescence recordings is an observable downtrend of signal strength, which is likely caused by substrate depletion (**5**). Several routines have been applied in order to remove this trend from the raw data series (see Chapter 38 and **refs. 4,11** and **15**).

Table 1
Luciferase Transgenics Related to Circadian Rhythms

Name	Reported clock gene	Expression level	Remarks	References
<i>plo</i>	<i>period</i>	Medium	<i>per</i> promoter sequences only; reflecting <i>per</i> transcriptional rhythms	3–5,8, 12–14,19
NOG- <i>luc</i>	<i>period</i>	Medium	Complete <i>per</i> 5'-UTR; reflecting <i>per</i> mRNA rhythms	12,19
BG- <i>luc</i>	<i>period</i>	High	<i>per</i> promoter sequences plus the N-terminal 2/3's of <i>per</i> coding region. Reflecting <i>per</i> mRNA rhythms	4,8,12, 14,19
XLG- <i>luc</i>	<i>period</i>	High	<i>per</i> promoter sequences plus almost entire <i>per</i> coding region	6
8.0- <i>luc</i>	<i>period</i>	Very low	Almost entire <i>per</i> coding region, but no 5'-UTR sequences. Depending on insertion line expressed in certain clock neurons only	6,19
<i>per</i> 69x3- <i>luc</i> or CEx3- <i>LUC</i>	<i>period</i>	Very high	Trimer of an 69-bp <i>per</i> enhancer sequence including an E-box. Does mediate robust luciferase cycling.	15,16
<i>tim</i> - <i>luc</i>	<i>timeless</i>	Very high	<i>timeless</i> 5-UTR; reflecting <i>tim</i> RNA rhythms	8,12,19
<i>tim</i> ^{FL-LUC}	<i>timeless</i>	Very high	756 bp of <i>tim</i> promoter; reflecting aspects of <i>tim</i> RNA rhythms; does not contain all information for proper phasing of the rhythms	12,17
<i>tim</i> ^{FL-LUC/TER1}	<i>timeless</i>	High	Same as <i>tim</i> ^{FL-LUC} , but containing a mutated TER1 element; luminescence ca. 50% lower compared to <i>tim</i> ^{FL-LUC}	17
<i>tim</i> ^{FL-LUC/PERR}	<i>timeless</i>	High	Same as <i>tim</i> ^{FL-LUC} , but containing a mutated PERR element; luminescence almost identical to <i>tim</i> ^{FL-LUC}	17
<i>tim</i> ^{FL-LUC/TER1-PERR}	<i>timeless</i>	High	Same as <i>tim</i> ^{FL-LUC} , but containing mutated TER1 and PERR elements; luminescence levels slightly reduced compared to <i>tim</i> ^{FL-LUC/TER1}	17
<i>tim</i> ^{FL-LUC/TER1-TER2}	<i>timeless</i>	High	Same as <i>tim</i> ^{FL-LUC} , but containing mutated TER1 and TER2 elements; luminescence ca. 70-80% lower compared to <i>tim</i> ^{FL-LUC} , rhythmicity almost abolished	17

<i>timenh-LUC</i>	<i>timeless</i>	Very high	150 bp <i>tim</i> enhancer fragment containing an E-box (subclone of the 756 bp fragment present in <i>tim^{FL-LUC}</i>). Leads to robust cycling of luciferase levels	15
<i>timenhmut-LUC</i>	<i>timeless</i>	Low	Same as <i>timenh-LUC</i> but with mutated E-box. Expression levels drastically reduced; no luciferase oscillations.	15
<i>TIM-luc</i>	<i>timeless</i>	Low	An Eag I fragment consisting of 664 bp <i>tim</i> promoter (not including the E-box which is present in <i>tim^{FL-LUC}</i>) and ca. 3.9 kb of <i>tim</i> cDNA. The potential TIM-LUC fusion protein reflects aspects of rhythmic <i>tim</i> expression	18
<i>CRE-luc</i>	<i>dCREB2</i>	Medium	3 cAMP response elements (CREs) placed upstream of the TATA box region of the <i>hsp70</i> promoter. Reports rhythmic expression of <i>dCREB2</i>	14
<i>mCRE-luc</i>	<i>dCREB2</i>	Low	Same as CRE-luc, except that CREs are mutated. <i>dCREB2</i> oscillations and expression levels are blunted	14
<i>to-luc</i>	<i>takeout</i>	High	3-kb promoter fragment. Reflects <i>to</i> transcriptional rhythms	16
<i>to-ΔE-luc</i>	<i>takeout</i>	?	Same as <i>to-luc</i> , 21bp containing an E-box are deleted. Phase and rhythmicity similar to <i>to-luc</i>	16
<i>to80x3-luc</i>	<i>takeout</i>	High	Trimer of an 80-bp <i>to</i> enhancer sequence including an E-box. Does not mediate robust luciferase cycling.	16
<i>to80ex3-luc</i>	<i>takeout</i>	high	Same as <i>to80x3-luc</i> , but carrying a mutated E-box. Nevertheless expression is identical as in <i>to80x3-luc</i> flies	16

Summary of all existing clock-gene related luciferase reporter lines that were analyzed for adult bioluminescence rhythms. In the reference column, all studies in which a particular transgenic was used for recording whole fly rhythms are listed. Note that some of the transgenics listed here were also applied to study body part or internal organ luciferase rhythms from tissue cultures (*see* Chapter 38).

3.6.3. Plate Jamming

One of the major curses of luminescence recordings are plate jams in the TopCount machine or in the stackers, which lead to an immediate cessation of the experiment. Several measures can be taken to minimize these events:

1. Use clean needles to poke holes in the adhesive seal (*see Note 8*).
2. Make sure plates do not stick to each other before loading them into the stacker (*see Note 9*).
3. Use new sample plates for each experiment. Clear spacer plates should be used no more than over the course of five separate runs.
4. If possible, place the computer monitor outside the room or incubator where the counter is situated. This allows you to check whether the experiment is running at all times, without disturbing the current experimental environmental condition (e.g., DD).
5. Do not connect the PC of the TopCount machine to any network. Since PerkinElmer moved from a DOS to a Windows-NT based version of the operating software, software crashes are problems that can occur in addition to the mechanical ones. Although the newest version of the supplied systems software seems to be stable, it is recommended to use the PC as an independent unit. Also make sure that you do not accumulate too much data on the hard drive, and to shut down and restart the PC after each run according to the manufacturer's instructions.
6. Although designed to handle and measure large sample numbers, the TopCount is not designed for circadian rhythm experiments, where it is often required to measure constantly for a week or longer. Consequently, mechanical or software failures occur frequently, and owing to the high costs of repair services, buying a service contract along with the machine is highly recommended.

4. Notes

1. Each light-emitting fly produces so-called "crosstalk," meaning that its light output is also detected in the neighboring well. To minimize crosstalk, two measures can be taken:
 - a. Use black microtiter plates instead of white ones. The black plates absorb more light and therefore reduce crosstalk. The drawback is that overall signal intensity is also decreased, but this is not a problem for lines with high expression levels (*see Table 1*).
 - b. Only every other well of a plate is loaded so that each fly-containing well is surrounded by four empty wells.
2. The "stop-plate" signals to the machine that one cycle of the counting protocol is completed and tells it to restack all the plates and to start with a new cycle. Labels to generate these plates (by putting the appropriate sticker at one side of the plate) are supplied with the machine.
3. To run the experiment, the bioluminescence counter should be placed in a light- and temperature-controlled room or incubator. It is very important that the room

is completely dark when performing experiments in DD. Even small amounts of light are sufficient to entrain molecular and behavioral rhythms in flies (8). The room should also be humidified, to avoid rapid drying out of food and flies.

4. An alternative to the I&A software (see **Subheading 3.5.1.**) was developed in the Hall lab. It also allows plotting of the raw data, as well as quantitative analysis of the obtained expression rhythms. Instead of FFT-NLLS, this software package uses autocorrelation, maximum entropy spectral analysis, and χ^2 periodogram functions to determine rhythmicity and estimate period length (10). Also, “phase” is calculated by a more accurate paradigm (compared with the FFT-NLLS method) (11). The FFT alternative seems especially useful when low-amplitude rhythms (e.g., emerging from antennae or other body part cultures) need to be scrutinized for rhythmicity. For high-amplitude rhythms, which are usually obtained from adult flies, FFT-NLLS estimates (which can easily be obtained using the more user-friendly I&A software) are reliable and similar to estimates obtained with the alternative but more sophisticated Hall-lab analysis.
5. It has been shown that the ability of the flies to move vertically in the well leads to noisy records (4). This is probably related to the fact that the light intensity detected by the photomultiplier tube (sitting on top of the well during counting) is proportional to the inverse square of the distance from the light source (fly). In other words, depending on where the fly “sits” during the brief time of counting within 1 h, the measured intensity can vary dramatically between two measurements, even if the actual expression level at both time points was similar or identical. Alternatively, the observed noisiness of the bioluminescence records might be caused by subsaturating levels of substrate within the fly. This was probably the case in early “fly-*luc*” studies, where the luciferin concentration in the food was only 1 mM (as opposed to 15 mM now routinely used; see **Subheading 2.1.1.** and **refs. 3 and 5**). Individual recordings in these studies showed wild, short-term fluctuations in LUC activity readouts that could not be explained by a circadianly regulated decrease in enzyme levels (i.e., these fluctuations occurred within 1 h, whereas the LUC-activity half-life is 2 to 5 h [3,5]). Because the flies could move freely around in the well, they often were away from the food, not eating the substrate and resulting in low cps values even though enzyme levels could still be high).

Probably both factors—distance from the photomultiplier tube and subsaturating substrate levels—contributed to the noisy records, because individual recordings became much cleaner when substrate concentrations were increased and movement was restricted simultaneously (4).

To prevent vertical movement, individual flies can be covered with a small plastic dome (see **Subheading 2.2.4.**). Alternatively, food levels can be increased in each well, so that there is only a little space for the fly between the food and the adhesive seal at the top. To prepare such plates: first, 250 μ L of food without luciferin (to save expensive substrate) are filled within each well (see **Subheading 2.1.1.** for preparation; omit luciferin); after the food is solid, it is topped with 100 μ L of luciferin-containing food. Although clean records can be obtained with

this method, several difficulties are connected with it: first, records are still noisier compared with the “dome” method. This can be overcome by putting up to three flies in one well, thereby generating a smoothed average for each well. Second, because the flies are so close to the adhesive seal, they often stick to it and die. To prevent this, the adhesive seal above each well can be covered with a clear plastic disk (cut out of overhead transparency film with a hole-puncher), but this procedure is extremely laborious (7). Nevertheless, the “no-dome” alternative can be the method of choice, for example, when performing mutant screens where less analytical precision is required (8).

6. Or each well, depending on the counting protocol you are using.
7. The author suggests collecting the flies that are to be analyzed a day before the experiment starts. Usually male flies produce much cleaner rhythms than females, probably due to arrhythmic clock gene expression in ovaries (9). Flies for analysis should be between 1 and 3 d of age to maximize the number of individuals that survive the entire experiment. Flies must not be too young because they will stick to the fly food if their cuticle and wings are still soft. Therefore, it helps to collect the flies a day early. Moreover, flies can then be loaded into plates without the use of a stereo microscope.
8. It is recommended to using a needle for only one experiment. We experienced cases of a given needle that was reused several times, became suboptimally sharp, and would “pull out” some of the adhesive material, thereby transferring it to the top of the adhesive seal. This can cause “sticking of plates,” which will lead to an interruption of the automated counting procedure.
9. Separation of sample plates by clear plates is crucial for experiments involving environmental LD cycles. In our experience normal lab illumination is sufficient to entrain clock gene expression of flies kept in sample plates covered with clear plates. In order to prevent plates sticking together and causing an experiment to halt prematurely, we test all plates that contact each other prior to loading them into the stacker. For this, we simply press the upper plate on the lower one by hand pressure and then lift the upper plate up. If the lower plate sticks to the ground and not to the upper plate, it is safe to load the plates into the stacker.
10. The number of sample plates can be defined by the user. We usually program our TopCount machine to read 5 or 9 plates within 1 h. To do this you have to enter the appropriate times for (a) counting each well, and (b) count delay (time the plate sits in the counting chamber before the counting starts; this should be at least 1 min) in the software provided with the machine. You also must consider the time that is needed for the machine to change plates and restack them after all plates have been counted. This has to be done empirically (i.e., with a timer), because stacking times vary between different machines.

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Monitoring Circadian Rhythms in *Arabidopsis thaliana* Using Luciferase Reporter Genes

Anthony Hall and Paul Brown

Summary

Arabidopsis thaliana is internationally adopted as the model plant species for molecular genetics. As such a huge range of resources are available for its study. *Arabidopsis* does not display any obvious circadian rhythms; however, rhythms in gene expression can be readily detected. For this reason the promoters of rhythmically expressed genes have been fused to the firefly *luciferase* gene. Using this reporter gene we have developed a number of automated techniques for monitoring luciferase activity in vivo in plants. This provides us with a robust and high-throughput assay for the circadian clock in *Arabidopsis*.

Key Words: Biological clock; luciferase; gene expression; *Arabidopsis*; plant transformation; genetic engineering.

1. Introduction

Circadian rhythms were identified in the plant *Mimosa pudica* in 1729 by De Mairan (1). Like cyanobacteria (see Chapter 8), *Arabidopsis thaliana*, the model plant species, has few readily measurable circadian rhythms. In the late 1980s and early 1990s a large number of studies identified that expression of numerous genes is controlled by the circadian clock in plants (2). These included studies demonstrating the circadian regulation of the chlorophyll a/b binding protein gene (*CAB*) in *Arabidopsis* (3). It is now clear that in *Arabidopsis* 10 to 15% of genes are regulated by the circadian clock (4,5). Measuring gene expression therefore provides a useful tool for assaying the circadian clock.

Measuring gene expression by assaying RNA abundance using Northern analysis or S1 protection assays is time-consuming, technically demanding, and destructive. Each time point requires the manual harvest of tissue and extraction of RNA. To get around this problem, the firefly luciferase reporter

gene has been utilized. Luciferase is a good marker for dynamic changes in gene expression. In plants both the *LUC* mRNA and the luciferase protein, in the presence of luciferin, have a short half-life (6). Luciferases are a class of enzymes that emit light. In the case of the firefly luciferases, light (560 nm) is emitted upon the adenosine triphosphate-dependent catalysis of luciferin and oxygen to oxyluciferin and carbon dioxide (7). By fusing luciferase to the promoter of a gene of interest and monitoring its activity, using either a scintillation counter or low-light-detecting charge-coupled device (CCD) camera, levels of promoter activity can be measured. In this way gene expression can be assayed *in vivo*, nondestructively, and repeatedly in the same plant over several days. By fusing the promoter of the circadian regulated *CAB* gene to the luciferase gene and transforming this reporter construct into *Arabidopsis*, rhythms in the *CAB* promoter activity can be measured *in planta* (6). This provides a simple method for assaying the circadian clock in *Arabidopsis*, given the required imaging facilities, and a method amenable to high-throughput screening approaches.

This chapter describes the luciferase vectors used and how these are transformed into *Arabidopsis*. The chapter discusses how we use either a cooled CCD camera or a liquid scintillation analyzer to assay circadian rhythms in *Arabidopsis* and how the promoter activity is analyzed to estimate period, amplitude, and phase values.

2. Materials

1. pSP-*luc+* Vector (Promega).
2. Plant binary vector (e.g., pPCV812).
3. *Agrobacterium tumefaciens* (e.g., strain GV3101).
4. 1 mM HEPES, pH 7.0.
5. 10% Glycerol.
6. YEBS media: beef extract 5 g/L, bacto-tryptone 5 g/L, yeast extract, 1 g/L MgSO_4 , pH 7.0.
7. Selective YEBS plates: YEBS, 15 g/L agar, 50 mg/L rifampicin, 100 mg/L carbenicillin.
8. Selective YEBS media: YEBS, 50 mg/L rifampicin, 100 mg/L carbenicillin.
9. Murashige and Skoog basal salt mixture (MS salts, Sigma-Aldrich).
10. MS plates: MS salts, 3% sucrose, 1.5% agar.
11. MS antibiotic plates: MS salts, 3% sucrose, 1.5% agar, 30 $\mu\text{g/mL}$ hygromycin.
12. MS solid media: MS salts, 3% sucrose, 1.2% agar.
13. Electroporator: Gene pulser (Bio-Rad, Hercules, CA).
14. Metamorph v5.0 software (Molecular Devices).
15. Electrically cooled CCD camera: ORCAII BT-1024G (Hamamatsu).
16. Light-emitting diode arrays (MD Electronics, Coventry, UK).
17. Parallel port switcher (MD Electronics, Coventry, UK).

18. Dark box.
19. 5 mM Luciferin (Biosynth AG; *see Note 1*).
20. Infiltration media: Vac-in-stuff (Silwet L-77, Lehle Seeds, Round Rock, TX).
21. Liquid scintillation analyzer: Packard TopCount (PerkinElmer Life Sciences).
22. Top-sealing tape for microplates: Packard TopSeal (PerkinElmer Life Sciences).
23. Black 96-well plates (DYNEX Technologies).
24. Reflector plates (*see Note 2*).
25. Lubricant: WD-40.
26. Packard results analysis software (PerkinElmer Life Sciences).
27. Toptemp, Biological Rhythm Analysis Software System (BRASS), and fast Fourier transform-nonlinear least squares (FFT-NLLS) software (*see Note 3*).

3. Methods

3.1. Description of LUC+ Vectors Used

Several luciferase reporter genes are currently available from Promega. We use the *LUC+* gene that has been modified to improve its function as a genetic reporter. These modifications include the removal of the peroxisomal translocation sequence, resulting in the transport of luciferase to the cytoplasm and the removal of glycosylation sites. Together these changes produce a severalfold increase in the light signal. The promoter luciferase fusions were constructed in the plant binary vector pPCV812. This plasmid contains a bacterial ampicillin resistance cassette for selection in *Escherichia coli* and *Agrobacterium* and a hygromycin resistance cassette for selection of transformed plants. To produce promoter: *LUC+* gene fusions, approx 1.5 kb of promoter sequence upstream of the ATG of the plant gene of interest was fused with the ATG of the *LUC+* gene. At the 3'-end of the *LUC+*, the *nos* termination sequence was attached (8,9).

3.2. Transformation of Arabidopsis

This section describes how we transform *Arabidopsis* with luciferase reporter genes using *Agrobacterium*.

3.2.1. Transformation of *Agrobacterium*

This section is adapted from **ref. 10**.

1. Inoculate 10 mL of YEBS media with the *Agrobacterium* strain GV3101 (rifampicin resistant). Incubate overnight at 28°C in an orbital incubator.
2. Use the overnight culture to inoculate 300 mL of YEBS and incubate in an orbital incubator at 28°C until an optical density at 600 nm (OD₆₀₀) of 0.5 is reached. Chill on ice and harvest by centrifugation. Wash three times in 10 mL of 1 mM HEPES, pH 7.0, once in 10% glycerol, and finally suspend in 3 mL of 10% glycerol. Snap-freeze electrocompetent *Agrobacterium* in 200-μL aliquots and store at -80°C.

3. Transform competent *Agrobacterium* with the *Promoter:LUC* fusions by adding approx 200 ng of vector to an aliquot of electrocompetent cells. Place in a 0.2-cm electroporation cuvet and electroporate in the Bio-Rad Gene Pulser with 2.5 kV using 25 μ F capacitance and 200 Ω resistance. After the pulse add 1 mL of YEBS and incubate for 1 h at 28°C.
4. Plate on selective YEBS plates and incubate for 48 h at 28°C (see **Notes 4** and **5**).

3.2.2. *Agrobacterium-Mediated Transformation of A. thaliana*

There are a number of methods available for the transformation of *Arabidopsis*; this is an adaptation of the method described in **ref. 11** (see **Note 6**).

1. Grow *Arabidopsis* in the greenhouse at a density of 6 plants per 3-in. pot. Remove the primary bolts when they are 1 to 5 cm tall. This will encourage secondary bolts. When the secondary bolts are approx 10 cm tall, they are ready to transform.
2. Prepare an inoculum of *Agrobacterium* transformed with the *promoter:LUC* construct of interest. Inoculate 10 mL of selective YEBS media with *Agrobacterium* and incubate in an orbital incubator overnight at 28°C. Use this seed culture to inoculate 500 mL of YEBS (with no antibiotics) and incubate for a further 24 h to produce an inoculum (see **Note 5**).
3. Add 250 μ L of Silwet L-77 to the inoculum and pour into a 250-mL beaker. Invert and dip the *Arabidopsis* plants into the inoculum, ensuring that no soil falls in and that all the floral stems are immersed.
4. Seal plants in biohazard bags for 24 h to maintain a high humidity, then remove. For high transformation rates, repeat **steps 2–4** after 7 d.
5. Allow plants to set seed, dry, and then harvest.
6. Sterilize seed (see Chapter 7), plate on MS antibiotic plates, vernalize at 4°C for 48 h, then transfer to constant light for approx 10 d. Transformed seedlings will grow and green normally, whereas untransformed seedlings will die.
7. Confirm the presence of a transgene by spraying hygromycin-resistant seedlings with 5 mM luciferin and taking a 20-min exposure with a cooled CCD camera. Plants containing the luciferase reporter gene will glow.
8. Grow transformed seedlings and harvest seed. Seed can then be used in experiments. Homozygous lines containing a single copy of the reporter gene should be selected.

3.3. Automated Imaging Using Cooled CCD Camera

For automated imaging we use an ORCA II camera (Hamamatsu) with a high-transmission lens (Xenon f/0.95, 25 mm). This camera is thermoelectrically cooled to -50°C and has low noise and a large dynamic range, making it ideal for luciferase imaging. Several other suitable cameras are available. The camera is set up as shown in **Fig. 1**. The camera is mounted on a dark box in a temperature-controlled room. The plants for imaging are placed approx 1 m away from the camera; at this distance, four 12 $\times$ 12 cm plates can be imaged,

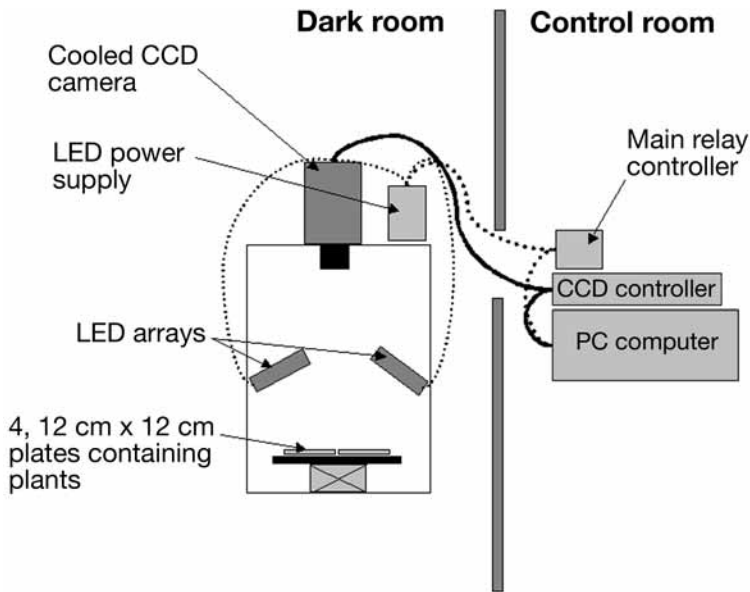


Fig. 1. Setup of cooled charge-coupled device system for the capture of luciferase bioluminescence images.

with each plate containing 100 seedlings. On each side of the dark box light-emitting diode (LED) arrays are fixed, providing between 10 and 30 $\mu\text{mol}/\text{m}^2/\text{s}$ of either red, blue, far red, or combinations of light. The LED arrays are switched on and off by a main relay switch, operated by the computer via the parallel port. The camera and LEDs are controlled by MetaMorph version 5 software. Using a set of journals (*see* **Note 7**), the computer turns on the LED arrays for 95 min, switches them off for 5 min, takes a image with an exposure time of 20 min, saves it to the hard drive, and then switches the LED arrays back on. This cycle can be repeated indefinitely to allow monitoring of luciferase levels, and hence promoter activity, for several days.

1. Sterilize seed (*see* Chapter 7) and sow on MS plates in a grid pattern, vernalize the seed at 4°C for 48 h, and then entrain for 6 d with light–dark cycles (12 h light/12 h dark).
2. Using a pump spray, spray the plate with 5 mM luciferin a full 24 h before the start of experiment; repeat spraying approx 12 h later (*see* **Note 8**).
3. Move the plates to be imaged at the end of the light period to the imaging system (**Fig. 1**), so that the first light signal the plants receive from the LEDs in the imaging chamber is at dawn. Image acquisition can be initiated at dusk or at dawn on the following day.

4. Image acquisition is controlled using MetaMorph version 5. Once communication with the parallel port and the correct camera driver have been installed, experiments are controlled from the “auto imaging” task bar (*see Note 7*). Buttons and journals are available for experiments in constant light or darkness, long photoperiods (16 h light/8 h dark), short photoperiods (8 h light/16 h dark), and 12 h light/12 h dark cycles. The program uses sequential file names to store images.

3.4. Analysis of Luminescence Data From Camera Experiment

The circadian analysis of luminescence data is achieved using MetaMorph version 5 and BRASS. MetaMorph allows numerical analysis of bioluminescent images, measuring integrated luminescence within defined regions of interest. BRASS is a Microsoft Excel workbook, providing an interface for running FFT-NLLS analysis, which estimates periods, amplitudes, and phase values from circadian data ([12](#)).

1. Open MetaMorph and load a stack of images. Using the region tool, draw circular regions of interest around each seedling, move through the stack to ensure that each seedling remains inside its circle. Also select a region to monitor the background level far from any luminescence signal, preferably beyond the plates (*see Note 9*).
2. Open a dynamic data exchange to log MetaMorph data into Microsoft Excel. Using the Graph Intensities function, choose to measure from a stack, over time, and measure integrated values. To ensure that the data are in the correct format for BRASS, configure the data log to save only “image name,” “image plane,” “image time and date,” “region name,” and “integrated.” Initiate the measurement.
3. The data for each region in each plane of the stack are logged in an Excel worksheet. This data can then be used directly to draw graphs ([Fig. 2](#)) or can be imported into BRASS to fit periods, amplitude, and phase values.

3.5. Automated Measurement of Luciferase Luminescence Using PerkinElmer TopCount

The TopCount is a scintillation counter using a set of photon-multiplier tubes to measure luminescence of samples in 96-well microtiter plates ([Fig. 3](#)). It automatically cycles through a stack of up to 20 plates, which are held on top of the instrument, and returns luminescence data in text or Excel files. Using the TopCount, luciferase activity of 960 plants free-running in constant darkness can be measured every 45 min for several days. LED arrays can be fixed alongside the stacks and reflector plates (*see Note 2*) inserted between microtiter plates to reflect light from the side, down into the wells. Using this setup, luciferase activity can also be measured under constant light or in light–dark cycles. The usefulness of the TopCount is limited by the amount of light that can be reflected into microtiter plates. Plants displaying interesting circa-

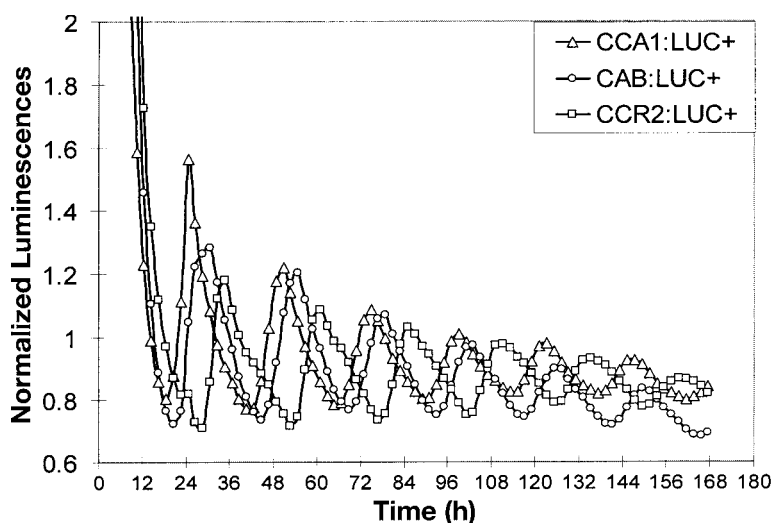


Fig. 2. Normalized luminescence of the circadian regulated promoters *CCR2*, *CCA1*, and *CAB* fused to the luciferase reporter gene. Luminescence levels were quantified using MetaMorph software from images captured using an ORCA II cooled charge-coupled device camera. Plants were entrained in 12 h light/12 h dark cycles and free-run in constant blue light. Data acquisition began at the start of the transfer to constant conditions.

dian phenotypes can be rescued from microtiter plates, planted in soil, and allowed to set seed.

1. Sterilize and entrain seedlings as described in **Subheading 3.3**.
2. Melt 200 mL of MS solid media. In a sterile flow hood add 300 μ L to each well of a black 96-well microtiter plate (*see Note 10*). Allow to dry and wrap plates in plastic film. Plates can be used immediately or stored at 4°C.
3. Transfer seedlings with sterile forceps to each well. Hold the seedling between root and hypocotyl and insert the root into the agar plug (*see Note 11*).
4. Pipet 15 μ L of 5 mM luciferin into each well (*see Note 8*).
5. Take two sheets of TopSeal; cut 5 mm off all the way around one sheet. Stick the two sheets together, sticky side to sticky side, creating a 5-mm sticky border. This can be stuck to the plate without plants becoming stuck to the TopSeal. Using a hypodermic needle, pierce the TopSeal above each well to allow gas exchange.
6. Stick an individual barcode label to the right-hand side of the plate and wipe the top of the plate with WD-40 to prevent plates jamming onto each other in the stack.
7. Return the plates to the entrainment cycle and, at dusk on the seventh day, load into the TopCount.

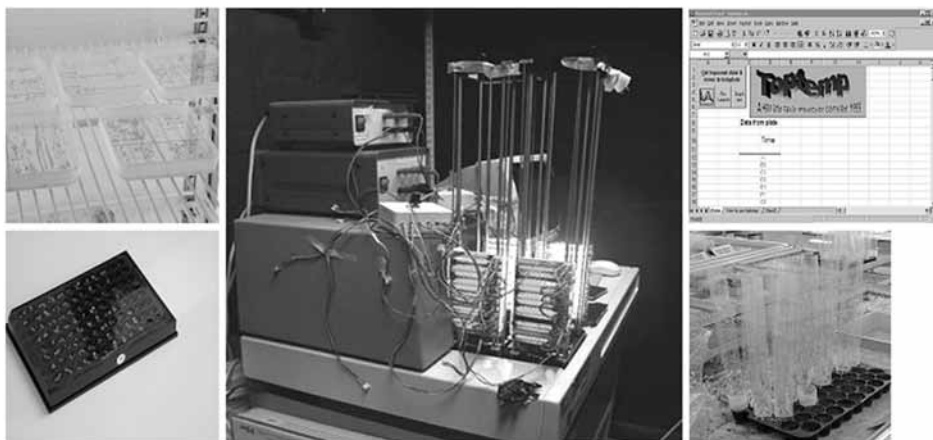


Fig. 3. Pictorial representation of the method used to analyze circadian rhythms in *Arabidopsis*. Top left: Seedlings entraining to light cycle on MS plates. Bottom left: Seedlings in a black microtiter plate ready to be assayed. Center: Packard TopCount with light-emitting diode arrays attached to the sides of the stackers. Top right: Excel worksheet program, TopTemp. Bottom right: Assayed plants transferred from microtiter plates to soil and allowed to flower.

8. Set TopCount to run indefinitely measuring luminescence; check periodically that the machine is still working and that plates have not become jammed. For constant light experiments, the lights are set to come on at dawn and remain on, using a commercial timer.

3.6. Analysis of TopCount Data

For the analysis of TopCount data, the Packard results analysis software is used to generate individual text files for each read of a plate. These are merged and an Excel-based program called TopTemp is used to convert these text files into an Excel spreadsheet and graph the data. This Excel spreadsheet can then be imported into BRASS (described in **Subheading 3.4.**), providing an interface with FFT-NLLS for the estimation of period, amplitude, and phase values.

4. Notes

1. Prepare a 50 mM stock solution of luciferin (D-luciferin, potassium salt) in 0.1 M Tris-phosphate, pH 8.0. This stock solution is divided into 1-mL aliquots, stored in the dark at -80°C . A working solution is prepared by diluting to a 5 mM solution in a solution of 0.001% Triton X-100. The luciferin is finally filter-sterilized before use.
2. Detailed information on the construction of reflector plates can be found at www.scripps.edu/cb/kay/ianda/spacer_plate.htm.

3. TopTemp and BRASS can be downloaded from www.amillar.org. FFT-NLLS can be obtained from Dr. Martin Straume (ms3g@virginia.edu). Alternative software with similar features, known as Import and Analysis (I&A), is available from the Kay lab homepage at the Scripps Institute.
4. All the promoters we have used drive a detectable level of expression of *LUC* in *Agrobacterium* and *E. coli*. This can be used to confirm that the *Agrobacterium* have been transformed with the luciferase gene. Incubate the strain for 48 h at 28°C to produce sizeable colonies, and spray them with 5 mM luciferin. Place the colonies under the camera and take a 10-min exposure. Transformed *Agrobacterium* should give a bright signal. Similarly, a small aliquot of *Agrobacterium* seed culture can be tested prior to the plant transformation.
5. *Agrobacterium* can be stored in 25% glycerol solution at –80°C.
6. Different accessions of *Arabidopsis* have differing efficiencies of transformation; we have found that Col-0 is very easy to transform and have had difficulty transforming *Ler*. It is very important to keep your plants healthy, as healthy plants transform well.
7. The task bar and journals for automated imaging using MetaMorph software can be downloaded from www.amillar.org.
8. Spraying with luciferin 24 h prior to the start of an experiment is essential, because luciferase protein is relatively stable in plants at room temperature. When the substrate luciferin is present, luciferase enzyme activity becomes unstable, probably a result of end-product inhibition (13).
9. Data from a region in the background are used to confirm that the camera signal was stable during the experiment. It is not usually necessary to use these data to estimate a background noise level for each image. There are several sources of noise in low-light imaging, which we cannot cover in detail here. Images have an offset level (which can typically be specified by camera control software and should be stable over time) with a few counts of readout noise on each pixel. The readout noise cannot be removed *post hoc*, because it has no spatial pattern, but in an appropriate camera it should be low and stable over time. The only image processing that is usually required is therefore to subtract the standard offset level from each pixel, which is most easily done in Excel. For integrated luminescence data, this requires that the pixel area of each region of interest is logged once.
10. Black plates are essential to avoid background phosphorescence from white plastic and to reduce signal crosstalk between wells.
11. Use a flow hood with a low air flow rate; a high air flow rate will cause the plants to wilt and will overdry the agar in the 96-well plates.

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III

IDENTIFICATION OF CLOCK GENES

Specialized Techniques for Site-Directed Mutagenesis in Cyanobacteria

Eugenia M. Clerico, Jayna L. Ditty, and Susan S. Golden

Summary

Synechococcus elongatus PCC 7942 is an excellent model system for studying the molecular mechanism of the circadian clock in cyanobacteria. The “plastic” genetic characteristics of this organism have facilitated the development of various methods for mutagenesis of its chromosome. These methods are based on homologous recombination between the chromosome and foreign DNA, introduced to the cyanobacteria by either transformation or conjugation. Here we describe different approaches to mutagenize the chromosome of *S. elongatus*, including insertional mutagenesis, hit-and-run allele replacement, *rps12*-mediated gene replacement, and regulated expression of genes from ectopic sites, the neutral sites of the *S. elongatus* genome.

Key Words: *Synechococcus elongatus* PCC 7942; hit-and-run allele replacement; in-frame deletion; *rps12*-mediated gene replacement; homologous recombination; transformation; neutral site.

1. Introduction

Synechococcus elongatus PCC 7942 is an excellent model system for understanding the molecular mechanism of the circadian clock in cyanobacteria. *S. elongatus* has a small genome size (~2.7 Mb, fully sequenced; see **Note 1**), which allows for easy saturation mutagenesis, and possesses an efficient system of homologous recombination to incorporate genetic information into its chromosomes. Also, *S. elongatus* is genetically transformable by two methods: natural transformation by the uptake of exogenous replicating or nonreplicating DNA (**1,2**), and conjugative transformation by receiving plasmid DNA capable of mobilization from an *Escherichia coli* donor (**3**).

The malleable genetic characteristics of *S. elongatus* have facilitated the development of methods and an array of cloning vectors for the production of

mutants in this organism. These approaches rely on homologous recombination between the chromosome and exogenous (not autonomously replicating) DNA that are linked to sequences homologous to the chromosome; by incorporating a selectable marker within the region of homology, engineered sequences can be directed to a particular locus. The simplest application is insertional inactivation of a gene, by replacing the gene of interest with an allele that has a heterologous cassette (generally an antibiotic-resistance cassette) inserted within its open reading frame. However, as prokaryotic organisms have polycistronic genetic operons, the insertion of a selectable marker within an open reading frame can exert polar effects on downstream genes. Additionally, when several genes within the same strain are to be inactivated, the use of many different antibiotics to select for various insertions can have cumulative, detrimental effects on cell culture viability. In light of these complications, two elegant allele replacement systems have been developed for allelic exchange, known as “hit-and-run” allele replacement (4) and *rps12*-mediated gene replacement (5,6). The advantage of these strategies is that they introduce the mutations without leaving residual genetic evidence of the mutagenic process, such as an antibiotic-resistance marker. These methods can be used to delete a gene by exchanging a wild-type chromosomal allele with an in-frame deletion construct that will not exert polar effects on neighboring genes. In addition, they can be used to introduce specific site-directed mutations within a gene (e.g., a single nucleotide mutation), or for the incorporation of additional nucleotides to a gene (e.g., adding sequence to encode an epitope tag).

S. elongatus genes can be expressed ectopically from alternative sites in the genome. Two sites on the chromosome have been developed as cloning loci, called “neutral sites,” where ectopic sequences can be homologously recombined without any apparent aberrant phenotype (see **Subheading 3.2.**). Any DNA of interest (e.g., reporter genes, overexpression constructs) can be cloned within the *S. elongatus* neutral site sequences and, by homologous recombination, moved into the cyanobacterial chromosome.

In this chapter, we describe the methods used in our laboratory for insertional mutagenesis, hit-and-run allele replacement, *rps12*-mediated gene replacement, and expression of genes from the neutral sites of the *S. elongatus* genome.

2. Materials

1. *E. coli* cloning vector, such as pUC18 or pBR322.
2. Modified BG-11 medium (BG-11M). Liquid medium (7): 1.5 g/L NaNO₃, 0.039 g/L K₂HPO₄, 0.075 g/L MgSO₄·7H₂O, 0.02 g/L Na₂CO₃, 0.027 g/L CaCl₂, 0.001 g/L EDTA, 0.012 g/L FeNH₄ citrate, and 1 mL of the following microelement solution: 2.86 g/L H₃BO₃, 1.81 g/L MnCl₂·4H₂O, 0.222 g/L ZnSO₄·7H₂O, 0.391 g/L Na₂MoO₄, 0.079 g/L CuSO₄·5H₂O, and 0.0494 g/L Co(NO₃)₂·6H₂O. Solid medium

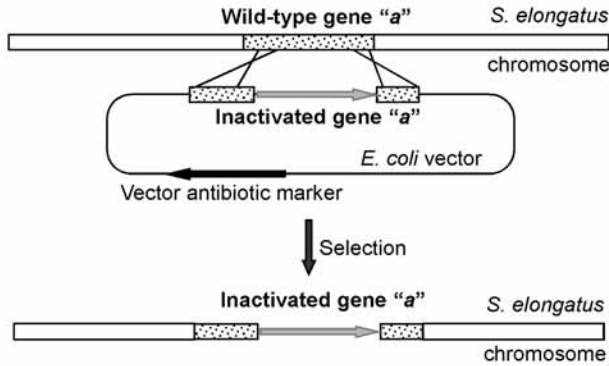
- (1): equal volumes of twice-concentrated BG-11M liquid medium and Difco agar solution (3% in sterile water) autoclaved separately and mixed together; add filter-sterilized Na_2SO_3 to 1 mM final concentration.
3. Antibiotics: kanamycin (Km), spectinomycin (Sp), chloramphenicol (Cm), streptomycin (Sm), and gentamicin (Gm) (see **Note 2**).
 4. 10 mM NaCl (sterile).
 5. 120 mM NaCl.
 6. 10 mM EDTA, pH 8.0.
 7. 25% Sucrose (w/v in water; sterile).
 8. 50 mM Tris-HCl, pH 8.0.
 9. 10 mg/mL Lysozyme in water.
 10. 20% Sarkosyl (w/v in water).
 11. 10 mg/mL Proteinase K in water.
 12. 5 M NaCl.
 13. 10% Cetyltrimethylammonium bromide in 0.7 M NaCl.
 14. 24:1 Chloroform:isoamyl alcohol.
 15. Equilibrated phenol (8).
 16. 100% Ethanol.
 17. 70% Ethanol.
 18. 10 mg/mL RNase A in water.
 19. Hit-and-run vector (see **Subheading 3.3**).
 20. *E. coli* strain carrying conjugal vector for conjugation (see **Subheading 3.1.4**).
 21. *E. coli* strain carrying helper vector for conjugation (see **Subheading 3.1.4**).
 22. MF-Millipore membrane filter (mixed cellulose esters), pore size 0.025 μm , diameter 25 mm (Millipore), autoclaved.
 23. LB culture media (8).

3. Methods

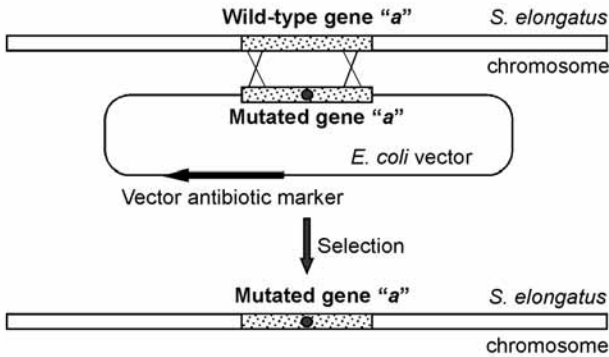
3.1. Mutagenesis by Homologous Recombination

By this method, any gene on the chromosome of *S. elongatus* can be replaced by a modified homologous allele (**Fig. 1A–D**). When inactivating a cyanobacterial gene, the recombinant allele of interest is constructed in an *E. coli* vector (which will not replicate in the cyanobacterium) by inserting an antibiotic-resistance cassette or a transposon into the coding region of the gene, making sure that the insertion is flanked on each side by at least 300 base pairs of homologous genomic DNA ([2,9] see **Note 3** and **Fig. 1A**). The recombinant mutant allele is introduced into *S. elongatus* by transformation or conjugation and subsequently crosses into the cyanobacterial genome by homologous recombination. Selection for a double crossover event and subsequent segregation of mutant chromosomes (because *S. elongatus* maintains multiple copies of its chromosome [10]) is based on growth of the strain on media containing the antibiotic to which the interrupting cassette confers resistance.

A Gene inactivation by insertion of an antibiotic resistance cassette



B Gene replacement by a mutated allele that confers a selectable phenotype



C Sequence addition or change at one end of a gene

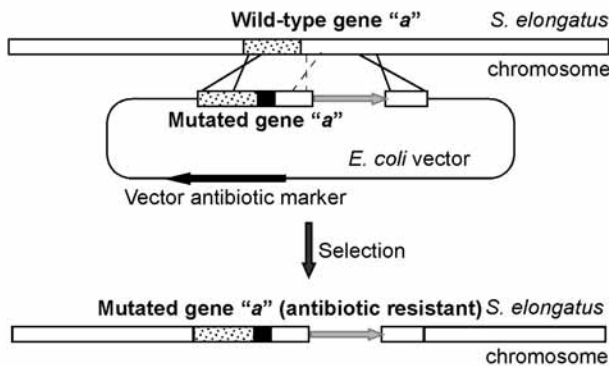


Fig. 1. Mutagenesis of the *S. elongatus* chromosome by homologous recombination. In all cases, the cyanobacterial chromosome is represented by an open bar; the wild-type gene to be replaced is named "a" and represented as a dotted box. Point mutation is symbolized as a black circle. Recombination events between the chromosome

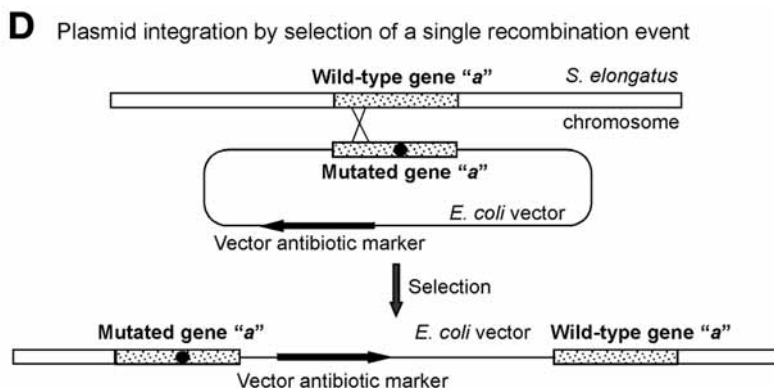


Fig 1. (*continued*) and the *E. coli* plasmid are denoted by an X. (A) Gene inactivation by insertion of an antibiotic-resistance cassette. The gene "a" is cloned into an *E. coli* vector, interrupted by an antibiotic-resistance gene (gray arrow), and moved into the cyanobacterium. The homologous sequences undergo double recombination and the inactivated copy replaces the original gene when the clones are selected on the antibiotic to which the mutated gene confers resistance. (B) Gene replacement by a mutated allele that confers a selectable phenotype. The cyanobacterial gene "a" carrying a mutation is cloned into an *E. coli* vector and moved into the cyanobacterium. As the mutation confers to the cyanobacteria a selectable phenotype, the recombinant clones are selected based on its phenotypic characteristics. (C) Sequence addition or change at one end of a gene. The cyanobacterial gene "a" is cloned into an *E. coli* vector and the sequence near one end is changed (or extra sequence added; black box). An antibiotic-resistance gene is cloned outside the open reading frame, as close as possible without disturbing likely regulatory sequences. After transformation and selection for clones resistant to the antibiotic, the presence of the mutation should be further confirmed because the recombination could occur between the selectable marker and the desired sequence change (dashed X). (D) Plasmid integration by selection of the single recombination event. The mutated gene "a" is cloned into an *E. coli* vector and moved into the cyanobacterium. The homologous sequences undergo single recombination and the plasmid becomes integrated into the chromosome by selection of clones on an antibiotic to which the vector confers resistance (black arrow).

This approach can also be used for purposes other than gene inactivation. If a cloned allele confers to the bacterium some selectable characteristic, the replacement of its chromosomal allele can be selected based on its new phenotype (ref. 9; Fig. 1B).

This same general strategy can be used to change or add extra sequence to a gene, such as to encode an epitope or 6-His tag. In addition to the mutation or added sequence, an antibiotic-resistance cassette is inserted outside the coding region for selection of transformants; the cassette is followed by additional

cyanobacterial sequence to direct integration to the appropriate chromosomal location (**Fig. 1C**).

A single crossover event between chromosome and an exogenous plasmid can be achieved by selection for the antibiotic-resistance marker on the *E. coli* cloning vector. The entire plasmid will integrate into the genome and subsequently cause a duplication of the gene at the site of the insertion: one wild-type allele and one mutated allele (**Fig. 1D** and **Note 4**).

In the following protocols cultures of cyanobacteria are always grown at 30°C and under constant light (300 $\mu\text{E}/\text{m}^2\text{s}$; *see* **Note 5**), and liquid cultures are shaken at 250 rpm unless otherwise indicated.

3.1.1. Plasmid Construction for Mutagenesis by Homologous Recombination

1. Using standard molecular biology techniques (**8**), clone the cyanobacterial sequence to be mutated into an *E. coli* cloning vector, such as pUC18 or pBR322.
2. Clone an antibiotic-resistance cassette within the sequence homologous to the cyanobacterial chromosome, making sure to leave at least 300 bp of homologous DNA flanking either side of the resistance gene for efficient recombination.

3.1.2. *Synechococcus* Transformation

1. Grow 100 mL of the *S. elongatus* strain to be mutated in liquid BG-11M to an OD_{750} of 0.7.
2. Harvest 15 mL of cyanobacterial cells by centrifugation for 10 min at 6000g (*see* **Note 6**).
3. Resuspend the cell pellet in 10 mL of 10 mM NaCl and harvest by centrifugation for 10 min at 6000g.
4. Resuspend the cell pellet in 0.3 mL of BG-11M and transfer to a microcentrifuge tube (*see* **Note 6**).
5. To each 0.3 mL of cells, add between 50 ng and 2 μg (typically, we use 1–2 μL from a preparation of 100–200 ng/ μL) of the recombinant plasmid that carries the mutagenized cyanobacterial gene.
6. Wrap the tubes in aluminum foil to shield the cells from light and incubate them overnight at 30°C with gentle agitation.
7. Plate the entire 0.3-mL cell suspension on a BG-11M plate containing the appropriate selective medium (*see* **Note 2**).
8. Incubate the plates at 30°C in constant light for approx 5 d until single colonies appear.
9. Restreak isolated colonies that have the appropriate phenotypes, maintaining the selection to favor complete segregation of mutant cyanobacterial chromosomes.
10. Grow mutant clones in 100 mL of BG-11M with the appropriate antibiotic to an OD_{750} of 0.7. Extract the chromosomal DNA (*see* **Subheading 3.1.3.**) and verify the presence of the mutation and its segregation on the cyanobacterial chromosome by PCR, restriction enzyme analysis, or other technique (*see* **Notes 4** and **7**).

3.1.3. Extraction of Chromosomal DNA From *S. elongatus*

This protocol, routinely used in our lab, is a modification of that described in **ref. (I)**.

1. Pellet approx 10 mL of a liquid culture of cyanobacteria or scrape cells from a plate.
2. Resuspend the pellet in 500 μL of 120 mM NaCl and 10 mM EDTA, pH 8.0, and transfer the suspension to a microcentrifuge tube.
3. Re-pellet the cells and resuspend them in 340 μL of 25% sucrose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0. Add lysozyme to a final concentration of 2 mg/mL. Incubate the cell suspension for 45 min at 37°C.
4. Add 2 μL of proteinase K (from a 10 mg/mL stock solution) and 20 μL of 20% sarkosyl, and vortex for 20 s. Incubate the mix at 55°C for 30 min.
5. Add 57 μL of 5 M NaCl and 45 μL of 10% cetyltrimethylammonium bromide in 0.7 M NaCl. Mix well and incubate for 10 min at 65°C.
6. Extract the suspension with 500 μL 24:1 chloroform:isoamyl alcohol.
7. Carefully transfer the upper aqueous phase to another tube and extract with 500 μL of equilibrated phenol. Vortex for 20 s and spin for 10 min at 16,000g. The high NaCl concentration may cause the phases to flip, placing the aqueous phase on the bottom after the centrifugation step; the aqueous solution can be identified by its pink hue.
8. Transfer the aqueous phase to another tube and extract with 500 μL of 24:1 chloroform:isoamyl alcohol mix. Vortex quickly and spin for 10 min at 16,000g.
9. Take the upper aqueous phase and precipitate the DNA by adding 2 v of 100% ethanol. Mix by inverting the tube several times.
10. Spin down the DNA for 15 min at 16,000g in a microcentrifuge. Carefully remove all the liquid (which contains significant salt) and wash the pellet with 1 mL of 70% ethanol. Spin again for 5 min and remove the ethanol from over the pellet.
11. Dry the pellet by leaving the tube open or by applying vacuum.
12. Resuspend the DNA in 50 μL of water and add 20 ng/ μL of RNase A (optional). From this 50- μL final solution, 0.5 to 1 μL is typically used for a standard polymerase chain reaction (PCR).

3.1.4. Transfer of Exogenous DNA From *E. coli* to *S. elongatus* by Triparental Mating

Although *S. elongatus* is easily transformable, its efficiency for incorporating foreign DNA is much higher when DNA is introduced by conjugation from *E. coli*. This increase in efficiency is particularly true for single recombination, which normally occurs with a much lower frequency than double recombination (**II**). Also, conjugation is sometimes the only way to introduce foreign DNA because isolates of *S. elongatus* can lose the ability to be successfully transformed at a reasonably high frequency, and sometimes this occurs in a desirable genetic background for which another isolate is not available. The

preferred protocol for conjugation between *E. coli* and *S. elongatus* involves a triparental mating procedure (3).

The plasmid that will be used for introducing a particular DNA sequence into the cyanobacterium is called the “cargo plasmid.” This plasmid (which in general should not replicate in cyanobacteria) should have the sequences necessary for replication in the *E. coli* strain used for conjugation, selectable markers for selection in the two hosts of interest, cloning sites, and a mobilizable replicon (e.g., pBR322, which carries a *bom* [basis of mobility] site). To be mobilized into the cyanobacterial host the cargo plasmid needs, additionally, the presence of a “conjugal plasmid” and a “helper plasmid,” the sources of *tra* genes and other *trans*-acting factors, respectively.

In the case of triparental mating, the conjugal and helper plasmids necessary for the transfer are in different strains (see **Note 8**). Normally, the helper plasmid is present in the *E. coli* strain bearing the cargo plasmid. The conjugal plasmid, in a second *E. coli* strain, will move naturally into the strain that carries the helper and the cargo plasmids. Then, the conjugal plasmid assists in transferring the cargo plasmid from *E. coli* to the cyanobacterium.

1. By using standard molecular biology procedures (8), introduce your cargo plasmid into the *E. coli* strain that carries the helper plasmid (see **Note 8**). Select for clones that carry both plasmids.
2. Prepare two overnight cultures in LB media: one from the *E. coli* strain that contains the conjugal plasmid and the other from the *E. coli* strain obtained in **step 1**.
3. Mix together 0.1 mL of each of the *E. coli* cultures from **step 2** with 1 mL of a fresh culture of the recipient cyanobacterial strain. Include a control that contains the cyanobacteria, the *E. coli* strain with the conjugal plasmid, and the *E. coli* strain with the helper plasmid but without the cargo plasmid.
4. Spin down the suspension for 1 min at 6000g. Aspirate the medium, leaving about 0.1 mL of liquid on top of the cells, and resuspend the pellet in that volume.
5. Place a sterile MF-Millipore membrane filter on the surface of a BG-11M agar plate that has been supplemented with LB medium 5% (v/v).
6. Place the cell suspension from **step 4** on the filter's surface in a large drop.
7. Incubate the plates in dim light for 24 h at 30°C (see **Note 9**).
8. Resuspend the cells from the filter in 100 μ L of BG-11M and plate them on a BG-11M plate that contains the antibiotic that will select for the desired recombination event.
9. Incubate the plates in standard light and temperature until green colonies appear (see **Note 10**) and restreak them on a fresh plate.

3.2. Homologous Recombination at Neutral Sites of *S. elongatus* Chromosomes

S. elongatus possesses at least two sites on the chromosome, called “neutral sites” ([NS] Neutral Site I and II; see **Note 11**), where ectopic sequences can be

inserted without generating any apparent phenotype. These loci have been developed as targeting sites for cloned genes. Any DNA of interest can be inserted into the *S. elongatus* NS sequences on a plasmid introduced into the cyanobacterium and, by homologous recombination, moved into the cyanobacterial chromosome.

The basic principles underlying recombination at the NSs are the same as those described for gene replacement (*see Subheading 3.1.*). In this case, the sequence to be inserted is directed to one of these specific sites in the *S. elongatus* chromosome by cloning it within NS sequences in specialized plasmids called “neutral site vectors.” (For details about the NS vectors, including a list of those vectors made by our lab and sample maps, refer to Chapter 8.) In brief, these vectors contain an antibiotic-resistance marker and a multiple cloning site where a gene of interest can be inserted, flanked by NS sequences from either NS1 or NS2. After introduction of plasmids into *S. elongatus*, the resulting recombinant clones are isolated by selection on media containing the proper antibiotic. Since they were developed, our laboratory has used *S. elongatus* NSs to introduce numerous kinds of engineered genes. Here, we describe some of these applications.

We have created an easily measurable reporter of circadian activity by expressing the genes that encode either bacterial luciferase (*luxAB*) or firefly luciferase (*luc*). Any *S. elongatus* promoter can be cloned upstream of the luciferase genes and its activity assayed. The genes coding for the enzymes that produce the substrate for the bacterial luciferase (*luxCDE* genes) can be cloned into a different neutral site to create autonomously bioluminescent strains. High-throughput monitoring devices are available to record and analyze bioluminescence in an automated manner (*see Chapter 8*).

When the inactivation or deletion of a single gene in cyanobacteria confers a particular phenotype, restoring the wild-type phenotype by complementation confirms that the observed phenotype is a consequence of only the deleted gene. This restoration can be accomplished by cloning the wild-type gene with its native promoter into an NS vector and targeting the construct to the chromosome of the strain that carries the mutated gene. As described in **ref. (12)**, *S. elongatus* mutants generated by chemical mutagenesis that showed altered circadian phenotypes were efficiently complemented by conjugation with *E. coli* cells that carried a library of wild-type *Synechococcus* DNA. The library was constructed in NS vectors to direct large fragments of wild-type DNA into NSs.

In addition, a gene can be expressed from a heterologous promoter to assay the phenotype that results from its altered expression. That heterologous promoter can be an engineered *E. coli* isopropyl- β -D-thiogalactopyranoside-inducible promoter (*see Note 12*).

3.3. Hit-and-Run Allele Replacement

A two-step selection protocol known as “hit-and-run” is used to introduce defined mutations (deletions, insertions, or point mutations) into specific genes on the chromosome of *S. elongatus* without leaving residual genetic markers (4). To accomplish this outcome, the mutant allele is cloned into a vector that carries both positive and negative selectable markers. The positive marker selects for the incorporation of the recombinant plasmid into *S. elongatus* through a single recombination event; this is a cassette that confers resistance to Km, Cm, or Sm, depending on the version of the vector chosen for cloning. The negative marker, which selects a second recombination event that excises the plasmid from the chromosome, is the *sacB* gene from *Bacillus subtilis*. The *sacB* gene encodes the enzyme levan sucrase, which, in the presence of sucrose, generates compounds that are toxic for many Gram-negative bacteria (see **Note 13**).

As shown in **Fig. 2**, after the introduction of the plasmid into cyanobacteria, the first step consists of selecting for integration of the entire plasmid into the chromosome via a single crossover event, by plating the cells on the proper antibiotic. This recombination event effectively results in the duplication of the gene of interest, as the bacterial chromosome now carries the wild-type copy and its mutant counterpart (both of which may be chimeric, depending on the site of crossover; **Fig. 2C**). Note that as the plasmid carries the *sacB* gene, those cells that successfully complete a single crossover would die on media that contain 5% (w/v) sucrose.

In the second step, the reverse of the integration event occurs. By culturing the cells in absence of antibiotic selection, cells are viable only when the plasmid “loops out” (removing the *sacB* gene) and leaves the chromosome by homologous recombination between the directly repeated sequences that flank the inserted vector. Depending on where the recombination takes place, and where the mutation lies in the duplicated target sequence, the mutant or the wild-type allele remains in the chromosome. A method of DNA analysis must be used to determine which allele has been retained in a given clone.

1. Clone the mutated gene to be inserted in the cyanobacterial chromosome into a hit-and-run vector by standard molecular biology methods (8).
2. Introduce the plasmid into *S. elongatus* either by transformation (as described in **Subheading 3.1.2.**) or by triparental mating (see **Subheading 3.1.4.**). Conjugal introduction of the plasmid will result in a much higher frequency of single recombinants.
3. Plate the cell suspension on BG-11M agar that contains the proper antibiotic to select for single recombinants and incubate at 30°C in constant light. Resistant colonies will appear in about 6 to 10 d (see **Note 10**).
4. Restreak the resistant colonies to a BG-11M plate with the proper antibiotic and to another BG-11M plate that contains 5% sucrose (w/v) but no antibiotic.

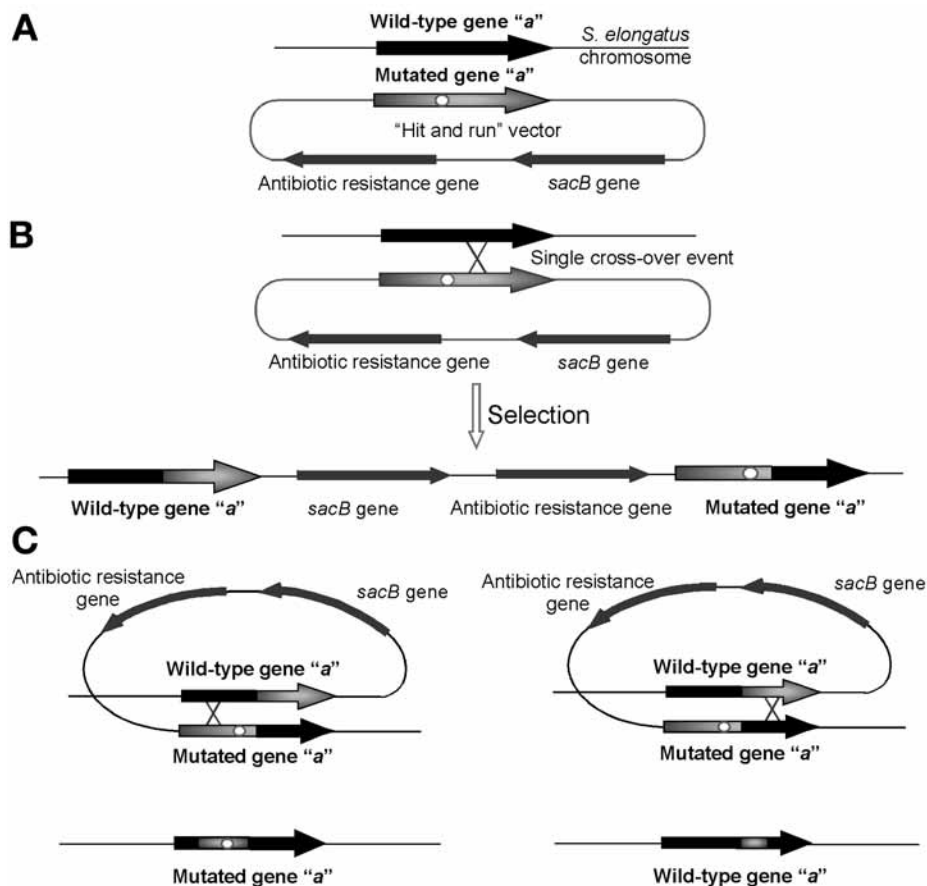


Fig. 2. Hit-and-run allele replacement. The *S. elongatus* chromosome is represented as a black line and a wild-type gene as a black arrow. A white circle denotes a mutation. Antibiotic-resistance cassette and *sacB* genes are represented as gray arrows. Recombination points are drawn as Xs. (A) Introduction of a hit-and-run vector carrying a mutated *S. elongatus* gene "a" (gray arrow) into *S. elongatus*. (B) Selection for antibiotic-resistant clones. The single crossover event is selected and the plasmid becomes integrated at that locus. The resultant clones are sensitive to sucrose because *sacB* is present. (C) In the absence of selective pressure, clones survive in which the plasmid loops out in the reverse of the integration process, yielding clones sensitive to the antibiotic and resistant to sucrose. The resulting population is a mixture of clones that carry the wild-type or the mutant copy of the gene, depending on where the recombination event took place relative to the mutation.

5. Choose the clones that are resistant to the antibiotic and sensitive to 5% sucrose (these clones have undergone the single crossover event; *see* Fig. 2). It is very important to confirm sucrose sensitivity before proceeding, as some sucrose-resistant clones arise that are not the result of resolution of the plasmid.
6. To promote the double recombination event (plasmid “loop-out”), grow the antibiotic-resistant/sucrose-sensitive clones in 2 mL of BG-11M medium without antibiotic or sucrose at 30°C in constant light until an approximate OD₇₅₀ of 0.7 (about 5 d).
7. Harvest 1 mL of the culture by centrifugation for 1 min at 6000g, and resuspend the pellet in 100 µL of fresh BG-11M.
8. Spread the entire cell suspension on BG-11M plates that contain 5% sucrose (w/v), and incubate in constant light until isolated colonies appear (about 7 d).
9. Replica plate isolated colonies to a fresh BG-11M plate that contains 5% sucrose (but no antibiotic) and a BG-11M plate that contains the original antibiotic used for selection in **step 3** (with or without sucrose). Incubate in constant light until colonies are fully grown on the sucrose plate (about 5 d).
10. Retain individual clones that are resistant to 5% sucrose and sensitive to the antibiotic. Based on phenotype, these clones should have undergone the double crossover event (*see* Fig. 2).
11. Grow the chosen clones in liquid BG-11M until they reach an OD₇₅₀ of 0.7.
12. Extract the DNA (*see* Subheading 3.1.3.) and verify the presence and complete segregation of the mutation by PCR analysis, restriction pattern, Southern hybridization, or sequencing.

3.4. *rps12*-Mediated Gene Replacement

A very clever method for gene replacement in cyanobacteria called *rps12*-mediated gene replacement was developed by T. Ogawa and colleagues ([5,6] *see* Fig. 3). With this technique point mutations, deletions, or insertions can be introduced into the cyanobacterial chromosome without leaving any markers of the process.

As a starting point to introduce the desired mutations, this procedure uses a cyanobacterial strain resistant to Sm; this resistant strain carries a mutation in the *rps12* gene (a single base change from A to G at position 128 of its open reading frame), which encodes the S12 subunit protein of the 30S ribosome. When the mutant and the wild-type alleles of this gene coexist in a cell, the *rps12* mutation is recessive and the strain is Sm-sensitive (5,6). The parent strain has only the *rps12* at its native locus, and is Sm-resistant.

Fig. 3. (*opposite page*) *rps12*-mediated gene replacement. The *S. elongatus* chromosome is represented as a black line; the wild-type *rps12* gene and its mutated version are illustrated as a black and a gray arrow, respectively. A white arrow represents the gene to be mutated and a gray circle denotes the mutation. Superscripts “S” and

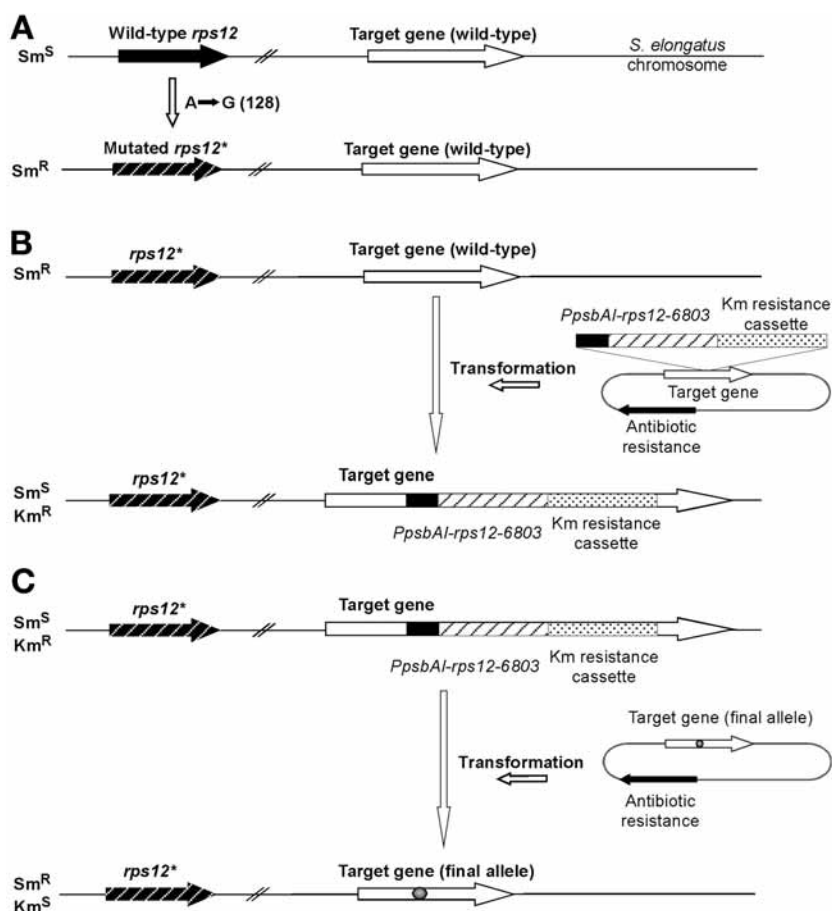


Fig 3. (continued) “R” indicate sensitivity or resistance, respectively, of that particular cyanobacterial strain to Sm or Km. (A) An A→G change is introduced in the sequence of the *rps12* gene in *S. elongatus* to yield a Sm-resistant strain. (B) The Sm^R strain is transformed with a plasmid that carries the sequence of the gene to be changed flanking a copy of the wild-type *rps12* gene from *Synechocystis* PCC 6803 (*rps12*-6803) driven by the *S. elongatus* *psbAI* promoter and a Km^R cassette. By homologous recombination, the construction will be inserted at the locus to be mutated and the strain will become Km^R (because of the presence of the cassette) and Sm^S (because the wild-type allele of *rps12* is dominant). (C) This strain is then transformed with the final copy of the cyanobacterial gene, which does not need to carry a selectable marker. Upon homologous recombination, the *rps12*-6803 allele driven by the *S. elongatus* *psbAI* promoter and the Km^R cassette will be eliminated from the cyanobacterial chromosome and the strain will be Km^S and Sm^R. This strain now carries intended allele at its native locus; the allele may be a point mutation, a tag-encoding allele, or an in-frame deletion of the target ORF.

To introduce a specific mutation in the cyanobacterial chromosome, the wild-type *rps12* gene, along with a Km-resistance cassette, is first inserted at the gene to be mutated in the background of the Sm-resistant *rps12* mutant strain. By homologous recombination, this “mother” strain becomes Km-resistant and Sm-sensitive. To minimize gene conversion between the homologous *rps12* genes (wild-type and mutant), a heterologous *rps12* gene from *Synechocystis* sp. PCC 6803 (about 75% similar to the *S. elongatus* PCC 7942 nucleotide sequence) is used as the “wild-type” gene (hereafter called *rps12*-6803). To get efficient transcription and translation of the PCC 6803 *rps12* gene in *S. elongatus*, the strong promoter of the *psbAI* gene and its ribosome binding site have been provided.

In a second transformation, the wild-type *rps12* gene and the Km-resistance cassette are removed from the cyanobacterial chromosome by recombination with the final version of the allele to be replaced. As *rps12*-6803 and the Km-resistance cassette are eliminated through recombination, the strain becomes Sm-resistant and Km-sensitive. Selection for Sm resistance occurs without need for a marker on the allele that replaces *rps12*-6803.

1. Create the Sm-resistant strain that will serve as the starting point to introduce the desired mutations. In our lab, this “mother” strain was constructed as follows: using chromosomal DNA from the Sm-resistant strain GRPS1 (kindly provided by Dr. M. Matsuoka [5,6]) as a template, the mutated *rps12* gene was amplified by PCR. This 850-bp fragment was cloned into a pCR™-Blunt vector (Invitrogen, Life Technologies) and sequenced using M13 forward and reverse primers. The plasmid generated (pAM3417) was used to transform wild-type *S. elongatus* (see **Note 14**). The Sm-resistant cyanobacterial strain was frozen in our laboratory as AMC1373.
2. Clone the mutated version of the DNA to be modified in the cyanobacterial chromosome in a proper vector (see **Note 15**) including flanking sequence around the mutation target site for efficient recombination (see **Note 3** and **Subheading 3.1.1.**).
3. Introduce, inside the DNA to be modified (cloned in **step 2**), the construction that contains *rps12*-6803 driven by the promoter of the *psbAI* gene (from *S. elongatus*) next to a Km-resistance cassette (5,6).
4. Use the plasmid constructed in **step 3** to transform the Sm-resistant *S. elongatus* created in **step 1** (see **Subheading 3.1.1.**). Select for the Km-resistant clones.
5. Make a replica of the Km-resistant clones on a fresh BG-11M plate containing 10 µg/mL Sm. Retain the Km resistant and Sm-sensitive clones.
6. Grow one Km-resistant and Sm-sensitive clone in liquid BG-11M; introduce by transformation (see **Subheading 3.1.1.**) the plasmid constructed in **step 2** and select for the Sm-resistant clones. By homologous recombination, this construction will replace *rps12*-6803 and the Km-resistance cassette by the final mutant product.

7. Make a replica of the Km-resistant clones in a fresh BG-11M plate containing Km. Retain the individual clones that are resistant to Sm and sensitive to Km.
8. Grow the colonies in liquid BG-11M, extract the DNA (see **Subheading 3.1.3.**), and confirm the presence of the mutation by PCR.

4. Notes

1. http://genome.jgi-psf.org/finished_microbes/synel/synel.home.html and <http://www.bio.tamu.edu/synecho/>.
2. The usable ranges of antibiotics concentrations in BG-11M for which *S. elongatus* cells are resistant are: 5–20 $\mu\text{g/mL}$ Km, 1–2 $\mu\text{g/mL}$ Gm, 7.5–10 $\mu\text{g/mL}$ Cm, 5–20 $\mu\text{g/mL}$ Sp, and 2–10 $\mu\text{g/mL}$ Sm. Because of the spontaneous occurrence of Sp-resistant cells, we use Sp and Sm together, e.g., 2 $\mu\text{g/mL}$ Sp + 2 $\mu\text{g/mL}$ Sm.
3. The efficiency of chromosomal recombination increases with the length of the homologous sequences flanking the desired insertion (2,9). The length of a homologous sequence on one side can be decreased by increasing the length of flanking sequence at the other side.
4. In *S. elongatus*, the single crossover event occurs at a much lower frequency than a double-recombination event; therefore, entire plasmid integration is not observed unless it is specifically selected. When single recombination occurs, the integration of the plasmid at the site of recombination causes duplication of the gene of interest (see Fig. 1D). If a gene intended to be inactivated via double recombination is essential for the cell, normally the single recombination event survives the selection, ensuring that a wild-type copy of the gene remains in the chromosome. Alternatively, sometimes the double-crossover event survives selection, but a mixed population of wild-type and mutant chromosomes persists in the cell. The frequency of single-recombination events can be boosted by orders of magnitude by introducing the plasmid via conjugation rather than transformation (11).
5. 1 Einstein = 1 mol of photons.
6. The volumes used for transformation are not critical and can be modified depending on the cell density. The number of transformants increases with the number of cells used for transformation (1).
7. According to our experience, segregation is normally complete when inactivating a gene in *S. elongatus* PCC 7942. If a particular gene is not segregated, it could mean that is essential and can not be eliminated from the cell.
8. We introduce the cargo plasmid into the *E. coli* strain AM179 (Cm-resistant) which contains the helper plasmid pRL528. The conjugal plasmid (RP-4) is in the strain AM076 (Ap-, Km-, and Tc-resistant) (3).
9. To create a dim-light environment, we wrap the plates with one layer of cheese-cloth and place them in the lighted incubator.
10. Sometimes a lawn grows within 2 d after the cells have been plated on BG-11M with the antibiotic. Normally, some very green colonies start showing up from the lighter background after a few days. Isolate these colonies and restreak them on a fresh plate containing the antibiotic. Also, take some colonies from the background and restreak them as well, to make sure that they die on fresh media.

11. NS I, NS1, GenBank accession number U30252; NS II, NS2, GenBank accession number U44761.
12. The *lacI-lacO* repression system is effective in *S. elongatus*. We typically use an *E. coli* *P_{trc}* promoter followed by one or two *lacO* operator sequences. The *lacI* gene is engineered in *cis*. Isopropyl- β -D-thiogalactopyranoside induces expression proportionately to inducer concentration. However, the promoter is not completely “off” in the absence of inducer. As a consequence, a basal expression from this promoter is always observed. Uninduced expression is typically sufficient for complementation of null mutants, and induced expression reveals dominant-negative effects of a cloned gene.
13. In our laboratory, three versions of the plasmid used for hit-and-run were obtained from Dr. C. P. Wolk. They carry Km (pRL278, GenBank accession number L05083), Sp (pRL277, accession number L05082), or Cm resistance (pRL271 accession number L05081) for positive selection.
14. In this case, the transformation was performed as described in **Subheading 3.1.2.**, except that after the transformation process the cells were plated in BG-11M without antibiotic and kept in the incubator overnight. The next day, the proper dilutions of Sm to yield final concentrations of 10, 5, and 2.5 $\mu\text{g/mL}$ were added under the agar and the plates were kept in the incubator until single colonies appear (additional information about underlying antibiotics is described elsewhere [2]). All plates yielded Sm-resistant colonies. Two colonies from each plate were grown in liquid BG-11M with 10 $\mu\text{g/mL}$ of Sm and their DNA was extracted as described in **Subheading 3.1.3.** The *rps12* gene from the clones was amplified by PCR and the product sequenced using the same reverse and forward primers. All clones showed the expected mutation (an A-to-G change at position 128 of the open reading frame) as the only change introduced into the *rps12* gene.
15. As *rps12-6803* along with a Km-resistance cassette will be introduced inside the gene to be mutagenized, is convenient that the vector chosen not have a Km-resistance marker to simplify the selection process in *E. coli*.

Acknowledgments

We thank C. P. Wolk for provision of vectors and protocols on which many of these methods are based, as well as past and present members of the S. S. Golden laboratory who contributed to protocol development, and M. Matsuoka for providing the plasmids and cyanobacterial DNA for *rps12*-based gene replacement. We especially thank S. R. Mackey for assistance with the manuscript. The research of the authors was supported by a postdoctoral fellowship to J. L. D. (NSF PA 99-025) and grants from the NIH (P01 NS39546 and R01 GM62419) DOE (DE-FG02-04ER15558), and NSF (MCB-0235292) to S. S. G.

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Novel Strategies for Identification of Clock Genes in *Neurospora* With Insertional Mutagenesis

Kruno Sveric, Moyra Mason, Till Roenneberg, and Martha Merrow

Summary

As the molecular mechanism of circadian clocks has reached high complexity, the fungal model system, *Neurospora crassa*, is increasingly important for clock research. It offers the possibility of extensive biochemical experimentation and thorough description of circadian properties. Realization of the full potential is dependent on efficient, high-throughput methods. We have combined several protocols to develop abundant and inexpensive production of mutants, and subsequent identification of the affected gene. We applied a novel screening protocol and, after screening several hundred mutants, identified a known clock gene, *frequency*. Furthermore, the methods described here can easily be adapted to various insertional constructs (e.g., those with alternative selection markers or that facilitate overexpression) or combined with strains carrying clock-regulated reporter genes.

Key Words: *Neurospora crassa*; fungi; circadian; mutagenesis; clock; entrainment.

1. Introduction

Neurospora has been a model organism for basic research on genetics for more than half a century. “She” is the mother of the “one gene, one enzyme” hypothesis (**1**). Accordingly, there are a number of well-described and easy ways in which to make mutants. Probably the most common methods include ultraviolet-light and chemically induced mutagenesis (*see* **Note 1**) of asexual spores, or conidia. Insertional mutagenesis, however, offers the possibility for rapid identification of the mutation through the rescue of the tagged DNA. Our method uses transformation of a resistance gene (to “Basta”) into conidia via electroporation. The mutants are selected for growth, screened for circadian properties, and “purified” to homokaryons. Once strains are confirmed to have single insertion sites, the location of the insertion is identified by a combina-

tion of mapping, partial cloning, and polymerase chain reaction (PCR). The identification of an insertion in a known clock gene has confirmed the general approach.

2. Materials

1. Solid minimal media: 1X Vogel's salts (**2**; *see also* Chapter 32), 2% glucose, with 2% agar (Difco).
2. 1 M Sorbitol.
3. 10X FIGS: 1 M L-sorbose, 10 mM D-fructose, 10 mM glucose.
4. Bottom agar: 1X Vogel's salts, 1.5% agar (Difco), 0.3% L-arginine, 1.5% Basta (also called ignite, glufosinate, or phosphinothricin), 1X FIGS. Add FIGS as a sterile solution following autoclaving of the rest of the formulation. About 25 mL of bottom agar is added per 87-mm Petri dish.
5. Top agar: 1X Vogel's salts, 1.0% agar (Difco), 1 M sorbitol, 1X FIGS. Add FIGS as a sterile solution following autoclaving the rest of the ingredients.
6. Slants containing minimal media and Basta: 70 × 10 mm glass test tubes with 0.5 mL minimal media and 0.4% Basta, autoclaved and cooled slanted so that the agar solidifies at an angle, leaving an increased surface area for growth.
7. Race tube media (**3**): 1X Vogel's salts, 0.3% glucose, 0.5% L-arginine, 10 mg/mL biotin, 2% agar (Difco).
8. Iodoacetate media (**4**): 0.1X Westergaard's salts (**5**; *see Note 2*), 0.1% sucrose, 2% agar (Difco), 1 mM iodoacetate. Add iodoacetate from a sterile 100 mM stock solution after autoclaving the rest of the formulation.
9. Sorbose media: 1X Vogel's salts, 0.05% glucose, 0.05% fructose, 2% sorbose, 2% agar.
10. Liquid minimal media: 1X Vogel's salts, 2% glucose.
11. Cetyltrimethylammoniumbromide (CTAB) buffer: 2% CTAB, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 1% sodium bisulfite.
12. C/IAA: 24:1 v/v, chloroform:isoamyl alcohol.
13. Primers: bar2.1, TCA AGC ACG GGA ACT GG; bar2.2, CAG CCT GCC GGT ACC GC; T7, TAA TAC GAC TCA CTA TAG GG; T3, AAT TAA CCC TCA CTA AAG GG.

3. Methods

The protocols described include (1) preparation of electrocompetent conidia; (2) their transformation and selection; (3) an example of a screening protocol; (4) purifying homokaryons; and (5) methods for rescuing the inserted DNA for identification of the mutation.

3.1. Preparation of Electrocompetent Cells

1. Grow the conidia on about 100 mL of solid minimal media after it has been autoclaved in a large (500-mL or 1-L) flask.

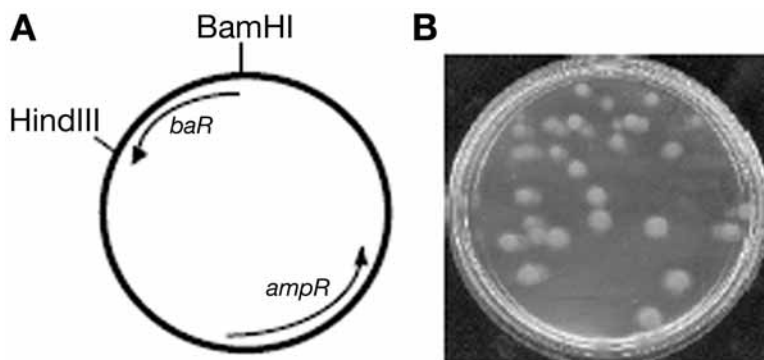


Fig. 1. Transformation of *Neurospora* with a plasmid that yields Basta-resistant colonies. (A) pKSbar2 was used for insertional mutagenesis. It is the standard Bluescript vector pKS containing a Basta resistance cassette (see **Note 5**). (B) Two (or more, as shown here) days following transformation, resistant colonies are picked and transferred to slants.

2. Harvest by adding 50 mL of sterile 1 *M* sorbitol and gently mixing on a shaker for 5 to 10 min (see **Note 3**).
3. Filter the conidia through several layers of sterile cheesecloth to remove hyphae.
4. Centrifuge the conidia at 2000*g* for 10 min at 4°C and resuspend in 50 mL of 1 *M* sorbitol. This process is repeated four times. The concentration of conidia is measured and adjusted to 2.5×10^9 /mL (see **Note 4**). Keep on ice until ready for use.

3.2. Transformation and Selection

1. Combine 40 μ L of the conidial suspension (about 10^8 conidia) and 1 μ L of linearized pKSbar2 (1 to 5 μ g; see **Note 5** and **Fig. 1A**) in a 1.5-mL microcentrifuge tube. Incubate on ice for 5 min. Linearized plasmid is inserted more efficiently in the genome than circularized plasmid (6).
2. Place this suspension in an ice-cold, 0.2-cm-wide cuvet (previously stored in a -20°C freezer) and electroporate using a Bio-Rad Gene Pulser with settings of 1.5 kV/cm, 25 μ F, and 600 Ω (7).
3. Recover the cells by gently combining with 1 mL of 1 *M* sorbitol for 10 to 20 min on ice.
4. Dilute 10 μ L of this solution in 10 mL of molten top agar, held at 50°C, and immediately pour over a Petri dish with bottom agar containing the selective agent Basta.
5. At 30°C, colonies will appear after 2 d (**Fig. 1B**). Pick colonies with a sterile needle and place them on slants containing minimal media and Basta. A negative control plate, containing conidia electroporated with no added DNA, should yield no colonies.

3.3. Screening Mutants

Following 7 d of growth on slants, the mutant colonies will have developed sufficient conidia for inoculation of race tubes for screening. For functional screening, Basta is generally excluded from the media. As for protocols, we noted that—to this point—*Neurospora* clock mutagenesis screens have used only constant conditions, thus limiting the results to the discovery of mutants with an altered free-running period or arrhythmicity. Screens utilizing entrainment theoretically allow for the possibility of identifying “phase of entrainment” mutants (such as so-called larks and owls). Some of these could have a normal free-running period and would be missed if the screen is performed in constant conditions, although they are potentially interesting mutants. We therefore determined to perform this screen under entraining conditions. Furthermore, as we are interested in looking for novel clock genes, and all the known *Neurospora* clock genes are involved in light signaling, we additionally introduced two unusual features: first, we screened in temperature cycles in darkness, and second, the cycle was 16 h long. With an amplitude of 5°C (between 22 and 27°C), with 8 h at both high and low temperatures, the *bd* strain entrains (Fig. 2; see Note 6), with the onset of conidiation occurring 2 to 3 h into the warm phase (8). In a cycle just 1 h shorter, entrainment occurs in only a few cases; rather, a free-run with relative coordination is observed. Thus, a 16-h cycle is close to the limit of the range of entrainment (9), and represents a challenge to the clock system.

1. Inoculate mutants and controls using a single race tube per isolate. Germinate overnight in the laboratory under constant light and transfer the tubes to the temperature cycle setup (8), screening under the entrainment protocol for about 1 wk. Mark the conidiation band on each race tube daily and finally analyze the circadian parameters with the CHRONO program (10).
2. Screen mutants with an abnormal phenotype (early or late conidiation compared with controls, or arrhythmicity) for a second time, using a pair of race tubes per mutant. About 10% of all isolates are usually rescreened.
3. To characterize the phenotype further, screen for free-running period under constant darkness and phase of entrainment in light–dark cycles.

3.4. Purifying Homokaryons

Once an interesting mutant is confirmed, its rescue is initiated. Most *Neurospora* conidia have two to three nuclei, so the transformed conidia can be heterokaryons. Growth on selective media (see Note 7) generally favors Basta-resistant nuclei, but mutants still must be purified to homokaryons. There are at least two methods that can be used for this purpose.

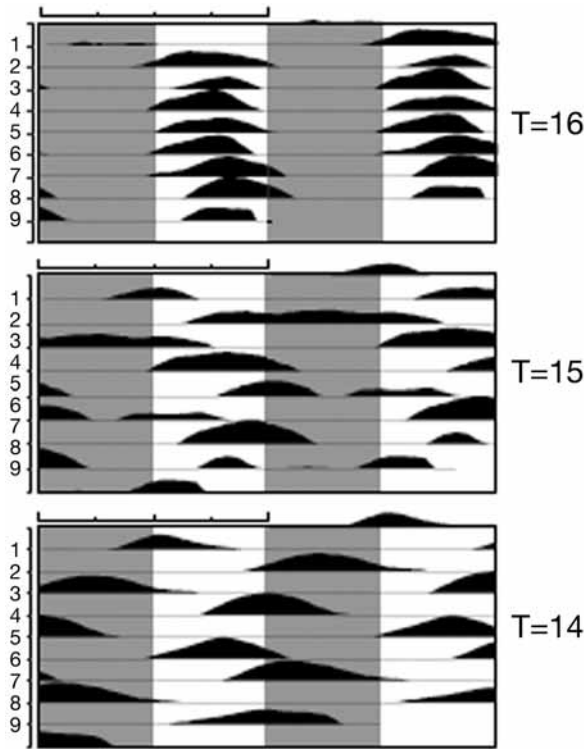


Fig. 2. Entrainment in short temperature cycles. In cycles with equal periods of cold (22°C) and warm (27°C) incubation, the *bd* strain entrains when the cycle length (T) is equal to or greater than 16 h. In a 15-h cycle ($T = 15$), stable entrainment rarely occurs, but sometimes relative coordination is observed. In still shorter cycles—but still substantially longer than half of the free-running period—the data show almost a free run, despite the imposition of a temperature cycle (a 14-h cycle is shown here).

3.4.1. Plating Assay

1. Suspend conidia to a concentration of about 1000 per mL.
2. Combine 100- μ L aliquots (i.e., 100 conidia) with 10 mL of top agar. Pour the top agar over plates that already contain bottom agar with and without Basta.
3. Grow colonies for 2 to 3 d. Compare the number of colonies on several sets of plates with and without the selective agent. If the numbers are equal, then the conidia are homokaryotic. If they are skewed (more colonies growing on plates with no selection), pick resistant colonies, grown on fresh slants, and reassay by this plating method until equal numbers of colonies grow with and without selective pressure.

3.4.2. Induction of Microconidia

Neurospora can be induced to produce microconidia, which contain a single nucleus.

1. Boil cellophane in 1% KOH to remove impurities, rinse thoroughly to remove salts, and autoclave between layers of filter paper.
2. Inoculate the heterokaryotic mutant strain underneath a layer of cellophane onto iodoacetate media (4). This is accomplished by puncturing the cellophane with a sterile needle in the center of the plate, and then inoculating through the hole. Incubate the Petri dishes in humidified conditions at 22 to 25°C for 10 d in the laboratory.
3. Remove the cellophane and wash the surface of the agar with 1 mL of sterile water prior to returning the dishes to the humidified incubation chamber for additional 24 h.
4. Harvest the microconidia with sterile water and plate on sorbose medium. Additional microconidia will develop with another 24 h incubation.

3.5. Rescuing Insertion to Identify Mutation

Once an interesting mutation is purified to homokaryonicity, identifying the gene that carries the insertion is initiated. An initial step in this regard is to determine how many insertions are found per mutant. This could be determined either by performing a sexual cross and following transmission of the trait using classical genetics, or more simply, with a Southern blot (described here).

1. Inoculate the conidia of each mutant line into liquid minimal medium. After achieving a tissue mass of about 1 cm³, blot the mycelia dry on paper towels and freeze in a -70°C freezer.
2. Grind the mycelia in liquid nitrogen using a mortar and pestle with the aid of a pinch of sand and suspend the powder in 0.5 mL of CTAB buffer.
3. Vortex and incubate at 60°C for 30 min.
4. Add 0.5 mL of C/IAA and extract the genomic DNA for 5 min with mixing.
5. Spin at 10,000g for 30 min at room temperature.
6. Collect the supernatant in a clean tube and repeat **steps 4 and 5**.
7. Add 1 µL of RNase A (10 mg/mL) and incubate the mixture for 10 min at 37°C.
8. Precipitate the DNA with isopropanol at room temperature for 30 min. Collect the precipitate by centrifugation for 10 min at 10,000g, and wash it once with 70% ice-cold ethanol. After drying the pellet, the DNA can be suspended in 50 µL water and quantitated by standard methods.
9. Digest 5 to 10 µg of genomic DNA with *Eco*RI, which does not cut within the pSKbar2 plasmid, thus yielding a single band per insertion.
10. Following digestion, separate the DNA on a 1% agarose gel, and blot according to standard methods.

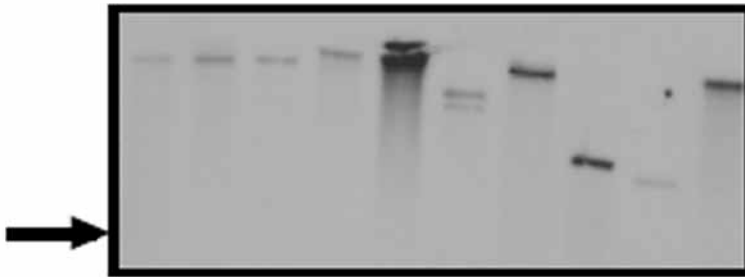


Fig. 3. Southern blot analysis of insertions. Genomic DNA of 10 mutants was digested with *Eco*RI, which does not cut within the inserted plasmid sequence. Strains with single inserts have one band (first and last four lanes). The mutants in the middle two lanes carry two copies of the Basta-resistant gene. The arrow indicates a molecular weight of 4 kbp, the size of the plasmid alone.

11. Using primers bar2.1 and bar2.2 and the pKSbar2 plasmid as template DNA, amplify the Basta resistance cassette by PCR.
12. Label the PCR product by random priming using either radioactivity or digoxigenin, and then use it to probe the blot.

Insertional mutagenesis often yields a single insertion, but there can also be several (see **Fig. 3**). In the latter case, there may be multiple insertions spread throughout the genome or at a single site. Mutants with a single insertion are suitable for rescue. To identify the insertion, we used a combination method, starting with size selection of genomic fragments, ligation, and PCR. The fragments are ligated into a vector and the mixture is subjected to PCR using known sequences from the Basta resistance gene and within the vector (see **Fig. 4** and **Note 8**).

1. Map the area of the insertion by standard methods (i.e., Southern blot), digesting the genomic DNA of the mutant with several restriction enzymes, which do not cut within the inserted plasmid, in conjunction with *Hind*III, which is known to cut within the Basta resistance gene (the minimal fragment that must be inserted for the selection). The primary goal is to identify a fragment that runs from the known *Hind*III site in the resistance gene (see **Fig. 1**) into the disrupted gene. A fragment with two distinct sticky ends (only one of which is *Hind*III) must be identified. Also, the size of the fragment should be big enough to deliver sequence information but small enough to ligate efficiently.
2. Gel-purify the restricted fragments of genomic DNA that run with the appropriate molecular weight (see **Note 9**) and ligate them overnight into a plasmid vector (i.e., pSK-II) using standard methods (see **Note 10**).
3. Use 1 μ L of the ligation reaction as the DNA template for a PCR reaction, which employs a primer for the Basta resistance gene on one end (bar2.1), and a primer

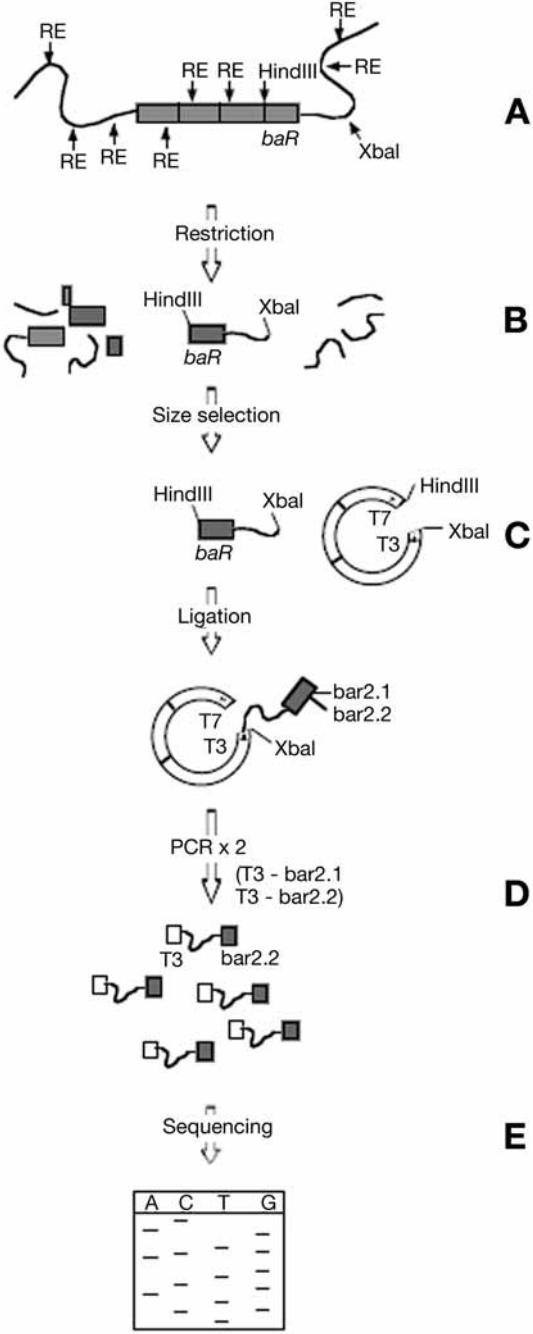


Fig. 4

for either T3 or T7 sites on the other. Thanks to the mapping work, the orientation of the fragment with respect to the vector is known, as well as the predicted size of the PCR product.

4. Utilize 0.5 μ L of the first PCR as a template for a second PCR reaction, using a primer nested in the Basta resistance gene (*bar2.2*). A major fragment of the expected size should be apparent. This can be purified and sequenced. The product can be compared to the known plasmid sequence, as well as to the mapped DNA.

3.6. Case Study

One test of a mutagenesis protocol is the rediscovery of previously described mutant alleles. On screening the first 800 Basta-resistant strains, a mutant (KOMO 303) showed a phase of entrainment that was opposite that of wild-type in the 16-h temperature cycle protocol (22 to 27°C). In a light–dark cycle using 3 μ mol of photons/m²/s, it completely failed to entrain, and in constant darkness, it was arrhythmic (*see* Fig. 5). These phenotypes resemble the null *frequency* mutant, *frq*⁰, and also the *white collar* (*wc*) mutants. The *wc* mutants are blind for light-induced mycelial carotenoids, in addition to having clock defects (11,12).

1. We determined that the mutation was not likely in the *wc* genes by a simple light-induced carotenoid assay using mycelial pads, showing near-normal levels. The *frq* locus was investigated for insertion of the Basta-resistance cassette with Southern analysis, revealing insertion in the open reading frame, in approximately the same location as the *frq*⁰ mutation is found (13). Furthermore, the Southern analysis showed a clear double insertion (i.e., two copies) of the drug resistance marker at this site.
2. One of the first insertions, KOMO 58, rescued as described in Subheading 3.5., was further characterized by amplifying the insertion site from the genomic mutant DNA. Primers were designed using the genomic DNA sequence predicted to flank the insertion. A positive result (a robust fragment of an appropriate molecular weight) would indicate that the drug-resistance marker is inserted with no large deletions or translocations. A negative result (no fragment) would indicate that genomic DNA was lost in the recombination leading to insertion, or that a translocation occurred. These possibilities can be finally determined with

Fig. 4. (*opposite page*) Flow diagram of insertion rescue protocol. (A) The area surrounding the insertion is mapped by restriction digestion and Southern blotting. (B) The genomic DNA is digested and the desired fragment is enriched by size selection. (C) The pool of fragments is ligated into pSK-II. (D) The appropriate ligation product is amplified with nested polymerase chain reactions, and gel-purified. (E) The fragment is sequenced. RE, restriction endonuclease site; *Xba*I and *Hind*III, restriction enzymes; T3 and T7, standard RNA polymerase sites used for primer binding; *bar2.1* and *bar2.2*, primers specific for the Basta-resistance gene, used in this protocol.

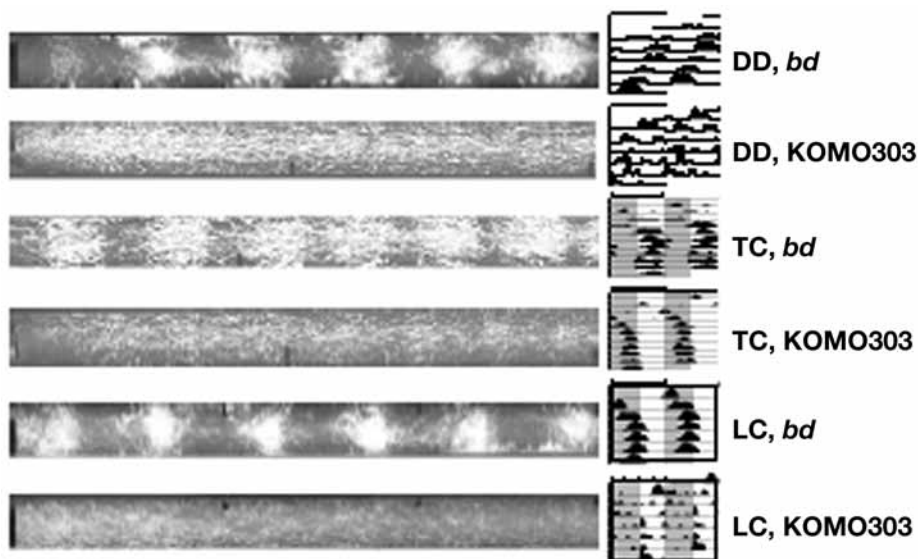


Fig. 5. A *frq* mutant was generated by insertional mutagenesis. Race tubes and double plots for a *frq* mutant generated in this study are shown here. The mutant is compared with the *bd* strain in constant darkness, a 16-h temperature cycle (TC), and a 12-h light cycle (LC). For each condition a control and a mutant race tube is shown, as well as a sample double plot. The gray areas in the plots indicate the cold (TC) or the dark phase (LC). Note the phase of entrainment of KOMO303 in the temperature cycle is approximately opposite that of *bd*. Furthermore conidiation is not stimulated by (or driven by) the onset of the cold phase. KOMO303 completely fails to entrain in light cycles. These phenotypes are consistent with those for the *frq*^o strain (8). KOMO303 was determined to be a *frq* mutant by Southern blotting (data not shown).

Southern blot or by rescuing the other end of the insertion. In either of the latter cases, the mutant is much less interesting, as it is almost impossible to determine where the mutant phenotype derives from. In the case of KOMO 58, the fragment generated in the PCR reaction was larger than would have been predicted. Sequence analysis showed that there was a duplication in the inserted plasmid (an extra copy of the ampicillin resistance gene was present), whereas the genomic DNA was intact except for a few nucleotides at the junctions.

3. To finally determine that the insertion is acting through a specific gene, the gene should be knocked out (13) and the null mutant strain then assayed for clock characteristics. If the gene is essential, an overexpression construct can be engineered using the inducible *qa-2* promoter (14) and transformed ectopically. After growing in the presence of an inducer, functional tests can be carried out in its absence.

4. Notes

1. Mutagenesis with ultraviolet light can be accomplished by diluting conidia in a sterile Petri dish and placing on a transilluminator for increasing amounts of time until 70% of the conidia do not survive (15). Chemical mutagenesis uses standard methods as described (16).
2. The formulation for 1X Westergard's media is: 10 mM KNO₃, 74 mM KH₂PO₄, 2 mM MgSO₄·7H₂O, 20 mM NaCl, 2.5 mM CaCl₂, and 0.5 mL of the trace elements used in preparation of Vogel's media. Whereas the stock solution is usually made up to 5X, the concentration required for microconidia production is 0.1X.
3. The conidia are hydrophobic; this step wets the conidia so that they will stay in solution better.
4. The concentration of conidia is determined by measuring the optical density at 420 nm. An optical density of 1.0 = 2.86×10^6 /mL.
5. The plasmid, pKSbar2, was donated by U. Schulte. For its construction, pBARKS1 (17) was cut with XbaI and SpeI restriction enzymes. Ends were filled and the fragment containing the *Bar* gene (for resistance to Basta) was inserted into pBluescript II KS, cut with the enzymes *Hinc*II and *Sma*I.
6. The *bd* gene is essential for formation of a clear conidiation band, which is the circadian phenotype under analysis.
7. We grow our selected transformants on solid media for convenience. Immediate transfer to liquid media, instead, results in more rapid growth (conidia can outgrow the liquid media in 2 d rather than 1 wk), thus decreasing the time needed for this step. Liquid media may also promote homokaryon formation.
8. We have developed this method to avoid certain pitfalls, including the possible loss of the ampicillin-resistance gene on insertion; otherwise we could simply cut the mutant DNA with EcoRI and circularize, transform bacteria, and select for ampicillin-resistant plasmids.
9. The fragment of interest is copurified together with many unrelated genomic DNA fragments, which will contribute to background. These "contaminating" fragments can be minimized by cutting with additional enzymes (those that do not disrupt the identified fragment, i.e., that cut outside this region). There will be fewer nonspecific fragments, and they will have different restriction sites, thus forming fewer ligation products that will amplify in the PCR reaction. The enzymes for this job are already known from the mapping work. This must be done strategically, as it could—under certain circumstances—actually increase contaminating fragments. See ref. 18 for help with the equations.
10. We have found that the efficiency of this ligation reaction is poor. By using PCR to amplify the ligation product we need only one side joined, increasing the efficiency of this step.

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Mutagenesis With *Drosophila*

Patrick Emery

Summary

Forward genetics in *Drosophila* has profoundly affected our understanding of circadian rhythms in this organism and, more generally, in the animal kingdom. Most *Drosophila* pacemaker genes were discovered through the isolation of gene variants affecting the free-running period of the circadian pacemaker. There are different ways to mutagenize flies. An alkylating agent can be used to randomly alter the fly genome, or transposable elements can be mobilized to disrupt or increase the expression of the targeted genes. The advantages of these different methods are complementary.

Key Words: Forward genetics; mutagenesis; ethyl methane sulfonate; P-element; misexpression.

1. Introduction

The use of *Drosophila* genetics to study animal behavior was pioneered by Benzer in the late 1960s. The isolation of three *period* alleles by Konopka in his lab marks the beginning of the elucidation of the molecular mechanisms generating circadian oscillations (1). Indeed, forward genetics has proven to be a method of choice to identify pacemaker genes (2–8). The principle is simple: flies are mutagenized randomly and mutants with abnormal circadian behavior period length in constant darkness are isolated.

Other types of circadian screens have been performed. For example, a luciferase-based screen has identified a photoreceptor mutant that would not have been identified with a regular constant-darkness screen (9). Most often, chemical mutagenesis has been used (see **Subheading 3.1.**). It has two great advantages: it is entirely random and can generate total loss-of-function and partial loss-of-function mutations. This was critical to the isolation of *double-time*, whose null alleles are homozygous lethal (3). Gain-of-function mutations can also be isolated with this approach. However, the disadvantage of the method resides

in the necessity to map a point mutation in the midst of 200 megabases of DNA. This is fortunately not as difficult as it used to be: the completion of the *Drosophila* genome sequencing, the male recombination method of mapping, and the still-growing number of gene disruption mutants and other transposon insertions facilitate this task considerably (*see* **Note 1**). Other mutagenesis approaches make use of P-elements. These transposons can be inserted pseudorandomly in the genome. P-elements prefer to target the 5'-region of genes, and target some regions of the genome preferentially. It is therefore not a totally unbiased approach. The great advantage of P-element mutagenesis is that there is no need for labor-intensive gene mapping (unless the P-element is not responsible for the mutant phenotype, as was the case for *tim*⁰; *see* **ref. 2**). Indeed, the site of insertion can easily be recovered by inverted PCR (*see* **Note 2**). P-elements are most frequently used as disruptive elements (*see* **Subheading 3.2.**). Their insertion in a target gene (regulatory or coding regions) usually results in a decrease or the complete suppression of its expression. These insertions are therefore frequently more disruptive than ethyl methane sulfonate (EMS)-generated point mutations. P-elements can also be used to misexpress the genes they are targeting (*see* **Subheading 3.3.** and **Fig. 4**; **ref. 10**). The P-element for this type of screen is called EP. It contains UAS binding sites upstream of a minimal promoter. These sites are recognized by the yeast transcription factor GAL4, which does not affect circadian rhythms when expressed in flies. GAL4 can be expressed only in tissues with circadian rhythms, for example with a *timeless* (*tim*) promoter (**6**). Using a tissue-specific GAL4 driver reduces the risk of lethality resulting from the misexpression of the targeted gene. In most cases, the EP-element is going to jump in the 5'-end of the targeted gene. If it is in the correct orientation, its activation by GAL4 will result in the overexpression of the targeted gene. In rare cases, the P-element is inserted in a reverse orientation, downstream of the transcriptional initiation site. This should result in downregulation of gene expression. This screen is thus for the most part a gain-of-function screen.

2. Materials

1. Incubator with light and temperature control.
2. Behavior monitoring system (for example, from Trikinetics, Waltham, MA): monitors, computer, data collection software, and data analysis software.
3. Ethylmethane sulfonate (EMS; *see* **Note 3**).
4. Fly strain with wild-type normal circadian behavior (e.g., *ry*⁵⁰⁶; *see* **Note 4**).
5. Attached-X female stock for X chromosome screen.
6. Balancer stocks (e.g., *TM3/TM6B* or *CyO/Sco*) for second or third chromosome screen.
7. Original P-element insertion (best on a balancer chromosome) with a *mini-white* gene marker (*see* **Notes 5** and **6**).

8. Fly line containing the transposase gene ($\Delta 2-3$) in a *w* genetic background.
9. Balancer strains in a *w* background.

3. Methods

3.1. Chemical Mutagenesis

3.1.1. EMS Treatment

1. Place three layers of blotting paper at the bottom of at least two empty fly vials (without food).
2. Add 30 male flies from the original “wild-type” strain (3 to 5 d old) into each vial.
3. Prepare 5 mL of a solution containing 10 mM EMS, 1% sucrose, and 1% red food color (*see Note 7*).
4. Use a syringe with a needle to soak the blotting papers with the EMS solution. There should be a little excess of liquid.
5. Leave the flies to feed overnight on the EMS solution.
6. In the morning, transfer the males to an empty vial (with food) and let them recover for 1 to 2 h.

3.1.2. X-Chromosome Screen (*F1 screen*; **Fig. 1**)

1. Cross the EMS-treated males with attached-X virgin females. In these females, the two X chromosomes cannot dissociate and segregate together during meiosis. The result is that their gametes contain either two X chromosomes or none. Hence, the male progeny receive their X from the father (instead of the mother), and the female progeny their attached X from the mother. Remove the males after 4 d.
2. The F1 males from this cross can be screened directly for the desired circadian phenotype (e.g., abnormal constant darkness behavior; *see Note 8*). Those that exhibit abnormal rhythms can then be crossed individually to attached-X females and the progeny of these crosses retested to confirm their mutant phenotype.

3.1.3. Recessive Second or Third Chromosome Screen (*F3 Screen*; **Fig. 2**)

1. Cross the EMS-treated males to second or third chromosome balancer female virgins. Balancer chromosomes do not undergo recombination owing to multiple chromosomal abnormalities. Some balancer chromosomes “balance” only a part of the chromosome. *CyO* and *TM3* are fully balanced second and third chromosome balancers, with easy marker for selection (curly wings and short bristles, respectively). Remove the males after 4 d.
2. Take individual males in the F1 progeny and cross them to three virgin balancer females.
3. For each individual cross, select and cross F2 males and virgin females (heterozygous for the mutated chromosome and a specific balancer).
4. Monitor the circadian behavior of F3 males homozygous for the mutated second or third chromosome.

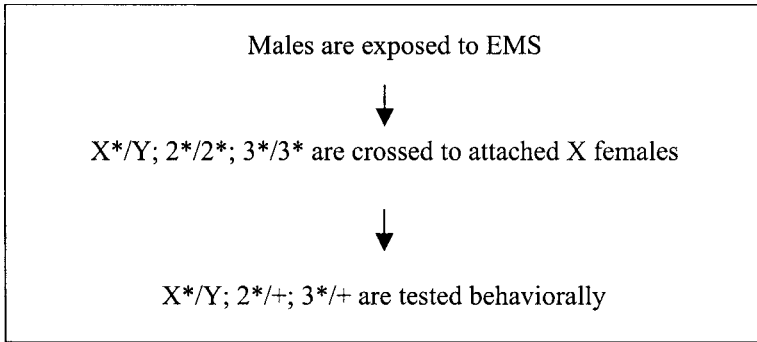


Fig. 1. F1 screen on the X chromosome. Note that a dominant mutation on autosomes can be detected too. The fourth chromosome, very small, is ignored. * indicates a mutagenized chromosome, + a wild-type chromosome.

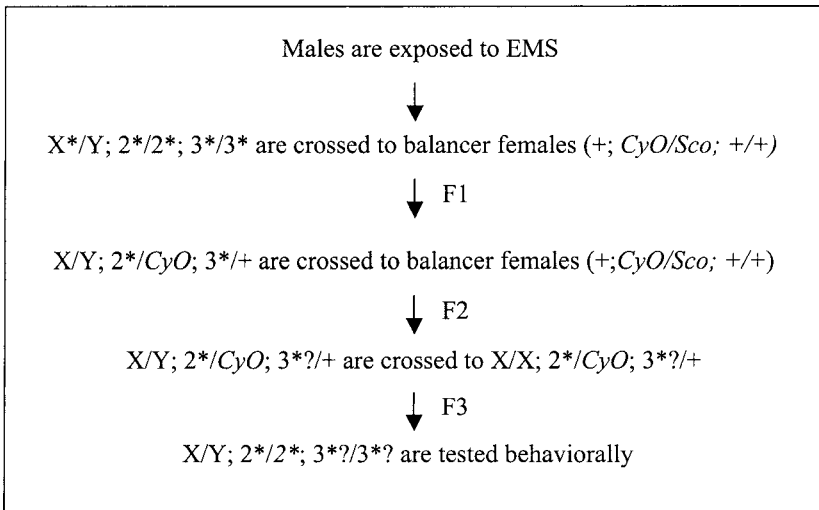


Fig. 2. F3 screen on the second chromosome. Note that the mutated X chromosome cannot be present in the F1 males, and that in rare cases a mutation on the third chromosome (or on the small fourth, not shown here) can be detected. *, mutagenized chromosome; *?, ambiguity on whether the chromosome is a mutagenized one or not; +, wild-type chromosome. Second and third chromosomes can also be screened simultaneously by using females with attached second and third chromosome balancers (see **Subheading 3.2.**).

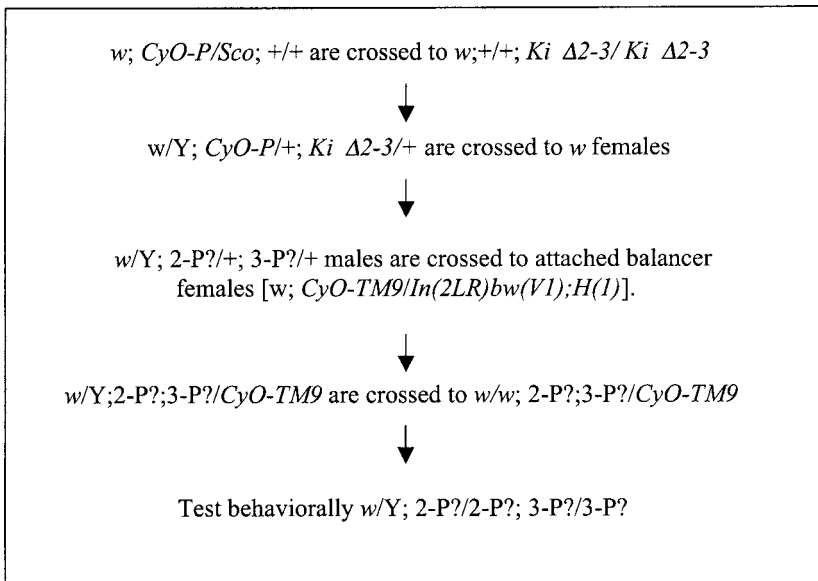


Fig. 3. Recessive P-element screen on the second and third chromosome: This screen strategy targets specifically the autosomes, but whether the second or third chromosome is targeted is not known (2-P? and 3-P? indicate the ambiguity on the P element location). The P-element location can be determined after the screen by inverse polymerase chain reaction (see **Notes 2** and **8**). For clarity, the small fourth chromosome is ignored.

3.2. P-Element Mutagenesis (Fig. 3)

The method presented here is an example of strategy for screening P-element insertions on the autosomes. Different strategies can be used, depending on the scope of the screen. However, the first steps are always the same: obtaining flies in which a P-element defective for transposition is in the presence of the transposase, and then removing the transposase gene at the next generation so that no further transposition occurs.

1. Cross males or virgin females containing a P-element insertion (with the *mini-white* marker) on a balanced chromosome (for example, *CyO*) in a *w* background with *w* virgin females or males expressing the transposase (see **Notes 5** and **6**). The transposase gene usually comes on the third chromosome—for example, with a dominant marker like *Ki* (short kinked bristles).
2. From this cross, take single males with the *CyO* chromosome (curly wings, mosaic orange eyes) and the *Ki* marker and cross them to *w* females (to keep the X chromosome insertions; see **Note 9**). In the gametes of these males, transposition is occurring.

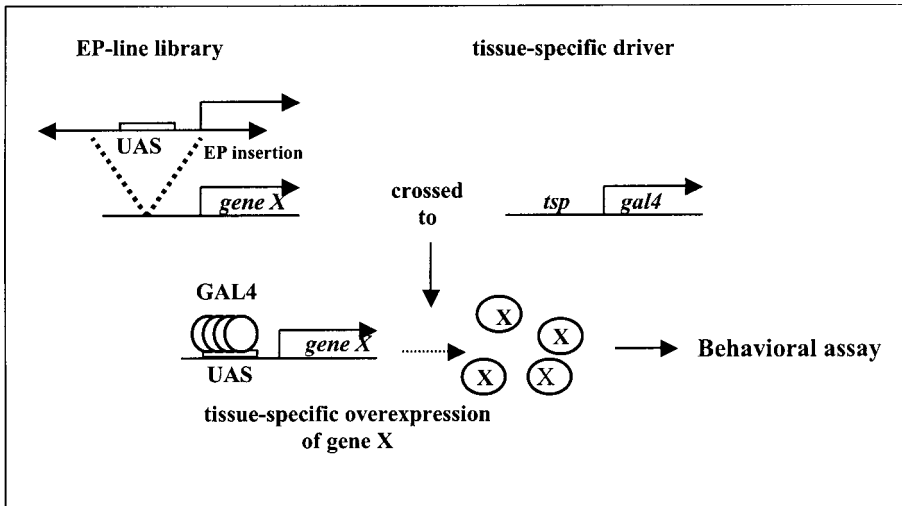


Fig. 4. Misexpression screen strategy. Flies containing the *GAL4* cDNA under the control of a tissue-specific promoter (*tsp*; for example, *tim*) are crossed to a library of flies with a pseudorandomly inserted EP element. In the progeny of these crosses the targeted gene is, in most cases, upregulated. However, if the EP element is inserted downstream of the transcription initiation site, and in an antisense orientation, downregulation should occur.

3. In the next generation, select the males with yellow, orange, or red eyes, straight wings, and normal bristles. These males contain the P-element in a new autosomal insertion site, and do not contain the transposase gene (the insertion is therefore stable). To avoid screening identical insertions, take only one male from each cross, unless the males have different eye colors. Cross these males individually to virgin attached *CyO-TM9* balancer females.
4. Cross males and virgin females with the same P-element insertion and the *CyO-TM9*-attached balancers.
5. Screen behaviorally the males homozygous for the insertion. Note that the *CyO-TM9* balancer contains a dominant temperature-sensitive lethal mutation (larval stage). If desired, the progeny of the cross can be kept at 29°C, and only flies homozygous for the P-element insertion will survive.

3.3. Misexpression Screen (EP Screen; Fig. 4)

1. Establish a collection of EP lines (similar methods as in Subheading 3.2.), or use an existing collection (e.g., Rorth collection; ref. 10).
2. Cross these lines to a *GAL4* driver line (for example, *tim[UAS]-GAL4*; ref. 6).
3. Select the flies with the EP element and the driver and monitor their circadian behavior.

4. Notes

1. A detailed description of the mapping methods for EMS mutations is beyond the scope of this chapter. Here is a list of the most commonly used methods:
 - a. Meiotic recombination mapping: recombination events between the mutation and several markers (preferentially visible under a dissecting microscope). The frequency of these events is used to estimate the position of the mutation on the chromosome. DNA sequence polymorphisms can also be used as markers (see <http://www.fruitfly.org/SNP/index.html>; ref. 11) Note that in *Drosophila*, only females recombine meiotically.
 - b. Deficiency mapping is a very effective method to narrow down the region where the mutation lies, if the mutation is recessive (or codominant). Mutant flies are crossed to flies with a chromosome containing a small cytologically mapped deletion called a deficiency. If the heterozygous flies (mutation/deficiency) behave like wild-type flies (in the case of a recessive mutation), then the mutation is not located in the region of the chromosome missing in the deficiency used. On the contrary, if the heterozygous flies behave like homozygous mutant flies, the mutation is located in the deleted region of the chromosome.
 - c. Male meiotic recombination: this relatively recent method is very powerful (12). As mentioned above, only female recombine their chromosome meiotically. However, if a male contains a P-element insertion and express the P-element transposase, recombination can occur (at low frequency) precisely where the P-element is located. This method is considerably more powerful than the classic meiotic recombination, because the location of the recombination is known. However, this method is limited to autosomes. A single recombination event is sufficient to determine whether the mutation lies on the right or the left of the P-element. The precision of the mapping is dependent on the density of P-elements available in the region of interest.
2. The protocol and primers for recovering the sequence flanking the P-element insertion can be found at the following link: www.fruitfly.org/about/methods/inverse.pcr.html.
3. EMS is carcinogenic and therefore needs to be manipulated with great care. EMS-treated flies should be handled separately from other flies.
4. An interesting alternative to a wild-type screen is to use a sensitized background. Looking for suppressor or enhancer of a pre-existing mutation is a classic approach to identify genes affecting the same biological process as the first mutation. For example, a screen in the *per^L* background was performed and resulted in the isolation of an allele of *tim* (*tim^{SL}*), which probably would have gone unnoticed in a regular wild-type screen (13). Indeed, the period length of *tim^{SL}* alone is 23.7 h, whereas *per^L* 29-h period is reduced to 25.5 h in combination with *tim^{SL}*.
5. P-elements carrying a *mini-white* gene marker are the easiest to use, as they give a yellow, orange, or red color to the eyes of *w* mutant flies. The color depends on the insertion site and the level of expression of the *mini-white* gene.

6. The w^{1118} background is commonly used. It is important to know that w^{1118} is frequently carrying a mutant allele of *per* (per^{SLH}). Before using any w^{1118} stocks, it is necessary to determine whether their circadian rhythms are normal, or have the 27-h period characteristic of per^{SLH} .
7. It is important to monitor the mutagenic efficiency of the EMS solution, as EMS is not a very stable compound. An overnight exposure to 10 mM EMS usually works well with a fresh stock of EMS. It is advisable, however, to first test different concentration of EMS before beginning the screen:
 - a. Feed males with different concentration of EMS.
 - b. Cross the males to FM7A females (FM7A is a homozygous viable X-chromosome balancer).
 - c. Cross individual females heterozygous for the mutated X chromosome and FM7A to FM7A males.
 - d. Determine the frequency of lethality by counting the number of tubes where the F2 progeny contains only FM7A males. A concentration of EMS that gives around 20 to 30% lethality is a good compromise between a relatively high exposure to the chemical and a reasonably low degree of lethality. Owing to its decay, the concentration of EMS may need to be adjusted after a few months.
8. For an X-chromosome screen, F1 males can be individually crossed to attached-X females to establish lines containing the mutant X chromosome, so that several males for each mutant X chromosome can be screened (F2 screen).
9. To screen for X-chromosome insertion as well as autosomes, cross the males in which the transposition is occurring to attached-X females. To determine whether the P-element is on the X or on an autosome, cross individual males to both attached-X and attached CyO-TM9 females. Separate the two types of females after 4 d of mating. If every male has colored eyes in the progeny of attached-X females, the insert is on the X chromosome.
10. With a P-element-based screen, it is important to confirm that the P-element is responsible for the mutation. Indeed, P-elements can jump several times before reaching their final insertion. Every time they jump, they might leave a small deletion behind them. A simple first test is to cross the mutant flies to flies with a deficiency chromosome. This chromosome has a deletion in the region of the targeted gene. The heterozygous flies (P-element targeted gene over deficiency chromosome) should behave like mutant homozygous flies. The next step is to excise precisely the P-element to verify that there is nothing else in the region of the gene that might be mutated. Flies with a precise excision should behave like wild-type flies.

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Mutagenesis in *Arabidopsis*

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Summary

Ethyl methane sulfonate (EMS) mutagenesis in *Arabidopsis* is the most widely used mutagenesis technique. EMS has high mutagenicity and low mortality and can be used in any laboratory with a fume hood. The chemical principle of EMS mutagenesis is simple; it is based on the ability of EMS to alkylate guanine bases, which results in base mispairing. An alkylated guanine will pair with a thymine base, resulting primarily in G/C to A/T transitions, which ultimately results in an amino acid change or deletion. There are several advantages to EMS mutagenesis compared with other mutagenesis techniques available for *Arabidopsis*. First, EMS generates a high density of nonbias irreversible mutations in the genome, which permits saturation mutagenesis without having to screen a large number of individual mutants. Second, EMS mutagenesis not only generates loss-of-function mutants, but can also generate novel mutant phenotypes, which include dominant or gain-of-function versions of proteins owing to alterations of specific amino acids. This chapter describes the use of EMS mutagenesis in *Arabidopsis* and how mutagenized plant populations should be handled after the mutagenesis event.

Key Words: Ethyl methane sulfonate; EMS; *Arabidopsis*; mutagenesis; genotypes; mutagen dose; M1 and M2 generation; pooling; pedigreeing.

1. Introduction

Mutagenesis is the process by which heritable alterations in the genome of an organism are produced. Forward genetic mutagenesis screens, in which genetic variation is artificially introduced and mutagenized plants are screened for phenotypes of interest, are a powerful tool for identifying genes involved in general developmental processes and signal transduction pathways. *Arabidopsis* mutagenesis has proved to be a powerful tool for the identification of components involved in the circadian rhythm in plants (1,2).

The most common mutagens available fall into three main categories: chemical, physical, and biological (3–6). The choice of mutagen depends on the desired type

of mutation, the frequency of mutation, and the appeal of facile cloning of the identified mutant gene(s). Each of the three main classes has its own advantages and disadvantages, which will be discussed briefly below.

The most popular chemical mutagen in *Arabidopsis* is ethyl methane sulfonate (EMS) because of its high mutagenicity and low mortality; a detailed protocol will be given for its use in this chapter (*see Note 1*). EMS alkylates guanine bases (addition of a methyl group), leading to base mispairing: an alkylated guanine pairs with thymine bases instead of cytosine bases, resulting primarily in G/C to A/T transitions. EMS mutagenesis allows for the generation of a high density of nonbias irreversible mutations, thus permitting saturation mutagenesis to be achieved using relatively few plants. Although the cloning of an EMS-mutagenized gene is time-consuming (the genetic positions of the mutations are unknown and must be cloned using positional cloning techniques), one major advantage is that whereas other mutagens commonly result in loss of function, EMS-generated point mutations often result in different degrees of functionality: loss of function, partially reduced function, qualitatively altered function, and constitutive function.

In *Arabidopsis* physical mutagenesis most commonly involves fast neutrons that create populations of plants with multiple small deletions (~1 kb; *see Note 1*). Such deletions can be beneficial, as they can provide true null mutations. As for EMS mutagenesis, positional cloning is often used to identify the mutation; however, by generating random deletion libraries, mutated loci can be identified using the polymerase chain reaction (PCR) with oligonucleotide primers flanking the target gene (7,8).

Biological mutagens disrupt genes by the insertion of foreign DNA (insertional mutagenesis) originating from primary transformation events or from transposable elements. In *Arabidopsis* this involves the use of either transfer DNA (tDNA) or transposable elements. The main disadvantage of mutagenesis by tDNA or transposable element insertion is that insertions occur at low frequencies per plant (~1.5 insertions per M1 plant) and thus relatively large numbers of plants must be screened in order to identify a mutant of interest: approx 500,000 plants would have to be screened to have a 99% probability of identifying an insertion in a 1-kb gene in *Arabidopsis* (4). In addition, the generation of large tDNA insertion collections/libraries is time-consuming and requires extensive genetic characterization (*see Note 1*). However, the main benefit is that if a mutation is caused by the insertion of a tDNA or transposable element the inserted sequence will act as a genetic marker, allowing the easy identification of the flanking genomic sequence using PCR-based methods (9–11).

This chapter focuses on the use of EMS to mutagenize *Arabidopsis* and will outline the methodology employed to achieve saturation mutagenesis in order to identify genes involved in circadian pathways in *Arabidopsis*.

1.1. General Principles of EMS Screen

In a typical EMS screen, seed of the parental genotype are treated with the mutagen. The plants grown from these seeds are the M1 generation and will be heterozygous for the induced mutations. The progeny derived from the self-fertilization of these plants is the M2 generation. The M2 is the first generation following mutagenesis in which homozygous recessive mutants can be detected. Because most mutations will be recessive and because M1 plants are chimeric, the M2 generation is most commonly used for phenotypic screening purposes.

The mutagenesis targets are the diploid cells of fully developed embryos covered by the seed coat. Because the genome of each cell of the embryo is mutagenized independently of the other cells, EMS will produce a particular mutation in only a single cell. Only mutations that occur in the cells that form the germ line will be detected in the plants grown from seed of the M1 generation. In *Arabidopsis* the number of cells contributing to the germ line, the genetically effective cell number, is typically two (12). If a mutation (m) occurs at a specific locus (M) in one of these two cells in an M1 seed, then 50% of the M2 progeny from the M1 plant will be derived from the nonmutated sector (MM) and 50% from the mutated sector (Mm). Consequently, the segregation ratio for that mutation in the M2 generation will be 7:1, obtained from 4:0 in the nonmutated sector and 3:1 in the mutated sector:

	Nonmutated sector	Mutated sector
M1 plant	MM	Mm
M2 plant	7 (MM + Mm) : 1 mm [4:0 MM + 3 (MM,Mm) : 1 mm]	

2. Materials

1. Seeds for *Arabidopsis* lines (see Note 2). Between 5000 and 50,000 seeds are required for a typical mutagenesis experiment.
2. Protective clothing and gloves.
3. Chemical fume hood that is fully functional and has been inspected.
4. Inflatable polyethylene glove bag (Atmos bag, Aldrich).
5. 0.1% Potassium chloride.
6. Disposable syringes.
7. Plastic beakers.
8. Filter tips.

9. Plastic gauze or Miracloth.
10. 100 mM Potassium phosphate, pH 5.0.
11. 1 M Sodium hydroxide (NaOH).
12. Dimethyl sulfoxide (DMSO).
13. EMS.
14. Sodium thiosulfate crystals.
15. 100 mM Sodium thiosulfate.
16. Whatman paper.
17. Trays with moist soil for plant growth.

3. Methods

3.1. Considerations When Conducting a Screen

There are several variables that should be considered before conducting an EMS mutagenesis screen. The following sections describe choice of parental genotype, the mutagen dose, and number of plants to be screened.

3.1.1. Choice of Parental Genotype

Critical to the success of a genetic screen is the selection of a suitable genetic background. A multitude of different *Arabidopsis* ecotypes and mutants are available that can be used as backgrounds for mutagenesis. It is useful to apply mutagenesis on commonly used standard “laboratory wild-types” such as Landsberg *erecta* (Ler), Columbia (Col), and Wassilewskija (Ws), as this facilitates downstream comparisons between different mutants. Moreover, genetic markers are available in these backgrounds, which is highly advantageous during the positional cloning of mutagenized loci. It should be noted, however, that laboratory wild-type lines do have inherent mutations, which may affect the isolation of certain mutant phenotypes (reviewed in refs. [13–15](#)). For example, *Ler* behaves as a mutant of the flowering inhibitor gene (*FLC*) and it has been shown that that *flc* loss-of-function mutations have shorter circadian periods ([16,17](#)).

EMS populations can be purchased from Lehle Seeds (www.arabidopsis.com) or obtained directly from laboratories where they were generated. Generation of your own EMS population has the advantage that you can mutagenize a specific genotype to screen for revertants or suppressor mutants (see **Note 3**). Additionally, several techniques are becoming available that enable screening of EMS populations for mutants that show no obvious biological phenotype. An example of this is the use of the *chlorophyll a/b binding protein* (*CAB*) promoter to drive expression of the firefly *luciferase* gene (*CAB2::LUC*) to isolate *timing of chlorophyll a/b binding protein* expression (*toc1*) mutants in the circadian clock ([1,2](#)). This approach demonstrates the power of combining EMS mutagenesis with inducible-promoter driven reporter genes.

3.1.2. Choice of Mutagen Dose

Ideally the dose of mutagen should be as high as possible to allow mutagenized populations to contain a high mutation frequency. This will allow screening of relatively small mutagenized populations that contain large numbers of mutations. However, too severe a treatment can cause sterility and loss of viability. In addition, “overmutagenizing” can also lead to an increase in unwanted mutations at multiple loci, which, in turn, complicates the downstream positional cloning process. At the other extreme, too mild a treatment will result in a low density of mutations and will therefore require extensive screening to obtain mutants of interest.

The efficiency of the mutagenesis procedure can be affected by many factors, including the physiological condition of the seeds, the temperature of the solution during the mutagenesis itself, and also the purity of the EMS, which can affect potency from batch to batch. Consequently, it is difficult to specify the exact amount of mutagen to use; we recommend using a gradient of doses (*see Subheading 3.1.2.*).

3.1.3. Number of Plants to Be Screened

When designing an EMS screen it is important to consider the absolute and relative size of the population to be mutagenized and subsequently screened (*see Note 4*). To decide this it is important to take into account how many plants it will be possible to screen, which depends largely on available growth space, and how many genes can mutate to cause the phenotype of interest.

The probability of a mutation occurring is a function of the size of the M1 generation. However, the probability of the mutation being observed is a function of the number of M2 individuals screened per M1 plant. As stated in the introduction, recessive mutants segregate at a frequency of one out of eight per M1 progeny and consequently, if the size of the M2 population is small relative to the size of the M1 population, some mutants will probably not be found. The relationship between the relative sizes of M1 and M2 populations has been well described (*18*) and will only be outlined here. Briefly, in *Arabidopsis*, where the cost of growing M1 and M2 plants is relatively low, it is most cost-effective to increase the number of mutants by screening a small number of M2 plants per M1 progeny. This leads to a scheme whereby researchers grow large numbers of M1 plants and reduce repetitive screening by screening just 2000 to 150,000 M2 plants, depending on the ease of the screen.

3.2. EMS Mutagenesis of *Arabidopsis* Seeds

EMS is an excellent mutagen and consequently is highly carcinogenic; your safety and that of others is therefore of utmost importance. EMS is volatile and

all necessary safety precautions should be taken when conducting a mutagenesis experiment. Dress in full safety gear, including lab coat, appropriate double gloves, and safety glasses. The experiment should be carried out in a fully functional and inspected chemical fume hood. Inform all members of the laboratory about the experiment and post appropriate warning signs. EMS is chemically inactivated by exposure to sodium thiosulfate or 1 *M* NaOH. Use disposable containers and plasticware where possible and wash with 1 *M* NaOH before disposal. Dispose of liquids in the same way using 1 *M* NaOH. Use 1 *M* NaOH to wash all surfaces that may be contaminated with EMS and then rinse them with water. Do not let anyone use the fume hood until the area is completely decontaminated. A material safety data sheet must be obtained from the manufacturer of the EMS and read before conducting the mutagenesis experiment to ensure that all local regulations are met. The following protocol describes a typical EMS mutagenesis experiment.

1. Prepare one 10-mL syringe per 2500 seeds by cutting off the end and covering it with either plastic gauze or Miracloth (*see Note 5*). This will allow solutions in the syringe to be changed without losing any seed. All solutions are prepared in beakers.
2. Batches of approx 2500 *Arabidopsis* seeds are soaked overnight in 0.1% potassium chloride inside the syringes. Preimbibing allows good uptake of the EMS.
3. The potassium chloride solution is then replaced with 20 mL of 100 *mM* potassium phosphate solution, pH 5.0. To do this, expel the first solution and replace it with the new.
4. To this add 1 mL of DMSO to permeabilize the seed.
5. At this stage all equipment is transferred into the glove bag (Atmos bag, Aldrich) in a fume cupboard and the glove bag is sealed (*see Note 6* and [Fig. 1](#)).
6. To each beaker containing potassium phosphate and DMSO, 135, 180, or 225 μ L of EMS is added and mixed thoroughly. This gives a final concentration of 60, 80, and 100 *mM* of EMS, respectively.
7. The seeds are then syringed up and down several times to ensure even exposure to the EMS. This is repeated every half hour.
8. After 3 h the solution of EMS is discarded into a beaker of sodium thiosulfate crystals to ensure chemical inactivation.
9. The seeds are then washed three times with 100 *mM* sodium thiosulfate solution to remove traces of EMS. Each wash is discarded into the beaker of sodium thiosulfate crystals.
10. The seeds are then washed with 100 mL of distilled water before being removed from the glove bag and washed from the syringes onto pieces of Whatman paper and left to dry for several hours (*see Note 7*).
11. After the seeds are dry they should be sown onto soil as soon as possible. The density of seeds is dependent on whether one is collecting in pools or individual plants (*see Subheading 3.3.* and *Note 8*). Also, sow a tray of nonmutagenized



Fig. 1. A researcher performing an EMS mutagenesis experiment inside an Atmos bag purchased from Aldrich. All equipment needed is inside the Atmos bag. The researcher is wearing full protective clothing, including two pairs of gloves.

seeds at the same density, which will act as a control. Keep the soil moist after planting.

12. It is valuable to know the size of the M1 population. Estimate the population size 10 to 14 d after sowing, as after the early rosette stage the plants will become too dense to allow counting.

At this stage it is important to estimate the degree of mutagenesis. Effective EMS treatment will lead to reduced germination speed and reduced seedling growth in addition to plants containing sectors with dominant color mutations and growth aberrations and sterility (*see* **Notes 9** and **10**).

3.3. Management of M1 Plants

Once the EMS mutagenized plants have grown to maturity, it is important to consider what harvesting strategy to adopt. The two harvesting strategies are to pool M1 plants to some extent (pooling), or to harvest seed from individual M1 plants and maintain these as discrete groups of siblings (pedigreeing). The extremes of the two strategies are illustrated by Lightner and Casper ([19](#)).

The pooling of seeds simplifies the handling and screening of the M2 seed. It is recommended that an M1 population containing 20,000 to 100,000 individuals be harvested as pools of 500 to 1000 M1 plants, which generally repre-

Table 1
Pedigreeing vs Pooling

Pedigreeing	Pooling
Save lethal/sterile mutants	Simplifies handling
Increase in effort	Less time
Increase in space (10 cm ² /plant)	Less space (1–2 cm ² /plant)
Certainty of different mutations	Same mutant can be identified twice

sent one seed tray. Allow for 1 to 2 cm² per plant (~500 seeds/tray) to provide adequate space for growth and to not too severely restrict more physiologically compromised plants in the M1 generation. The disadvantage of this strategy is that if two plants with the same phenotype are recovered from the same pool it is often assumed that they are derived from the same mutation event and only one of these is used for further analysis. In contrast, plants from different pools will carry independent mutations. In addition, sterile or lethal mutants will be lost when using the pooling strategy.

Harvesting the M1 as discrete individuals involves substantially more effort, time, and growth space (allow 10 cm²/plant). However, when an interesting mutant that is sterile or lethal is found among the progeny of a particular M1 plant, spare seed will allow for the recovery of heterozygous sister plants. Also, when two mutants are recovered with similar phenotypes from different M1 plants, they can be assumed to have resulted from independent mutation events.

A table showing the advantages and disadvantages of pedigreeing vs pooling EMS mutagenized M1 plants is shown in [Table 1](#).

4. Notes

1. Collections of EMS, fast neutron, and γ -ray mutated seeds in common laboratory backgrounds can be purchased from Lehle Seeds (www.arabidopsis.com/) if the researchers do not wish to perform their own mutagenesis experiment. Also, several large collections of insertion mutant lines are available from Arabidopsis Biological Resource Center at Ohio State University (www.arabidopsis.org/abr) and Nottingham Arabidopsis Stock Centre at the University of Nottingham (<http://arabidopsis.info/>).
2. Common laboratory backgrounds can be purchased from Lehle Seeds. Lines are also available from the main stock centers (*see* **Note 1**) but will have to be bulked up before a mutagenesis experiment can be performed.
3. In order to screen for suppressor mutants it is common practice to use an M0 plant (parental mutant line) with a visible marker to avoid confusing a wild-type pollen or seed contamination with a new mutant. Often the *gll* mutation, which

causes loss of trichomes, is used for this purpose. Alternatively, if the original mutation has been cloned, then molecular markers for this can be developed.

4. As a guide to estimate the number of seeds, generally 10,000 seeds weigh approx 0.2 g. Allowances should also be made for the loss of 10 to 20% of the seed during washing and for up to 25% lethality. It is advisable to mutagenize as many seeds as possible, as they can be stored for future experiments or shared with other laboratories.
5. Some researchers pack the seeds into cloth bags, which facilitates rinsing by transferring the packs of seeds to the wash solutions. This method can lead to problems with effective diffusion of the EMS into the entire seed mass and difficulty with rinsing. Alternatively, the seeds can be placed in a 50-mL Falcon tube, sealed with sealing film, and rotated with a tube rotator or rocked on a rocking platform. The seeds can then be allowed to settle to the bottom of the tube and the EMS solution pipetted off.
6. The Atmos bags are available in a variety of sizes; this reflects the size of the working area, rather than the glove size. We recommend using a medium-size bag to comfortably fit all of the necessary equipment and safety precautions in the bag. To provide a stable working area, place a tray in the bottom of the bag and cover it with paper towels so that spills can be easily spotted.
7. Even after significant rinsing, EMS will still remain on the seeds, so it is important to wear gloves during handling.
8. The density of the M1 plants is critical to good growth. It is advisable to practice sowing with untreated seeds before the mutagenesis experiment is undertaken.
9. It is a good idea to sow a tray of control, unmutagenized seeds along with the mutagenized population. During growth of the plants, compare the degree of silique elongation between the mutagenized and unmutagenized populations: if the M1 plants' siliques fail to elongate then the mutagenesis may have been too severe and the plants sterile.
10. The effectiveness of a mutagenesis experiment is best monitored by measuring the frequency of one or more classes of known mutants. The easiest way is to assess the number of plants with a pigment phenotype (white, yellow, pale green). In M1 plants 0.1 to 1% of plants should have chlorotic sectors and this is useful to confirm that the mutagenesis experiment has worked. In the M2 plants 2 to 10% of young seedlings grown on minimal media should have pigment phenotypes. This method is most useful for comparing different mutagenized populations, rather than estimating an absolute mutation rate. A detailed discussion of the estimation of mutation frequency is dealt with by Li and Redei (12).

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Yeast Two-Hybrid Screening

Jodi Maple and Simon G. Møller

Summary

Yeast two-hybrid screening represents a sensitive *in vivo* method for the identification and analysis of protein–protein interactions. The principle is based on the ability of a separate DNA-binding domain (DNA-BD) and activation domain (AD) to reconstitute a functional transactivator when brought into proximity. In the MATCHMAKER yeast two-hybrid system, a bait protein is expressed as a fusion to the GAL4 DNA-BD, whereas the prey protein is expressed as a fusion to the GAL4 AD. When a bait and a prey protein interact, the DNA-BD and AD form a functional transactivator, resulting in activation of reporter gene expression in yeast reporter strains. The method described in this chapter can be used to identify novel protein interactions, analyze protein–protein interactions between two known proteins, as well as dissect interacting protein domains.

Key Words: Yeast two-hybrid; MATCHMAKER system; GAL4 DNA-binding domain; GAL4 activation domain; reporter gene; yeast transformation; auxotrophy; bait protein; prey protein; cDNA library; protein expression; yeast mating; diploids; LacZ.

1. Introduction

It is becoming increasingly clear that most proteins do not function in isolation but rather act together with protein partners mediated by direct protein–protein interaction. There is therefore a need to identify such interactions in order to fully understand protein function. Although several complementary methods exist for protein–protein interaction analysis, the yeast two-hybrid system represents a relatively rapid and sensitive *in vivo* assay that enables the direct identification of genes encoding interacting proteins.

The yeast two-hybrid system is based on the modular nature of eukaryotic transcriptional activators that consist of two discrete domains: one domain interacts with DNA (DNA-binding domain [DNA-BD]) and a second domain recruits the transcriptional apparatus (activation domain [AD]). These domains

are nonfunctional in isolation but can function noncovalently via protein–protein interactions. Thus, a pair of proteins that interact with each other will bring together a separate DNA-BD and an AD to reconstitute a functional transactivator. In a typical yeast two-hybrid assay a bait protein, expressed as a fusion with a DNA-BD, and a prey protein fused to an AD, are coexpressed in yeast cells. In the event of the two proteins interacting, the reconstituted transactivator is recruited to DNA-BD-containing reporter gene(s) ultimately resulting in reporter gene induction. Using this approach individual known proteins can be screened for direct interaction or entire cDNA libraries can be screened to identify new interacting proteins.

This chapter will describe the MATCHMAKER GAL4 yeast two-hybrid system (Clontech, Palo Alto, CA), which takes advantage of the DNA-BD and AD of the yeast GAL4 transcriptional activator ([1,2](#)). The chapter is focused on methods enabling the reader to perform entire cDNA screens to identify novel interacting proteins. However, methods are also provided for protein–protein interaction analysis of two known proteins.

2. Materials

1. MATCHMAKER Library Construction and Screening Kit (Clontech).
2. Oligonucleotide primers.
3. Restriction enzymes, DNA polymerase, and T4 DNA ligase.
4. *Escherichia coli* strain DH5 α .
5. Agarose and DNA sequencing gel equipment.
6. Ampicillin, kanamycin, LB medium.
7. Whatman filter paper no. 5 and nitrocellulose filter paper.
8. pPCR-Script[®] Cam Cloning Kit (Stratagene, La Jolla, CA).
9. PLATE mixture: 40% polyethylene glycol 4000 (w/v), 100 mM lithium acetate, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA.
10. 1 M 3-amino-1,2,4-triazole (3-AT; Sigma cat. no. A-8056). Dissolve in deionized H₂O and filter-sterilize. Store at 4°C.
11. YPDA: 20 g/L Difco peptone, 10 g/L yeast extracts, 15 g/L Bacto-agar (for solid media). Add H₂O to 950 mL. Adjust pH to 6.5 if necessary. Autoclave and cool to 55°C and add 15 mL/L 0.2% adenine hemisulfate solution (final concentration 0.003% in addition to the trace amount of adenine present in yeast peptone dextrose) and dextrose to 2% (50 mL of a sterile 40% stock solution). Adjust the final volume to 1 L if necessary.
12. Synthetic dextrose minimal medium (SD): 6.7 g/L yeast nitrogen base without amino acids, 20 g/L Bacto-agar. Adjust pH to 5.8 if necessary. Autoclave and cool to 55°C before adding dextrose to 2% (50 mL of a sterile 40% stock solution), 100 mL of the appropriate 10X dropout solution (*see* **Note 1**), and 3-AT as necessary (*see* **Subheading 3.1.4**).
13. 10X Dropout solution. For 1 L of 10X dropout solution combine the following ingredients minus the appropriate nutrients used to maintain selection (*see* **Note 2**):

200 mg L-adenine hemisulfate, 200 mg L-arginine HCl, 200 mg L-histidine (His) HCl monohydrate, 300 mg L-isoleucine, 1000 mg L-leucine (Leu), 300 mg L-lysine HCl, 200 mg L-methionine, 500 mg L-phenylalanine, 2000 mg L-threonine, 200 mg L-tryptophan (Trp), 300 mg L-tyrosine, 200 mg L-uracil, and 1500 mg L-valine. Grind the nutrients with a pestle and mortar to ensure that they are mixed. The dropout mix can then be stored as powdered stocks or dissolved in water, autoclaved, and stored at 4°C for up to 1 yr.

14. Carrier DNA.
15. 1 M 1,4-Dithio-DL-threitol.
16. STET buffer: 8% sucrose (w/w), 50 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 5% Triton X-100.
17. Glass beads (0.45 μ m).
18. 7.5 M Ammonium acetate.
19. Ethanol.
20. X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) stock solution: 20 mg/mL in *N,N*-dimethylformamide. Store at -20°C in the dark.
21. Z buffer: 16.1 g/L Na₂HPO₄·7H₂O, 5.5 g/L NaH₂PO₄·H₂O, 0.75 g/L KCl, 0.246 g/L MgSO₄·7H₂O. Adjust to pH 7.0 and autoclave. Can be stored at room temperature for up to 1 yr.
22. Blue test substrate mix: 2.5 mL Z buffer, 6.5 μ L β -mercaptoethanol, 125 μ L X-gal stock solution.
23. Liquid nitrogen.
24. Protein extraction sample buffer: 0.06 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-mercaptoethanol, 0.0025% (w/v) bromophenol blue. Buffer should be made fresh before use and can be stored at -20°C for approx 6 mo.
25. SDS-polyacrylamide gel electrophoresis equipment.
26. c-Myc and hemagglutinin antibodies, or GAL4 AD and GAL4 DNA-BD monoclonal antibodies (Clontech).

3. Methods

The methods outlined below describe (1) the construction and testing of the bait plasmid; (2) the construction of the yeast two-hybrid library; (3) yeast two-hybrid screening by yeast mating and analysis of positive clones; (4) assaying interactions between two known proteins; and (5) preparing yeast two-hybrid data for publication.

3.1. Construction of Bait Plasmid and Yeast Transformation

The first step in a yeast two-hybrid screen is to construct a bait plasmid that expresses the protein of interest as a fusion to the DNA-BD. This plasmid is transformed into the appropriate yeast reporter strain and a series of control experiments are then performed to establish whether the bait is suitable for use in a library screen or interaction studies between two known proteins.

The construction of a bait plasmid expressing the protein of interest is described in **Subheadings 3.1.1–3.1.4**. This includes a description of (1) the DNA-BD fusion vector; (2) cloning procedures; (3) transformation of yeast; and (4) testing the DNA-BD fusion construct.

3.1.1. *pGBKT7 DNA-BD Fusion Vector*

The pGBKT7 (**Fig. 1**) DNA binding domain fusion vector (Clontech) expresses bait proteins fused to the C-terminus of the GAL4 DNA-BD. The multiple cloning site (MCS) in pGBKT7 contains unique restriction sites in frame with the 3'-end of the GAL4 DNA-BD allowing for the generation of GAL4 DNA-BD/bait protein fusions. In this configuration the bait protein is also expressed as a fusion to a c-Myc epitope tag (*see Fig. 1 and Note 3*).

3.1.2. *Cloning*

DNA manipulations were performed by standard recombinant DNA methods described by Sambrook and Russell (**3**) and are not described in detail here because of space limitations. The target gene encoding the bait protein was PCR-amplified using a high-fidelity thermostable DNA polymerase and oligonucleotide primers containing *NdeI* and *BamHI* restriction sites (*see Note 4*). The blunt-ended amplified DNA fragment was subcloned into the *SrfI* site of the pPCR-Script vector (Stratagene, La Jolla, CA) followed by DNA sequence analysis using gene-specific and vector-specific oligonucleotide primers to ensure that no PCR-generated mutations had occurred. The cloned gene was then cut out from pPCR-Script using *NdeI* and *BamHI* and ligated into the same restriction sites of the pGBKT7 vector. The DNA was transformed into *E. coli* DH5 α cells by standard methods (**3**), plated onto LB medium containing kanamycin (50 μ g/mL), and incubated overnight at 37°C. Single colonies were selected and grown overnight in LB liquid medium containing kanamycin (50 μ g/mL). The plasmid DNA was then isolated (**4**) and analyzed for the presence of the insert using restriction enzyme digestion. Using the T7 primer and the 3' DNA-BD sequencing primer (Clontech) flanking the MCS site in pGBKT7, the plasmid DNA was subjected to DNA sequencing to ensure that the gene of interest was in-frame with the GAL4 DNA-BD fusion partner.

3.1.3. *Transformation of Bait Plasmid Into Yeast*

The next step is to test the activity of the bait protein in yeast reporter strains. The bait plasmid and the empty DNA-AD (prey) vector (*see Fig. 2 and Subheading 3.1.4.*) must be transformed into the appropriate yeast reporter strain. The authors use AH109 and Y187 as the mating strains (*see Subheading 3.2.2.*) and it is recommended that the same tests (*see Subheading 3.1.4.*) be per-

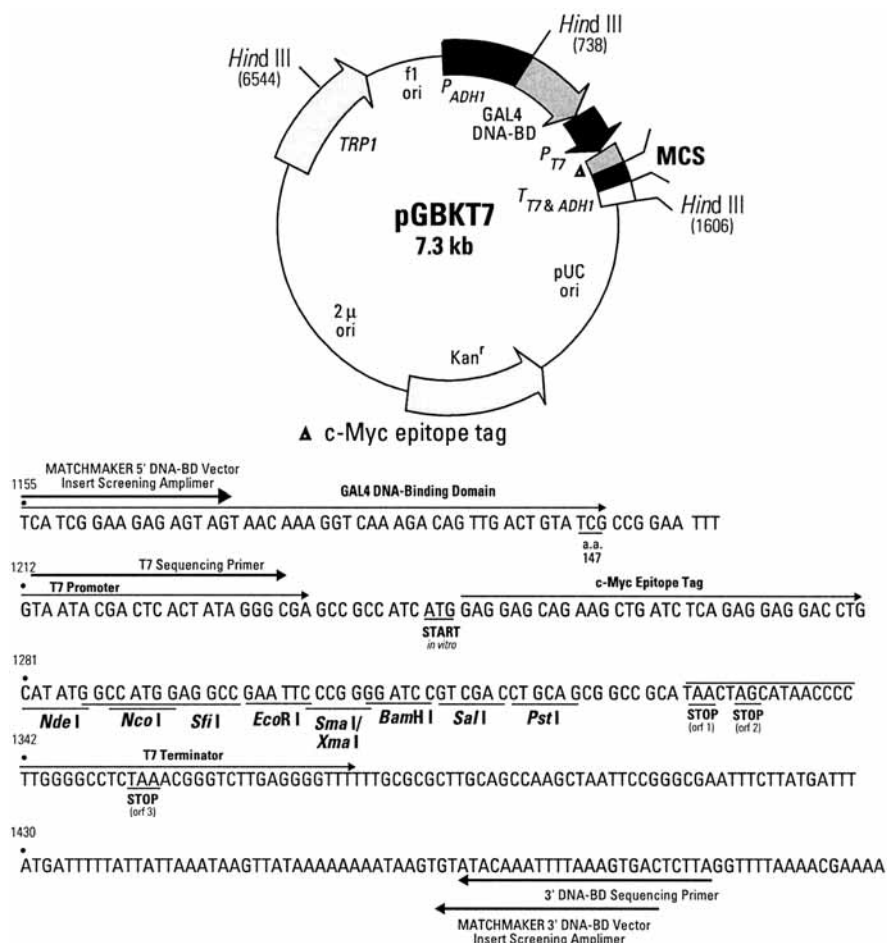


Fig. 1. Schematic diagram of the DNA-binding domain bait vector pGBKT7. The multiple cloning site is shown. (Printed with permission. ©1999 Becton, Dickinson and Company.)

formed in both strains. To do this only a few yeast transformants are required and the protocol described below can be used.

1. Inoculate 10 mL of liquid YPDA medium with a yeast colony and grow overnight at 30°C at 250 rpm in a rotary shaker.
2. Harvest 1.5 mL of yeast cells for each transformation in a microcentrifuge tube by pulse centrifugation for 5 s (*see Note 5*). Decant the supernatant by inverting the tube and shake once, leaving the cells in 50 to 100 μ L of the remaining liquid.
3. Add 2 μ L of 10 mg/mL carrier DNA and resuspend the cells with a pipet tip.
4. Add in order 1 μ g of bait plasmid DNA, 0.5 mL of PLATE mixture, and 20 μ L 1 M DTT. Vortex briefly after each addition.

3.1.4. Testing the Bait Strain

Before starting any yeast two-hybrid screen it is important to test the DNA-BD/bait fusion for (1) transcriptional autoactivation, (2) protein expression, and (3) toxicity to the yeast cells.

3.1.4.1. AUTOACTIVATION

Autoactivation of His reporter gene expression may be a problem with some proteins fused to the DNA-BD, which may lead to the identification of false-positive clones. It is therefore important to determine the level of leaky His reporter gene activity by the DNA-BD/bait fusion protein itself and to test whether this background autoactivation can be suppressed. Suppression of background growth on SD medium lacking His can be performed using 3-AT, a competitive inhibitor of the yeast HIS3 protein. Conditions are sought where the AH109 reporter strain expressing only the DNA-BD/bait fusion protein does not grow on SD medium lacking His.

1. Using the yeast transformation protocol outlined in **Subheading 3.1.3.**, cotransform AH109 yeast cells with the DNA-BD/bait plasmid and the empty AD prey plasmid pGADT7 (**Fig. 2**).
2. Select for transformants on SD/–Trp/–Leu plates (*see Notes 1 and 2*) and grow up a colony overnight in 10 mL of the same SD media.
3. Plate 100 μ L of cells onto SD/–Trp/–Leu/–His plates containing increasing concentrations of 3-AT (0, 5, 10, 20, 30 mM). In addition, plate cells on SD/–Trp/–Leu as a control for growth.
4. Incubate plates at 30°C.
5. After 4 to 6 d examine the growth of colonies on each plate. If the bait strain exhibits background growth on SD/–Trp/–Leu/–His medium then it will be necessary to eliminate/reduce the background by adding 3-AT to the selection medium for the screen. We have found that concentrations of 5 to 30 mM are sufficient to select against autoactivation (*see Note 7*).

3.1.4.2. VERIFICATION OF PROTEIN EXPRESSION

The expression of the fusion protein should be verified by Western blotting (*see Note 8*). pGBKT7 encodes a c-Myc epitope tag (**Fig. 1**) that can be used for immunological detection of the expressed fusion protein. Alternatively, commercially available antibodies raised to the DNA-BD may be used.

Total yeast protein extracts are prepared as described below:

1. Grow Y187 (bait) overnight to $OD_{600} = 0.7$ and harvest 1.5 mL of cells in a microcentrifuge tube.
2. Pulse-spin for 5 s in a microcentrifuge, discard supernatant, and wash once with water.

3. Pulse-spin for 5 s in a microcentrifuge and resuspend cells in 100 μ L of protein extraction sample buffer.
4. Heat at 95°C for 5 min
5. Centrifuge at 13,000g for 5 min.
6. Load 15 μ L per lane on a standard SDS polyacrylamide gel. Methods for pouring and running SDS gels as well as Western blotting can be found in **ref. 3**.

3.1.4.3. TEST FOR TOXICITY

Compare the growth rate in liquid culture of yeast containing the bait–DNA-BD fusion and yeast containing the empty DNA-BD fusion vector. If growth is noticeably slower then the bait, fusion protein may be toxic to the yeast cells (*see Note 9*).

3.2. Library Screening

3.2.1. Library Construction

A variety of cDNA libraries are available commercially or directly from the laboratories where they have been constructed. Although constructing your own cDNA library does offers the opportunity to choose an mRNA source where your protein of interest may have a relevant biological role, cDNA library construction can be both time-consuming and costly.

The authors used the MATCHMAKER library construction kit (Clontech) to generate a cDNA library from *Arabidopsis thaliana* seedlings. In this protocol, the cDNA library is constructed in one strain of yeast (AH109) by in vivo cloning and the transformants are collected and frozen at –80°C in aliquots. For each yeast two-hybrid screen, the pretransformed cDNA library strain is mixed with the bait strain and diploids are selected (*see Note 10*). This protocol is advantageous in that only one high-efficiency cDNA library transformation is required for multiple screens with different protein baits. The transformation efficiency is often the bottleneck when performing yeast two-hybrid experiments. A yeast mating protocol leads to more reproducible results and increases the chances of finding rare protein–protein interactions.

3.2.2. Screen by Yeast Mating

A medium-stringency screen by yeast mating is described in which interacting clones are selected by virtue of their ability to grow on medium lacking His and containing necessary levels of 3-AT (*see Subheading 3.1.4.*) Putative positives are then identified as those that also turn blue in β -galactosidase tests.

1. Inoculate a single, fresh (<2 mo old) Y187 (bait) yeast colony into 10 mL of SD/-Trp medium and grow overnight at 30°C at 250 rpm in a rotary shaker. The OD₆₀₀ should be higher than 0.8.

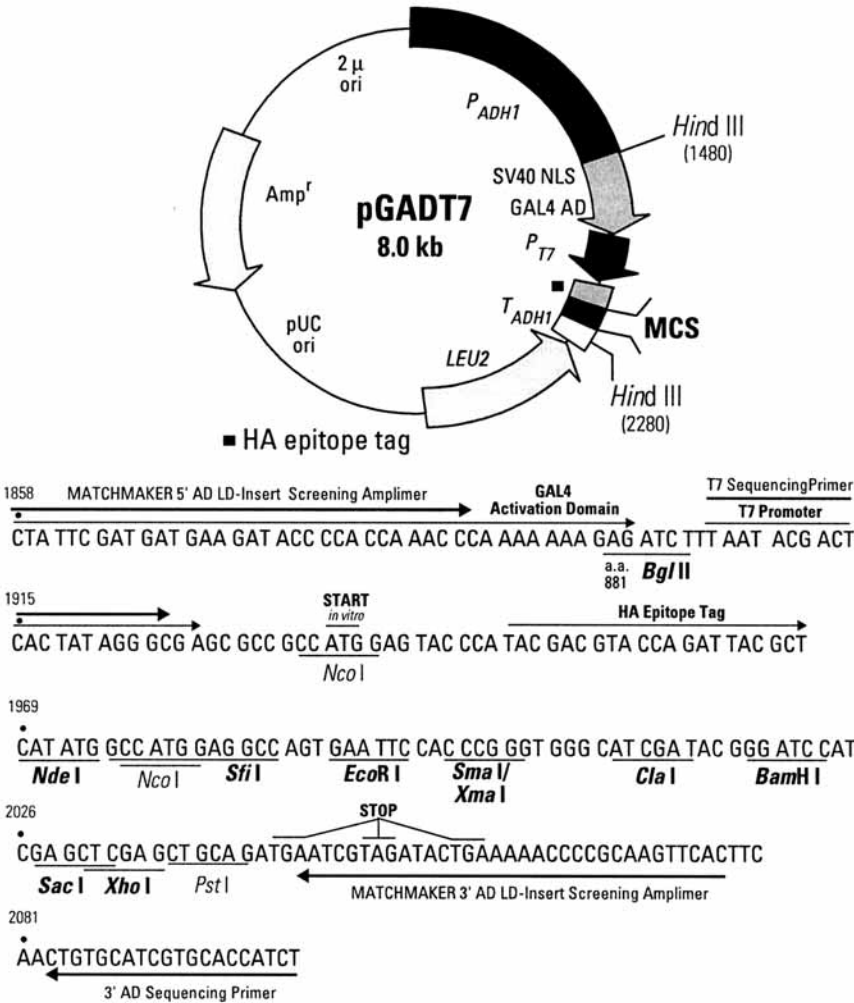


Fig 2. Schematic diagram of the activation domain bait vector pGADT7. The multiple cloning site is shown. (Printed with permission. ©1999 Becton, Dickinson and Company.)

5. Incubate at room temperature for 6 to 8 h (*see* **Note 6**). The cells will have settled to the bottom of the tube.
6. Heat-shock the cells for 10 min at 42°C.
7. Plate between 5 and 50 μL of heat-shocked cells onto selective SD media (*see* **Notes 1** and **2**) to select for the presence of the plasmid(s).
8. Incubate the plates for 2 to 4 d at 30°C until colonies appear.

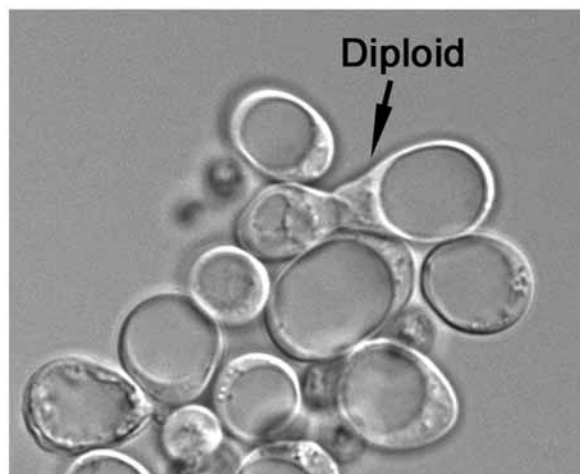


Fig. 3. Mating of AH109 (library) and Y187 (bait) yeast cells. A diploid is indicated with an arrow.

2. Thaw a 1-mL aliquot of your AH109 (library) strain in a water bath at room temperature.
3. In a flat-bottomed 100-mL flask inoculate (a) 9 mL of YPDA, (b) 1 mL of the Y187 (bait) overnight culture, (c) 100 μ L of AH109 (library), and (d) 50 μ g/mL kanamycin.
4. Incubate at 30°C at 40 to 50 rpm in a rotary shaker.
5. After 22 h examine an aliquot using standard light microscopy to confirm the presence of diploids (**Fig. 3**). If zygotes are present, allow mating to continue for four more hours.
6. To determine the mating efficiency spread 100 μ L of a 1:1000, 1:100, and 1:10 dilution of the mating mixture onto 90 mm plates containing:
 - a. SD/–Leu.
 - b. SD/–Trp.
 - c. SD/–Leu/–Trp.
7. Store the remaining mating mixture at 4°C.
8. Incubate the mating efficiency plates at 30°C until colonies appear (usually after 2 to 3 d), count the number of colony-forming units (cfu), and calculate the number of viable cfu growing on each type of SD medium:

$$\frac{\text{cfu} \times 1000 \mu\text{L} / \text{mL}}{\text{Vol. plated} (\mu\text{L}) \times \text{dilution factor}} = \text{no. viable cfu/mL}$$

no. cfu/mL on SD/–Leu = viability of the AH109 (library) strain

no. cfu/mL on SD/–Trp = viability of the Y187 (bait) strain

no. cfu/mL on SD/–Leu/–Trp = viability of the diploids (*see Note 11*)

To calculate the mating efficiency:

$$\frac{\text{no. cfu/mL of diploids}}{\text{no. cfu/mL of limiting partner (see Note 12)}} \times 100 = \% \text{ diploid}$$

Mating efficiencies in our laboratory are typically 40 to 60% (see Note 13).

9. Plate 50 μL of the mating mixture onto 150 mm SD/–Leu/–Trp/–His/+3-AT plates (approx 30 plates per mating) and incubate at 30°C for 3 to 8 d.

3.2.3. Selection of Potential Positive Clones

After 2 to 3 d colonies will be visible. Incubate for at least 5 d to allow slower-growing colonies (weak positives) to appear (see Note 14). Choose His⁺ colonies for further analysis and replica plate onto fresh SD/–Trp/–Leu/–His plates to dilute out extra AD domain plasmids. Grow at 30°C for 2 to 4 d, seal plates, and store at 4°C for up to 1 mo (see Note 15).

3.2.4. Blue (β -Galactosidase) Testing Yeast

Colonies that grow under His selection (His⁺) are then tested for β -galactosidase expression. Colonies that are His⁺ lacZ⁺ will be characterized as first-round positives (see Note 16).

Here we describe the colony-lift filter assay used in the laboratory to identify first-round positives.

1. Streak out yeast colonies onto selective plates (SD/–Trp/–Leu/–His) and grow for 3 to 4 d. Best results are obtained with fresh growing colonies.
2. Prepare fresh blue test substrate mix. For each plate to be assayed, presoak Whatman no. 5 filter paper in 1.8 mL of blue test substrate mix in a 90-mm plate.
3. Using forceps, place a clean dry filter paper over the surface of the plate of yeast to be assayed. Press the paper onto the cells with a spreader to help the colonies stick to the filter. Use a syringe needle to punch holes through the filter paper and into the agar to orient the filter on the plate.
4. Carefully lift the filter off the agar plate and place into liquid nitrogen face up for 10 s.
5. Allow to thaw and transfer the filter, colony side up, to the plate containing the presoaked substrate filter, being careful to avoid trapping air bubbles.
6. Incubate the filter at 30°C and check periodically for the appearance of blue color (see Note 17).
7. Identify the β -galactosidase-producing colonies by aligning the filter paper using the orienting marks.

3.4. Analyzing Positive Interactions

False-positives are often generated in yeast two-hybrid experiments, and secondary criteria for distinguishing true positives should always be considered (see Note 18).

Described below are protocols for (1) analysis of cDNA library inserts, (2) retransformation of the reporter yeast strain with an isolated cDNA library vector, and (3) DNA sequence analysis of unique clones. It may also be advantageous, although not strictly necessary, to place the bait protein in the prey vector and the prey protein in the bait vector, followed by retesting for a positive interaction.

3.4.1. Plasmid Recovery From Yeast

1. Harvest 1.5 mL of yeast grown overnight in SD/–Trp/–Leu/–His by pulse-spinning for 10 s in a microcentrifuge.
2. Resuspend cells in 100 μ L of STET buffer. Add the same amount of glass beads and vortex for 5 min.
3. Add another 100 μ L of STET buffer, vortex briefly, and boil for 3 min.
4. Cool on ice briefly and spin for 10 min at 4°C at 13,000g.
5. Transfer 100 μ L of the supernatant to a fresh tube containing 100 μ L of 7.5 M ammonium acetate.
6. Incubate at –80°C for at least 20 min and then spin for 10 min at 4°C at 13,000g.
7. Add 100 μ L of supernatant to 400 μ L precooled ethanol and spin for 10 min at 13,000g.
8. Resuspend the DNA pellet in 10 μ L of sterile water. Use 5 μ L of this solution to transform 100 μ L of competent DH5 α *E. coli* cells using standard techniques (3). Plate the cells on LB medium containing ampicillin (50 mg/mL) to select for the pGADT7 library vector and incubate at 37°C overnight.

3.4.2. Analysis of cDNA Library Plasmids Recovered From Yeast

Isolate plasmid DNA using standard protocols from a single bacterial colony (4). The plasmid DNA can then be subjected to endonuclease restriction digest analysis to determine the size of the insert (*see* **Note 19**) using a common restriction enzyme such as *Hind*III. Analyze the DNA insert sizes and pattern from the different clones by agarose gel electrophoresis to identify both different and identical cDNA library plasmids and classify these into groups (**Fig. 4**). Continue your analysis with one representative clone from each group.

3.4.3. Retest Protein Interactions in Yeast

Retransform purified cDNA library plasmid into the yeast reporter strain containing the original bait plasmid or into a yeast reporter strain containing a bait plasmid encoding an unrelated “control” protein. Plate on SD/–Trp/–Leu and assay the resulting colonies for growth on SD/–Trp/–Leu/–His/+3-AT and for β -galactosidase activity to eliminate false-positives. True-positives will activate the reporter genes of AH109 yeast cells only when the original bait plasmid is also present.

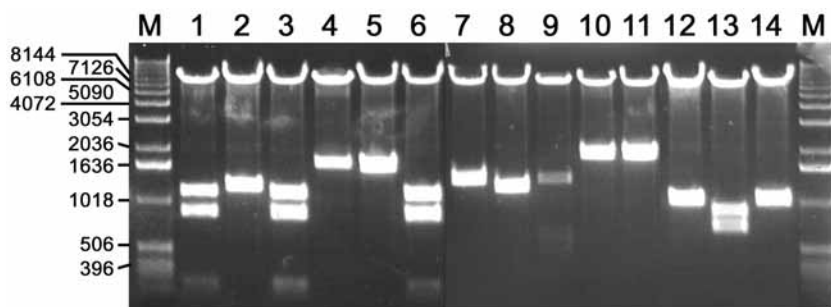


Fig. 4. An agarose gel showing *Hind*III restriction digest analysis of cDNA library plasmids recovered from 14 different positive clones. Based on their restriction pattern the clones were classified into different groups. Group A, clones 1, 3, 6; Group B, clones 2, 8; Group C, clones 4, 5; Group D, clones 7, 9; Group E, clones 10, 11; Group F, clones 12, 14; Group G, clone 13.

3.4.4. DNA Sequencing of cDNA Library Inserts

Perform initial DNA sequencing of the inserts in the positive cDNA library plasmids using the T7 sequencing primer. Verify the presence of an open reading frame fused in-frame to the GAL4 AD domain sequence and design new oligonucleotide primers to allow for further sequence determination. Compare the obtained DNA sequence to those in databases such as that of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) using BLAST algorithms and identify the predicted encoded protein. Because many proteins are inherently “sticky” and will interact with any bait protein, we have supplied a summary of commonly found false positive bait-interacting proteins ([Table 1](#)).

3.5. Testing for Protein Interactions Between Two Known Proteins

The yeast two-hybrid system can also be used for the analysis of protein–protein interactions between two known proteins or for determining what protein domains or amino acid residues mediate protein–protein interactions.

To test for protein–protein interactions between two known proteins the MATCHMAKER pGADT7 AD vector ([Fig. 2](#)) is used in conjunction with the pGBKT7 DNA-BD vector. The pGADT7 harbors the T7 promoter, a hemagglutinin epitope tag, and an MCS and expresses proteins fused to the GAL4 AD.

The following protocol outlines how to test for protein–protein interactions between two known proteins:

1. Clone the gene(s) of interest (gene X and gene Y) into pGBKT7 and pGADT7 respectively as outlined in **Subheading 3.1.2**.

Table 1
List of Most Common False-Positives Identified by Researchers Conducting Yeast Two-Hybrid Screens

Protein	Number of times found as a false-positive
Heat shock proteins	16
Ribosomal proteins	14
Cytomchrome oxidase	5
Other mitochondrial proteins ^a	3
Proteasome subunits	4
Ferritin	4
Transfer-RNA synthase	3
Collagen-related proteins	3
Zinc finger containing proteins	3
Vimentin	2
Inorganic pyrophosphatase	2
Proliferating cell nuclear antigen	2

^aFive other mitochondrial proteins are among other categories.
(See **ref. 6**, www.fccc.edu/research/labs/golemis/).

2. The clones should then be tested for suitability in interaction studies. Cotransform the bait vector (pGBKT7/Gene X) and prey vector (pGADT7/gene Y) into the reporter strain with the empty AD and DNA-BD vectors, respectively, using the methods described in **Subheading 3.1.3.**, selecting for positive transformants on SD/–Trp/–Leu (see **Note 2**).
3. Test the autoactivation properties of the bait and prey vectors, possible toxicity problems, and expression of the fusion proteins (see **Subheading 3.1.4.**).
4. Once the bait and prey vectors have been tested, cotransform the bait (pGBKT7/gene X) and prey (pGADT7/gene Y) vectors into a yeast reporter strain (see **Subheading 3.1.3.**).
5. Select yeast colonies from each transformation and test for restoration of His auxotrophy (see **Subheading 3.2.3.**) and β -galactosidase activity (see **Subheading 3.2.4.**).

3.6. Preparing Yeast Two-Hybrid Data for Publication

As with all scientific experiments, the ultimate aim is to publish the data. Below we describe two of the most popular methods (streak and spot) used to present data obtained from yeast two-hybrid screens and interaction studies. The method chosen will depend on the amount of data to be presented and the way that most clearly demonstrates the protein–protein interaction differences detected. Additional methods include colony filter lift assay described in **Subheading 3.2.4.** and quantitative analysis of β -galactosidase activities (**5**).

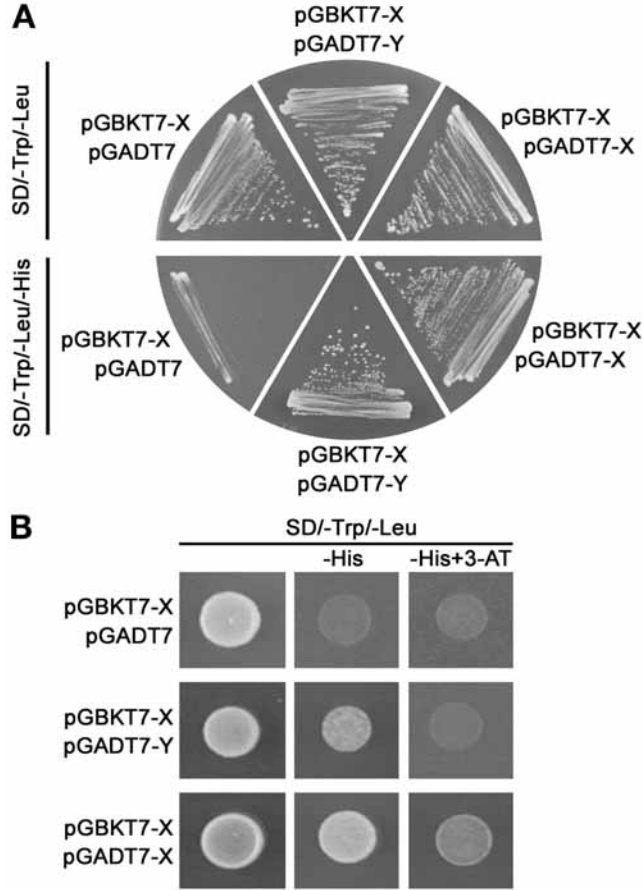


Fig. 5. A yeast two-hybrid assay testing whether protein X can interact with protein Y and whether protein X can form homodimers. **(A)** SD plates lacking Trp and Leu (top) or lacking Trp, Leu, and His (bottom). Note that protein X interacts relatively weakly with protein Y (some growth on SD/-Trp/Leu/-His), whereas protein X interacts strongly with itself (good growth on SD/-Trp/Leu/-His). **(B)** The same SD plates and yeast strains as in (A) plus SD/-Trp/-Leu/-His/+5 mM 3-AT plates. The yeast was spotted onto the plates and allowed to grow for 4 d. The same result was obtained as in (A).

To streak yeast plates, use a sterile inoculating loop or toothpick. Load approximately equal amounts of yeast material onto the loop from a freshly grown colony (2–3 d) and streak in a triangular shape onto a SD/-Trp/-Leu plate as a control for growth and onto a SD/-Trp/-Leu/-His plate to select for interactions. Incubate at 30°C for 2 to 6 d (**Fig. 5A**).

To spot yeast material, grow a colony overnight in SD/–Trp/–Leu media at 30°C to OD₆₀₀ = 0.8. From this overnight culture pipet 5 µL of culture onto a SD/–Trp/–Leu plate as a control for growth and onto a SD/–Trp/–Leu/–His plate to select for protein interactions. Incubate at 30°C for 2 to 6 d (**Fig. 5B**). For each method it is recommended that photographs be taken over several days to record the growth differences of the yeast before it reaches saturation.

4. Notes

1. Dropout solution is a mixture of specific amino acids and nucleosides used to supplement the SD base to make SD medium. Dropout solutions are missing one or more of the nutrients required by untransformed yeast cells to grow on SD medium. Yeast cloning vectors carry nutritional markers to allow for selection of yeast transformants plated on the appropriate SD media lacking specific nutrients. For example, to prepare a –Trp, –Leu double dropout powder combine all the amino acids listed in **Heading 2.** except Trp and Leu.
2. All amino acids listed in **Heading 2., item 13** should be mixed, omitting (a) Trp for pGBKT7 selection, (b) Leu for pGADT7 selection, (c) Trp and Leu for pGBKT7/pGADT7 double selection, and (d) Trp, Leu, and His for double selection and protein–protein interaction analysis.
3. Both the DNA-AD and DNA-BD vectors in the Clontech Matchmaker III kit include T7 promoters downstream of the GAL4 coding sequences. This offers the opportunity to transcribe and translate the epitope-tagged gene fusions in vitro.
4. The PCR-mediated incorporation of restriction endonuclease sites ensures the correct reading frame and gene orientation to allow for subsequent GAL4 DNA-BD/bait fusion protein expression. Any of the restriction endonuclease sites in the MCS which are absent in your gene of interest can be used. The authors favor a combination such as *NdeI* and *BamHI*, which enable genes to be subcloned easily into both pGBKT7 and the AD vector pGADT7 (see **Figs. 1** and **2**).
5. If an overnight culture of yeast is not available, scrape a toothpick of colonies from a plate and resuspend in 50 to 100 µL of YPDA. Transformation is most efficient if cells are used from a fresh plate but will work, albeit at a lower efficiency, using yeast on older plates (up to 8 wk).
6. The number of transformants increases linearly over time. For example, 2 h is sufficient for single transformations, whereas 24 h gives fourfold more than 6 h.
7. If adding 3-AT fails to suppress the autoactivation activity of your bait, then it will be necessary to modify the bait protein or to screen using only selected domains of the bait protein. For example, cellular localization signals or strong hydrophobic domains may cause problems, and in such cases truncations of the problematic proteins may render them suitable.
8. Expression from certain promoters leads to low or very low levels of fusion protein expression that may not be detectable using Western blotting. However, low-level expression does not prevent a fusion protein from working well in a screen and the authors have found this to be the case.

9. If the bait strain is toxic to yeast then it may be possible to counter this problem by using a DNA-BD vector that has a lower level of expression, such as pGBT9 (Clontech).
10. Combinations of yeast strains other than AH109 and Y187 can be used in a yeast two-hybrid screen by mating. The key to choosing the strains is to ensure that the bait and prey strains are of the opposite mating types (a and α), and that both have auxotrophies that allow for selection of the appropriate plasmids and reporter genes.
11. The viability of diploids is useful information because it can be used to calculate how many clones will be screened. This will of course be dependent on the quality of the cDNA library.
12. The “limiting partner” is the mating partner with the lower viability. Ideally this should be the AH109 (library) strain to ensure that the maximum number of library cells can find a mating partner.
13. Although mating efficiencies higher than 50% are observed in our laboratory, it is noteworthy that the manufacturer’s protocol states that mating efficiencies should be $>2\%$ (Clontech).
14. Check plates every day and mark colonies with the day number on which they appear. On the final day pick all colonies and streak in order of appearance. This can facilitate the decision of which clones to analyze further.
15. For long-term (indefinite) storage, yeast strains should be stored at -80°C as glycerol stocks. To prepare a glycerol stock of yeast use freshly cultured cells; pellet and resuspend in yeast peptone dextrose/20% glycerol. Ensure that the yeast cells are distributed in the medium by mixing well before freezing. To recover frozen strains streak a small amount of the frozen glycerol stock onto the appropriate SD plate and incubate at 30°C for 2 to 3 d.
16. Although we prioritize $\text{His}^+/\text{LacZ}^+$ colonies for our initial characterization, $\text{His}^+/\text{LacZ}^-$ colonies should never be discarded because the *lacZ* reporter gene is less sensitive than the His reporter gene.
17. Check for the development of blue color at regular intervals. Strong-positives will develop a blue color in more than 30 min. Weaker interactions may take up to 8 h to develop. Prolonged incubation may give rise to false-positives.
18. Once positive interactions have been identified using the yeast two-hybrid system they should be confirmed either *in vivo* using a different technique such as fluorescence resonance energy transfer analysis or by *in vitro* immunoprecipitation approaches.
19. In cases where many positive colonies are obtained, it may be helpful to initially group these using a rapid PCR screening test. The DNA prepared from the yeast can be used as a PCR template together with the AD vector-specific T7 primer and 3' DNA-BD sequencing primer (Clontech). The PCR products should be separated on an agarose gel to identify identical and different clones.

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Microarrays

Quality Control and Hybridization Protocol

Ken-ichiro Uno and Hiroki R. Ueda

Summary

Microarray technology is an exciting and promising tool, and is increasingly employed for studying circadian rhythms. To obtain optimal results from this technology, it is important to perform quality control experiments before engaging in genome-wide expression analysis. In this chapter, we provide an overview of quantitative polymerase chain reaction experiments using the ABI PRISM® 7900HT system for quality control of samples, and micorarray experiments using the Affymetrix GeneChip system for genome-wide expression analysis.

Key Words: Circadian rhythm; quantitative PCR; microarray; quality control.

1. Introduction

The past decade has seen a growing number of model organisms that have either draft or complete genome sequences, including *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens*. The availability of these resources and the development of tools such as DNA microarrays that allow high-throughput experimental biology have been driving a paradigm shift in the life sciences, from the molecular “one-gene-at-a-time” level to a network or systems level (**1–5**).

Genome-wide experimental biology approaches offer the opportunity to work more efficiently, and also the possibility to explore universal governing principles underlying physiology. Several recent microarray studies have investigated the large-scale organization of gene expression, revealing complex networks that capitulate physiological processes such as the cell cycle, responses to environmental change, circadian rhythms, and developmental and tissue specific gene regulation (**6–8**). In microarray studies of circadian rhythms, we,

along with others, performed genome-wide gene expression analyses using DNA microarrays to identify clock-controlled genes in *Arabidopsis*, *Drosophila*, and mammals (6,9–25). In this chapter, we describe the overall procedures of quantitative polymerase chain reaction (Q-PCR) experiments using the ABI PRISM® 7900HT system for quality control of RNA samples, and also micorarray experiments using the Affymetrix GeneChip® system for genome-wide expression analysis.

2. Materials

2.1. Quantitative PCR

1. ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).
2. Primer Express® (Applied Biosystems).
3. Microsoft Excel® (Microsoft).
4. Random primer (PROMEGA, cat. no. C1181); store at –20°C.
5. Super Script II RNaseH–Reverse Transcriptase 200 U/μL, 5X first-strand buffer, 0.1 M dithiothreitol (DTT; Invitrogen, cat. no. 18064-014); store at –20°C.
6. 10 mM dNTP mix (Invitrogen, cat. no. 18427-013); store at –20°C.
7. 2X SYBR® Green PCR Master Mix (Applied Biosystems, cat. no. 4309155); store at 4°C in dark.
8. 384-Well clear optical reaction plate (Applied Biosystems, cat. no. 4309849).
9. Optical adhesive covers (Applied Biosystems, cat. no. 4311971).

2.2. GeneChip

1. Affymetrix GeneChip technology equipment: hybridization oven, operating software (GCOS), fluidics station 400, scanner 3000.

2.2.1. Preparation of Double-Stranded cDNA

1. T7-(dT)₂₄ Primer (Amersham, cat. no. 72-0591-01); dissolve in water to 100 pmol/μL and store at –20°C.
2. Super ScriptII RNaseH–Reverse Transcriptase 200 U/μL, 5X first-strand buffer, 0.1 M DTT (Invitrogen, cat. no. 18064-014); store at –20°C.
3. 10 mM dNTP mix (Invitrogen, cat. no. 18427-013); store at –20°C.
4. 5X Second-strand buffer (Invitrogen, cat. no. 10812-014); store at –20°C.
5. 10 U/μL DNA ligase (Invitrogen, cat. no. 18052-019); store at –20°C.
6. 10 U/μL DNA polymerase I (Invitrogen, cat. no. 18010-025); store at –20°C.
7. 2 U/μL RNaseH (Invitrogen, cat. no. 18021-071); store at –20°C.
8. 5 U/μL T4 DNA polymerase (Invitrogen, cat. no. 18005-025); store at –20°C.
9. 0.5 M EDTA (Sigma, cat. no. E7889).
10. Phenol:chloroform:isoamyl alcohol (25:24:1; Invitrogen, cat. no. 15593-049); store at 4°C.
11. 7.5 M Ammonium acetate (Sigma, cat. no. A2706); store at 4°C.
12. Glycogen (Ambion, cat. no. 9510); store at –20°C.

2.2.2. Preparation of Biotin-Labeled cRNA

1. Enzo® BioArray™ HighYield™ RNA Transcript Labeling Kit (T7; Enzo Life Science, cat. no. 42655-10); store at -20°C .
2. RNeasy® Mini Kit (QIAGEN, cat. no. 74103, 74104, or 74106).

2.2.3. Fragmentation of cRNA

1. 5X Fragmentation buffer: 4 mL of 1 M Tris-acetate, pH 8.1, 0.64 g of magnesium acetate, 0.98 g of potassium acetate, DEPC-treated water to 20 mL. Sterilize with a 0.2- μm filter and store at room temperature.

2.2.4. Hybridization Cocktail and Hybridization

1. Eukaryotic hybridization control kit, including control oligonucleotide B2 (3 nM) and 20X eukaryotic hybridization controls (Affymetrix, cat. no. 900299); store at -20°C .
2. Herring sperm DNA (Promega, cat. no. D1811); store at -20°C .
3. Acetylated BSA solution (Invitrogen, cat. no. 15561-020); store at -20°C .
4. GeneChip Probe Array (Affymetrix).
5. 12X 2-morpholinoethanesulfonic (MES) stock (1.22 M MES, 0.89 M $[\text{Na}^+]$): 70.4 g MES-free acid monohydrate (Sigma, cat. no. M5287), 193.3 g MES sodium salt (Sigma, cat. no. M5057), H_2O to 1 L. Prepare under RNase-free conditions, sterilize through a 0.2- μm filter, and store in the dark at 4°C .
6. 2X Hybridization buffer: 8.3 mL of 12X MES stock, 17.7 mL of 5 M NaCl, 4.0 mL of 0.5 M EDTA, 0.1 mL of 10% Tween-20, 19.9 mL of H_2O . Prepare under RNase-free conditions and store in dark at 4°C .

2.2.5. Wash and Stain

1. Nonstringent wash buffer (Wash A): 300 mL of 20X SSPE (Invitrogen, cat. no. 15591-043), 1 mL of 10% Tween-20, 699 mL of H_2O . Prepare under RNase-free conditions, sterilize through a 0.2- μm filter, and store in the dark at 4°C or room temperature.
2. Stringent wash buffer (Wash B): 83.3 mL of 12X MES stock buffer, 5.2 mL of 5 M NaCl, 1.0 mL of 10% Tween-20, 910.5 mL of H_2O . Prepare under RNase-free conditions, sterilize through a 0.2- μm filter, and store in the dark at 4°C .
3. R-phycoerythrin-streptavidin (SAPE; Molecular Probes, cat. no. S-866); store at 4°C in the dark.
4. 2X Stain buffer: 41.7 mL of 12X MES stock buffer, 92.5 mL of 5 M NaCl, 2.5 mL of 10% Tween-20, 113.3 mL of H_2O . Prepare under RNase-free conditions, sterilize through a 0.2- μm filter, and store in the dark at 4°C .
5. SAPE stain solution (stains 1 and 3): 600 μL of 2X stain buffer, 48 μL of acetylated bovine serum albumin (BSA; 50 mg/mL), 12 μL of SAPE (1 mg/mL), 540 μL of H_2O . Divide into two aliquots of 600 μL each to be used for stains 1 and 3.
6. Goat IgG, reagent grade (Sigma, cat. no. 15256); resuspend to 10 mg/mL in phosphate-buffered saline (PBS) and store at 4°C .

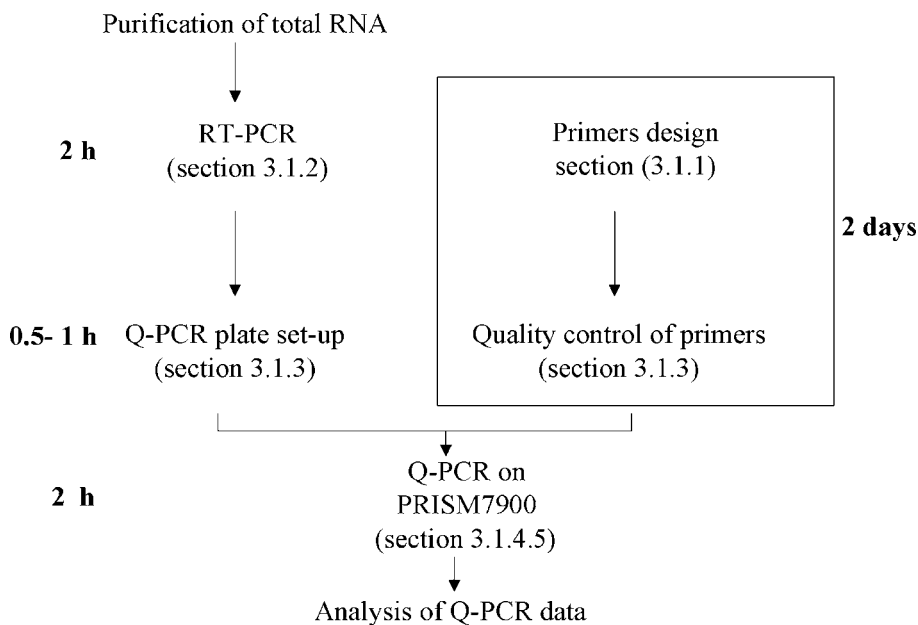


Fig. 1. Quantitative polymerase chain reaction flow chart.

7. Antistreptavidin antibody (goat), biotinylated (VectorLaboratories, cat. no. BA-0500); resuspend to 0.5 mg/mL in PBS and store at 4°C.
8. Antibody solution (stain 2): 300 μ L of 2X stain buffer, 24 μ L of acetylated BSA (50 mg/mL), 6 μ L of normal goat IgG (10 mg/mL), 3.6 μ L of biotinylated antibody (0.5 mg/mL), 266.4 μ L of H₂O.

3. Methods

3.1. Q-PCR: Quality Check of Sample RNA

Quality control of samples prior to microarray analysis can be performed by Q-PCR. This method requires only a small amount (250 ng) of total RNA and results in excellent reproducibility. Moreover, Q-PCR can be used for independent verification of the data following microarray analysis. Here we describe the overall procedure for Q-PCR (**Fig. 1**).

3.1.1. Primer Design

The design of primer sets for Q-PCR begins with obtaining the DNA sequence for the gene of interest via a public database such as “UCSC Genome Browser” (<http://genome.ucsc.edu/>), “Ensembl” (www.ensembl.org/), and “LocusLink” (www.ncbi.nlm.nih.gov/LocusLink). We usually design Q-PCR primer sets

within a single exon at the 3'-untranslated region (UTR) of a target gene. There are several reasons for this choice:

1. A relatively large exon can be usually found at the 3'-UTR of genes.
2. DNA sequences at the 3'-UTR are relatively unique.
3. Probes used in the GeneChip expression analysis system are designed at the 3'-UTR.
4. Designing primer sets within a single exon allows the use of genomic DNA as a standard for absolute quantification.

We do not design primers across exon boundaries and we recommend avoiding repetitive sequences, which are not suitable for Q-PCR analysis. For exons longer than 200 bp, the primer design is not very difficult. We usually use Primer Express® software, which is included with the PRISM® 7900HT system. Here we describe the basic procedure for primer design using Primer Express.

1. Start the software Primer Express.
2. For designing new primers, select "DNA PCR Document" from the "File/New" menu.
3. Enter the DNA sequence either by going to "File/Import" and selecting the "Import DNA File" button in the "Sequence" Tab, or by copying and pasting the sequence through the "Edit" menu.
4. Click the "Params" tab and then click the "Defaults" button. You will not need to alter the majority of the parameters, as the defaults follow the Probe and Primer design guidelines established by Applied Biosystems. However, you should change "Min length" at "Amplicon Requirements," we usually use a "Min length" value between 50 and 80. The default parameters that we use are as follows: At "Primer Tm Requirements," "Min Tm" is 58°C, "Max Tm" 60°C, "Optimal Tm" 59°C, and "Maximal Tm difference" 2°C. At "Primer GC content Requirements," "Min %GC" is 20, "Max %GC" 80, and "3'-GC clamp of residue" 0. At "Primer Length Requirements," "Min length" is 9, "Max length" 40, and "Optimal length" 20. At "Amplicon Requirements," there is no other parameter than "Min length" as described above. "Min Tm" is 0°C, "Max Tm" 85°C, and "Max length" 150.
5. Select "Find Primers/Probes Now" from the "Options" menu. Wait for the end of operation.
6. If the software finds suitable primers, click the "Primers" tab to display a list of suggested primer sets. If the software cannot find suitable primers, try again by changing the "Min length" parameter of the "Params" tab.
7. Select a primer pair from the list that will produce the shortest amplicon while satisfying all design guidelines.
8. Click on the line containing the chosen primer and probe set, then click on the "Order" button at the bottom of the "Primers" tab. This order form does not actually place electronic orders. The "Order" document is a text file that enables the editing of the sequence information for ordering. The sequences within the order document can now be copied and pasted into electronic orders.
9. Test the quality of the primers before extensive use in Q-PCR analyses (*see* **Note 1**).

Below we provide the name, accession number, and primer sequence for six mouse genes commonly used for circadian expression analysis. *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapd*), *b-actin* (*Actb*) and *TATA box binding protein* (*Tbp*) are used as internal (noncycling) controls. *Period2* (*Per2*), *Cryptochrome1* (*Cry1*), and *Brain muscle arnt like1* (*Bmal1*) are used as positive controls.

Gapd NM_008084

mGapd-1039F CAAGGAGTAAGAAACCCTGGACC

mGapd-1109R CGAGTTGGGATAGGGCCTCT

Tbp NM_013684

mTbp-722F GTGATGTGAAGTTCCCCATAAGG

mTbp-782R CTAAGTGAAGTCTGGTGGGTCA

Actb NM_007393

mActb-1765F TTGTCCCCCAACTTGATGT

mActb-1835R CCTGGCTGCCTCAACACCT

Per2 NM_011066

mPer2-5643F TGTGCGATGATGATTCGTGA

mPer2-5713R GGTGAAGGTACGTTTGGTTTGC

Cry1 NM_007771

mCry1-1740F TGAGGCAAGCAGACTGAATATTG

mCry1-1805R CCTCTGTACCGGGAAAGCTG

Bmal1 NM_007489

mBmal1-2407F CCACCTCAGAGCCATTGATACA

mBmal1-2477R GAGCAGGTTTAGTTCCACTTTGTCT

3.1.2. Reverse Transcription

Q-PCR experiments start with the synthesis of cDNA from total RNA. In this step, random hexamers prime total RNA for reverse transcription (RT). Here we describe the detailed protocol for RT.

1. Add the following components to a nuclease-free tube:
 - a. Total RNA (0.1 µg/µL): 2.5 µL.
 - b. Random primer (25 ng/µL): 2.5 µL.
 - c. DEPC-treated H₂O: 7.0 µL.
2. Incubate at 70°C for 10 min in a thermal cycler.
3. Quickly chill on ice.
4. Add the following components:
 - a. 4.0 µL 5X first-strand buffer.
 - b. 2.0 µL 0.1 M DTT.

- c. 1.0 μL 10 mM dNTP mix.
- d. 1.0 μL 200 U/ μL Super Script II RT.
5. Gently mix the contents of the tube.
6. Incubate the reaction at 25°C for 15 min, at 42°C for 50 min, and then at 70°C for 15 min using a thermal cycler.
7. Chill on ice, then add 180 μL (or 380 μL) of H_2O and transfer to a new 1.5-mL tube.
8. Store at -20°C.

3.1.3. Preparation of Q-PCR Reaction Plates

In this section, we describe the preparation of reaction plates (384-well format) for Q-PCR using the ABI PRISM 7900HT Sequence Detection System and SYBR Green I dye. In this system, PCR products are monitored in real time by measuring the increase in fluorescence caused by the binding of SYBR Green I to double-stranded DNA.

The quantification of gene expression via RT Q-PCR requires normalization of the data according to the amount of cDNA added to each PCR reaction. For this purpose we use amplification of *Gapd*, *Tbp*, or *Actb* (depending on the organism and tissue) as an invariable internal control. Because absolute quantification requires that absolute quantities of the standard should be known by independent means, we use genomic DNA to prepare these absolute standards. The DNA concentration is measured by absorbance at 260 nm and it is converted to genome copies per microliter using the molecular weight of the genomic DNA. **Figure 2** shows the plate setup for quantification of the expression of 10 genes. Fivefold dilutions of genomic DNA are used to construct the standard curve for each gene. We usually use six dilutions (1/5, 1/25, 1/125, 1/625, 1/3125, and 1/15625) of mouse genomic DNA (0.1 $\mu\text{g}/\mu\text{L}$), which correspond to 6667, 1333, 267, 53, 11, and 2 genome copies per microliter, respectively (*see Note 2*). Genomic DNA standards and cDNA samples are usually assayed in duplicate. The arrangement of standards and samples on the plate is dependent on the number of genes and samples. We usually design the plate setup with spreadsheet software such as Microsoft Excel, and then prepare the Q-PCR reaction plate.

1. Add 4.0 μL of each cDNA sample (prepared as in **Subheading 3.1.2.**) into the designated wells of a 384-well clear optical reaction plate, in duplicate. Do the same for each gene tested.
2. Add 4.0 μL of each dilution of genomic DNA into the designated wells of the plate, in duplicate. Do the same for each gene tested.
3. Prepare a PCR reaction mixture as follows:
 - a. 2X SYBR Green PCR master mix: 5.0 $\mu\text{L}/\text{well}$.
 - b. Forward primer (100 mM): 0.5 $\mu\text{L}/\text{well}$.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0-1		4-1		8-1		12-1		16-1		20-1		0-2		4-2		8-2		12-2		16-2		20-2	
B	1/5		1/25		1/125		1/625		1/3125		1/15625		1/5		1/25		1/125		1/625		1/3125		1/15625	
C	0-1		4-1		8-1		12-1		16-1		20-1		0-2		4-2		8-2		12-2		16-2		20-2	
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Fig. 2. Quantitative polymerase chain reaction design on a 384-well plate. Samples collected every 4 h over 2 d generate 12 cDNA to be analyzed for each gene tested. Moreover, for each gene, six dilutions of genomic DNA are required to build the standard curve used for absolute quantification. Each assay is performed in duplicate; hence 36 wells (12 for standards, 24 for samples) are required for the analysis of each one gene. On a 384-well plate, 10 genes can be analyzed simultaneously. Wells from A1 to B12 are used for gene 1, wells from B13 to C24 are used for gene 2, ...wells from N13 to O24 are used for gene 10. A sample name such as “0–1” indicates time 0 on day 1, “4–2” means time 4 on day 2, and so on. “1/5,” “1/25,” and so forth indicate fivefold dilutions of genomic DNA standards.

- c. Reverse primer (100 mM): 0.5 µL/well.
- d. H₂O: 0.9 µL/well.
- 4. Dispense 6.0 µL of the reaction mixture into each well with template DNA.
- 5. Seal the plate with an optical clear seal and mix the plate well.
- 6. Spin down. The plate can be kept at 4°C for several hours.

3.1.4. Q-PCR

Every run on the 7900HT SDS instrument requires the creation of a plate document within the SDS software.

- 1. Launch the SDS software SDS 2.1.
- 2. Select “File/New” to open New Document dialog box.
- 3. Configure the New Document dialog box with the following settings:
 - a. “Assay” drop-down list—select “Absolute Quantification (Standard Curve).”
 - b. “Container” drop-down list—select “384 Wells Clear Plate.”

- c. "Template" drop-down list—select "Blank Template."
- d. "Barcode" field—leave blank.
4. Click "OK."
5. Select "Tools/Detector Manager" (*see Note 3*).
6. In the Detector Manager dialog box, click "New."
7. Configure the Add Detector dialog box:
 - a. "Name" field—enter a name for the detector (e.g. "SYBR-GAPD").
 - b. "Description" field (optional)—enter a brief description of the assay.
 - c. "Reporter" drop-down list—select "SYBR Green."
 - d. "Quencher" drop-down list—select "Non Fluorescent."
 - e. "Color" box (optional)—click the box then use the Color Picker dialog box to select a color to represent the detector, and click "OK."
 - f. "Note" field (optional)—enter any additional comments for the detector.
8. Click "OK." The software saves the new detector and displays it in the detector list.
9. Repeat **steps 6–8** to create detectors for all remaining assays on the plate.
10. In the Detector Manager dialog box of the SDS software, copy the detectors to the plate document:
 - a. While pressing and holding the "Ctrl" key, select the detectors you want to apply to the plate document. The software highlights the selected detectors.
 - b. Click "Copy to Plate Document." The software adds the detectors to the well inspector (right window) of the plate document.
11. Click "Done" to close the Detector Manager.
12. In the plate grid (upper left window), select the wells containing the assay for the first detector.
13. Apply detector to the selection by clicking the check box for the detector in the "Use" column of the well inspector (right window).
14. Repeat **steps 12** and **13** to apply the remaining detectors to the plate grid (upper left window).
15. Using the "Ctrl" and "Shift" keys select the wells of the plate grid (upper left window) containing samples for a particular task such as sample, standard or negative control (*see Note 4*).
16. In the well inspector (right window), click the field in the "Task" column for each detector entry and select the appropriate task from the drop-down list. A task "Unknown" will be applied to cDNA samples, "Standard" to genomic DNA standards, and "NTC" to negative control wells that contain PCR reagents but lack template DNA. The SDS software labels all selected wells with the task.
17. Repeat **steps 15** and **16** until all tasks are applied to the plate document.
18. In the plate grid (upper left window) of the SDS software, select the replicate wells containing the first standard, i.e., the first dilution of genomic DNA.
19. In the well inspector (right window), click the field in the "Task" column for the appropriate detector entry, and select the "Standard" from the drop-down list. Click the "Quantity" column for the appropriate detector, enter a quantity for the

- standard in the appropriate unit of measurement (e.g., copy number), and press “Enter.” The software labels the selected standard wells with the specified quantity.
20. Repeat **steps 18 and 19** to configure the plate with the other sets of replicate standard wells on the plate.
 21. In the “Instrument” tab of the plate document select the “Thermal Profile” tab (*see Note 5*).
 22. Deselect the “9600 Emulation” checkbox (*see Note 6*).
 23. Modify the default thermal profile as follows:
 - a. Delete Stage 1.
 - b. Change the “Repeats” in Stage 3 from 30 to 45.
 - c. Change the “Temperature” in the second step of Stage 3 from 60°C to 59°C (*see Note 7*).
 - d. Click the “Sample Volume (mL)” field and enter 10 μ L.
 - e. If you would like to check that your primers can amplify the target sequence correctly, click “Add Dissociation Stage” (*see Note 8*).
 24. In the SDS software, select the “Instrument” tab of the plate document.
 25. In the “Real-Time” tab of the “Instrument” tab, click “Open/Close.” The instrument tray rotates to the OUT position.
 26. Place the prepared optical plate into the instrument tray as shown below.
 27. In the “Real-Time” tab of the “Instrument” tab, click “Open/Close.” The instrument tray rotates to the IN position.
 28. Select “Files/Save As” to save the ABI PRISM SDS Document file.
 29. Click “Start” to perform a Q-PCR.
 30. After the Q-PCR run, you can save the run data to the ABI PRISM SDS Document file by selecting “Files/Save As.”
 31. Click “Open/Close” in the “Instrument” tab to eject the optical plate.

3.1.5. Q-PCR Data Analysis

1. Click “OK” in the small window that opens after successful conclusion of the run.
2. Select “Analysis/Analyze.” The SDS software analyzes the run data and displays the results in the “Results” tab.
3. Select the “Results” tab and view “Standard plot” and “Amplification plot” for each gene analyzed.
4. If you have added the dissociation stage to your protocol, you can view the curve in the “Dissociation Curve.” Correct amplification will result in a single peak in the dissociation plot.
5. Select “File/Export” to export the results to a text file.
6. Close the SDS 2.1 software and turn off ABI PRISM 7900HT system.

3.1.6. Quality Control of RNA Samples for Microarray by Q-PCR

To evaluate the quality of an RNA sample for microarray experiments by Q-PCR, we usually measure the expression of three housekeeping genes (*Gapd*, β -*actin*, and *Tbp*) as internal controls, and of three clock genes (*Per2*,

Table 1
Outline of Affymetrix GeneChip Expression Analysis Protocol

Day 1	
Preparation of double-stranded cDNA (Subheading 3.2.1.)	
First-strand cDNA synthesis	2 h
Second-strand cDNA synthesis	2.5 h
Cleanup of double-stranded cDNA	1.5 h
Day 2	
Preparation of biotin-labeled cRNA (Subheading 3.2.2.)	
Synthesis of biotin-labeled cRNA	5 h
Cleanup of biotin-labeled cRNA	1 h
Fragmentation of cRNA	1 h
Hybridization (<i>see Subheading 3.2.3.</i>)	
Preparation of hybridization cocktail	0.5 h
Hybridization of probe array	0.5 h
Day 3	
Stain and wash (Subheading 3.2.4.)	1.5 h/4 arrays
Scan and analysis (Subheading 3.2.5.)	0.5 h/1 array

Cry1, and *Bmal1*) as positive controls of circadian expression. We first check whether the expression of the housekeeping genes is really constant or if there is rather a noticeable difference among samples. We usually observe that the expression of at least one of the above genes shows less than a twofold difference among samples. For example, we usually use *Tbp* or *Gapd* as an internal control for expression analysis of mouse liver. Conversely, β -*actin* shows more than a twofold variation in this tissue. We then confirm the rhythmic expression of *Per2*, *Cry1*, and *Bmal1* and their sequential phase of expression in this order.

3.2. Genome-Wide Expression Profiling With Microarrays

After the quality check, the samples can be used for microarray experiments. The major steps using Affymetrix GeneChip Expression Analysis are highlighted in **Table 1**. A brief outline of the protocol follows below.

1. On the first and second days of the microarray experiment, total RNA is isolated from tissue or cells and used to produce double-stranded cDNA (**Subheading 3.2.1.**; *see Note 9*). An in vitro transcription reaction is then performed to produce biotin-labeled cRNA from the cDNA (**Subheading 3.2.2.**).
2. At the end of second day, the cRNA is fragmented and a hybridization cocktail is prepared, which includes fragmented cRNA, probe array control, BSA, and herring sperm DNA. This cocktail is then hybridized to the probe array during a 16-h incubation (**Subheading 3.2.3.**).

3. On the third day of the experiment, immediately after hybridization, the probe array undergoes an automated washing and staining protocol on the fluidics station (**Subheading 3.2.4.**).
4. Finally, the probe array is scanned and images are stored in separate files identified by the experiment name and are saved with a data file (.dat) extension. Data are analyzed, using Microarray Suite, for probe intensity; results are reported in tabular and graphical formats (**Subheading 3.2.5.**).

3.2.1. Preparation of Double-Stranded cDNA

3.2.1.1. FIRST-STRAND cDNA SYNTHESIS

1. Mix the following components:
 - a. Total RNA sample: 5.0 to 10.0 μg .
 - b. T7-(dT)₂₄ primer (100 pmol/mL): 1 μL .
 - c. DEPC-treated H₂O: to 12 μL final volume.
2. Incubate for 10 min at 70°C.
3. Cool the sample at 4°C for at least 2 min.
4. Add the following first-strand master mix:
 - a. 5X first-strand reaction buffer: 4 μL .
 - b. DTT (0.1 M): 2 μL .
 - c. dNTP mix (10 mM): 1 μL .
5. Incubate for 2 min at 42°C.
6. Add 1 μL of Super Script II (200 U/ μL) to each RNA sample for a final volume of 20 μL .
7. Incubate for 1.5 h at 42°C.

3.2.1.2. SECOND-STRAND cDNA SYNTHESIS

1. Add 130 μL of the following second-strand master mix to each 20- μL first-strand synthesis sample for a total volume of 150 μL .
 - a. 5X Second-strand buffer: 30 μL .
 - b. dNTP mix (10 mM): 3 μL .
 - c. DNA ligase (10 U/ μL): 1 μL .
 - d. DNA polymerase I (10 U/ μL): 4 μL .
 - e. RNaseH (2 U/ μL): 1 μL .
 - f. DEPC-treated water: 91 μL .
2. Incubate for 2 h at 16°C in a cooling water bath.
3. Add 2 μL of T4 DNA polymerase (5 U/ μL) for blunting.
4. Incubate for 5 min at 16°C in a cooling water bath.
5. Stop the reaction by adding 10 μL of 0.5 M EDTA (final volume 162 μL).

3.2.1.3. DOUBLE-STRANDED cDNA CLEANUP

1. Add 162 μL of phenol:chloroform:isoamyl alcohol (25:24:1) to the double-stranded cDNA sample for a total volume of 324 μL .

2. Mix briefly by vortexing.
3. Centrifuge for 5 min at maximum speed ($>12,000g$).
4. Transfer the aqueous upper phase to a fresh 1.5-mL tube.
5. Add 1 μ L glycogen, 80 μ L of 7.5 M NH₄Ac, and 400 μ L of 99.5% ethanol (stored at -20°C) and mix by vortexing.
6. Centrifuge for 20 min at maximum speed ($>12,000g$) at room temperature.
7. Remove the supernatant. Wash the pellet with 0.5 mL of 80% ethanol (stored at -20°C).
8. Centrifuge for 10 min at maximum speed ($>12,000g$) at room temperature.
9. Remove the 80% ethanol very carefully.
10. Repeat the 80% ethanol wash, **steps 7–9**.
11. Air-dry the pellet.
12. Resuspend the pellet in 12 μ L of DEPC-treated H₂O.
13. Store at -20°C .
14. Check 1 μ L of the cDNA by electrophoresis on a 1% agarose gel (TAE or TBE).

3.2.2. Preparation of Biotin-Labeled cRNA

3.2.2.1. SYNTHESIS OF BIOTIN-LABELED cRNA

We usually use the Enzo BioArray HighYield RNA Transcript Labeling Kit in this step.

1. Mix the following components:
 - a. Template cDNA: 10 μ L.
 - b. DEPC-treated H₂O: 12 μ L.
 - c. 10X HY reaction buffer: 4 μ L.
 - d. 10X biotin-labeled ribonucleotides: 4 μ L.
 - e. 10X DTT: 4 μ L.
 - f. 10X RNase inhibitor mix: 4 μ L.
 - g. 20X T7 RNA polymerase: 2 μ L.
2. Incubate immediately for 5 to 6 h at 37°C . Gently mix the contents by tapping and spin down every 1 to 1.5 h during the incubation.

3.2.2.2. CLEAN UP OF BIOTIN-LABELED cRNA

We usually use the QIAGEN RNeasy mini kit in this step.

1. Add 60 μ L of DEPC-treated H₂O to the cRNA sample (40 μ L) for a total volume of 100 μ L.
2. Add 350 μ L of buffer RLT to the cRNA sample and mix thoroughly.
3. Add 250 μ L of 95 to 100% ethanol to the cRNA sample and mix thoroughly by pipetting.
4. Transfer the cRNA sample (700 μ L) to the RNeasy mini column.
5. Centrifuge for 15 s at more than 12,000g.
6. Reapply the flow-through onto the RNeasy mini column.
7. Centrifuge for 15 s at more than 12,000g and discard the flow-through.

8. Transfer the RNeasy column into a new 2-mL collection tube.
9. Add 500 μL of buffer RPE to the RNeasy column.
10. Centrifuge for 15 s at more than 12,000*g*. Discard the flow-through.
11. Add another 500 μL of buffer RPE to the RNeasy column.
12. Centrifuge for 2 min at maximum speed.
13. Transfer the RNeasy column to a new 1.5-mL collection tube.
14. Add 30 μL of RNase-free H_2O to the RNeasy column (directly to the silica-gel membrane).
15. Wait for 1 min and centrifuge for 1 min at more than 12,000*g*.
16. Measure the concentration of biotin-labeled cRNA with a spectrophotometer. The concentration of biotin-labeled cRNA should be more than 0.6 $\mu\text{g}/\mu\text{L}$.
17. Check 0.5 to 1 μg of biotin-labeled cRNA by electrophoresis on a 1% agarose gel (TAE or TBE).

3.2.2.3. cRNA FRAGMENTATION

1. The suggested fragmentation reaction mix for cRNA sample at a final concentration of 0.5 $\mu\text{g}/\mu\text{L}$ is shown below.

Components	49 Format (standard) array	100 Format (midi) array
Biotin-labeled cRNA sample	20 μg (1–21 μL)	15 μg (1–21 μL)
5X Fragmentation buffer	8 μL	6 μL
DEPC-treated H_2O	To 40 μL final volume	To 30 μL final volume

2. Incubate for 35 min at 94°C. Put on ice immediately afterward.
3. Check the fragmentation of the cRNA by electrophoresis on a 1% agarose gel (TAE or TBE).

3.2.3. Hybridization

3.2.3.1. PREPARATION OF HYBRIDIZATION COCKTAIL

1. Mix the following components to prepare the hybridization cocktail. The final concentration of the fragmented biotin-labeled cRNA is fixed at 0.05 $\mu\text{g}/\mu\text{L}$.

Components	49 Format (standard) array	100 Format (midi) array
Fragmented biotin-labeled cRNA	15 μg	10 μg
Control oligonucleotide B2 (3 nM)	5 μL	3.3 μL
20X Eukaryotic hybridization controls	15 μL	10 μL
Herring sperm DNA (10 mg/mL)	3 μL	2 μL
Acetylated BSA (50 mg/mL)	3 μL	2 μL
2X Hybridization buffer	150 μL	100 μL
DEPC-treated water	To final volume of 300 μL	To final volume of 200 μL

2. Incubate at 99°C for 5 min in a heat block.
3. Incubate at 45°C for 5 min.
4. Spin the hybridization cocktail at maximum speed for 5 min to remove any insoluble material from the hybridization mixture.
5. The hybridization cocktail can be stored at -80°C.

3.2.3.2. HYBRIDIZATION OF PROBE ARRAY

1. Equilibrate the probe array at room temperature immediately before use.
2. Fill the array with an appropriate volume of 1X hybridization buffer (200 µL for standard array, 130 µL for midi array, 80 µL for mini/micro array) and incubate at 45°C for 10 min in a hybridization oven with rotation at 60 rpm.
3. Remove the 1X hybridization buffer.
4. Fill the array with an appropriate volume (as above) of hybridization cocktail. If the hybridization cocktail has been stored at -80°C, it is necessary to incubate at 99°C for 5 min and at 45°C for 5 min before use.
5. Hybridize in the oven at 45°C for 16 h at 60 rpm.

3.2.4. Stain and Wash (Third Day)

In this section, we describe the stain and wash procedures using GeneChip Operating Software (GCOS), GeneChip Fluidics Station 400, and Affymetrix GeneChip Scanner 3000.

3.2.4.1. SYSTEM SETUP

1. Turn on the workstation, Fluidics Station, and GeneChip Scanner.
2. Start GCOS (e.g., select “Start/Programs/Affymetrix/GeneChip Operating Software”) from the workstation.
3. Select “Run/Fluidics” from the menu bar of GCOS to open the Fluidics Station dialog box.
4. To prime the fluidics station, select “Protocol” in the Fluidics Station dialog box and choose “Prime.”
5. Change the intake buffer reservoir A to “Non-stringent Wash Buffer” (wash buffer A) and intake buffer reservoir B to “Stringent Wash Buffer” (wash buffer B).
6. In GCOS, select the “All Modules” check box, and then click “Run.”
7. Exchange tubes according to the instruction by the LCD window on each module of the fluidics station.

3.2.4.2. ENTER EXPERIMENT INFORMATION

To wash, stain and scan a probe array, an experiment must be registered in GCOS.

1. Select “Run/Experiment info” from the menu bar to open new experiment information.

2. Input the fields of experimental information including “Experimental Name,” “Probe Array Type,” “Sample Name,” “Sample Type,” and “Project.” “Sample name” is especially important as it is used as a saved file name.
3. Select “File/Save” from the menu bar to save experiment information.

3.2.4.3. PROBE ARRAY WASH AND STAIN

1. After 16 h of hybridization, remove the hybridization cocktail from the probe array and transfer to a new 1.5-mL tube (reusable if stored at -80°C).
2. Fill the probe array with 200 to 250 μL of nonstringent wash buffer (wash buffer A).
3. Prepare 1200 μL of SAPE stain solution (stains 1 and 3) and 600 μL of antibody solution (stain 2).
4. In the Fluidics Station dialog box on the workstation, select the correct experiment name in the “Experiment” drop-down list. The probe array type will appear automatically.
5. In the “Protocol” drop-down list, select the appropriate antibody amplification protocol to control the washing and staining of the probe array format being used (e.g., choose EukGE-WS2vX for Standard Array).
6. Choose “Run” in the Fluidics Station dialog box to begin washing and staining.
7. Insert the probe array into the designated module of the fluidics station.
8. Engage the probe array and exchange tubes according to the instructions on the LCD window on each module of the fluidics station.

3.2.5. Scan and Analysis

3.2.5.1. PROBE ARRAY SCAN AND DATA ANALYSIS

1. After washing and staining, remove the probe array from the fluidics station. If you are unable to scan the arrays immediately, keep the probe array at 4°C and in dark conditions until ready for scanning.
2. Select “Run/Scanner” from the menu bar to open the scanner dialog box.
3. Select the experiment name that corresponds to the probe array to be scanned from the “Experiment Name” drop-down list.
4. Once the experiment has been selected, click the “Start” button.
5. Load the probe array into the scanner according to the message on a dialog box. Click “OK” in the Start Scanner dialog box.
6. After scanning, check the grid alignment at the four corners.
7. Select “Run/Analysis” from the menu bar to analyze the scanned image file.
8. Verify the .chp file name, edit if necessary, and click “OK.”
9. Verify the “Probe Array Type” in the subsequent pop-up “Expression Analysis Settings” window, Click “OK” to begin analysis and generate the analysis results file (.chp).
10. After analysis, select “File/Save” from the menu bar. The displayed data can be saved as a text file and used for the subsequent analysis.

3.2.5.2. SHUTTING DOWN THE FLUIDICS STATION

1. After washing and staining, change the intake buffer reservoir A and B to “DD Water”
2. Select “Shutdown” for all modules from the drop-down “Protocol” list in the Fluidics Station dialog box. .
3. Click the “Run” button for all modules.

4. Notes

1. The most reliable quality test for primers is to perform Q-PCR using several dilutions of genomic DNA (and water as a negative control) to demonstrate a linear relationship between quantity of template and amplification. We also usually check the dissociation curve for each primer set. Typically, 60 to 80% of the primers give satisfactory results. If you have to design a new primer pair because of a failure in this quality test and have difficulty in designing new primers with a standard parameter set described above, please design new primers by changing the “Min Length” or the DNA sequence used for primer design.
2. Mouse genomic DNA (0.1 ng) corresponds to about 333.33 copies.
3. Before you can use a plate document to run a plate, it must be configured with detector information (e.g., SYBR Green double-stranded DNA binding dye I) for all assays present on the plate.
4. You must assign a “task” to the detectors applied to each well of the plate document that defines their specific purpose on the plate: “Unknown” for cDNA samples, “Standard” for genomic DNA standards, and “NTC” for negative control wells.
5. During a run, the SDS software controls the instrument based on the instructions encoded in the routine of the plate document. The procedure described from 21 to 31 shows how to configure the basic features of the routine: thermal cycler conditions, sample volume, and data collection options.
6. When selected, the SDS software reduces the ramp rate of the 7900HT instrument to match that of the ABI PRISM 7700 Sequence Detection System instrument.
7. The overall PCR cycle is as follows: 95°C 10 min: (95°C for 15 s, 59°C for 1 min) for 45 cycles.
8. The dissociation stage is added at the end of the PCR run. The amplified DNA is dissociated by increasing temperature and the process is monitored in real time as a decrease in fluorescence. As each double-stranded DNA produces its own dissociation profile (resulting in a single peak in the dissociation plot), this technique highlights the presence of primer dimers or nonspecific PCR products.
9. If only a small amount of RNA is available, we recommend the Two-Cycle Target Labeling Assay method (Affymetrix).

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Microarrays

Statistical Methods for Circadian Rhythms

Rikuhiko Yamada and Hiroki R. Ueda

Summary

Microarrays are promising tools that are increasingly being applied to the study of circadian rhythms. The large and complex datasets they generate, however, mean they require a new approach on how to design experiments, handle datasets, translate results, and derive conclusions. This technology also requires statistical methods for the correct interpretation of data generated by the microarrays. In this chapter, we provide an overview of analytical methods applied to microarray experiments for the identification of genes with circadian expression.

Key Words: Circadian rhythm; microarray; *p*-value; *fp*-value.

1. Introduction

One of the most remarkable advances in molecular biology over the past decade is the availability of genomic sequence information and the development of high-throughput and genome-based technologies such as microarrays (DNA chips). Microarray studies look at the mRNA expression of tens of thousands of genes and simultaneously measure the fluorescence emitted by hybridized gene-specific probes. One of the main purposes of these analyses is to identify genes with characteristic expression patterns that recapitulate the observed physiology.

One particular aim in applying microarray studies to circadian rhythms is to identify clock-controlled genes that exhibit circadian rhythmicity in their level of expression. The purpose of this chapter is to provide a general overview on the analytical methods used for the identification of clock-controlled genes from the tens of thousands of genes on the microarray. First, the hybridization intensities of multiple microarrays are normalized to balance them appropriately so that meaningful biological comparisons can then be made. The actual circadian rhythmicity is then assessed by calculating correlation coefficients

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between experimental expression profiles and theoretical cosine waves. Finally, statistical significance and the probability of false positives are evaluated by calculating the p -value and fp -value, respectively.

In this chapter, we do not intend to give a comprehensive and detailed description of microarray statistical methods available for circadian studies because of their rapid evolvement and because no clear consensus yet exists on which method is best for identifying circadian rhythmicity in gene expression levels. Rather, we will focus on a method based on basic concepts but that is open to further development.

As a prerequisite to reading the chapter, we assume that readers have some experience of spreadsheet applications such as Microsoft Excel, and some knowledge of Mathematica (Wolfram Research, Champaign, IL). It is also advisable to have a basic knowledge of statistical tests (1,2).

2. Materials

1. Wolfram Research Mathematica (preferably version 5.0 or later).
2. Microsoft Excel (or other spreadsheet software).

3. Methods

In this section, we provide a step-by-step guide to the statistical analysis of microarray data for the identification of genes that exhibit circadian expression. After formatting, the expression data are normalized and then assayed by crosscorrelation with cosine waves cycling with circadian rhythmicity. Finally, p -value and fp -value are calculated to evaluate statistical significance and the probability of false-positives.

3.1. How Many Chips?

In circadian studies, animals or other organisms are first entrained to a 12 h:12 h light–dark (LD) cycle for days and then are released into free-running constant dark (DD) conditions. RNA is harvested during LD and/or DD cycles, most commonly at 4-h intervals over 48 h (3–8). Twelve microarrays are therefore generally used for one experiment. Several studies, however, have suggested that it would be preferable to use more arrays over the course of an experiment. Panda et al. (6) used two arrays for each time point over 2 d in DD condition (24 arrays in total), and Claridge-Chang et al. (9) used three arrays for each time point over 2 d of LD followed by DD condition (36 arrays in total). Using fewer arrays may be more appropriate for more specific purposes where, for example, the effects of mutations or light stimuli are measured (10–12). Further information regarding this and other studies may be found in Table 1 (3–22) and in some excellent reviews (23,24). In this chapter, we assume that only one array has been used for each of the 12 datapoints over 2 d.

3.2. Preparation of Data Files

All expression data should be placed into a table consisting of number of probes (rows) $\times$ number of chips (columns) (**Table 2**). This can be performed through basic manipulation in a spreadsheet application such as Microsoft Excel. The subsequent data table should be saved as a tab-separated text file. Here, we save this file as C:\work\data.txt.

3.3. Normalization

In spite of great care in keeping experimental conditions constant, random effects are unavoidable. In circadian research we usually use multiple chips (12 chips in our case) to measure temporal changes of mRNA expression. As stochastic variability is inevitable, proper mathematical procedures must be implemented to allow for cross-chip comparisons. “Normalization” is a term used to describe processes that reduce the impact of random effects on the data, with many methods having been proposed (**25,26**). In this section we adopt the following: we scale the average expression level on each chip so as to be equal among all chips, as we assume that all chips have been stained with roughly the equal amount of total mRNA. Another popular technique is to scale the expression levels so as to have equal medians for all the chips. Although more sophisticated techniques are now currently available (**25**), normalization of the average or the median are still first-choice methods (**Fig. 1**). In the following subheading, we describe the program codes for Mathematica to perform these normalization steps.

3.3.1. Load Packages and Expression Profile Data

1. Before starting, load the Mathematica packages required for the subsequent analyses.

```
Needs["Statistics'MultiDescriptiveStatistics'"]
Needs["Statistics'ContinuousDistributions'"]
```

2. Load the previously prepared raw expression data, using the following Mathematica code:

```
dataTable =
ReadList["C:\\work\\data.txt",{Word, Number, Number,
Number, Number, Number, Number, Number, Number,
Number, Number, Number}];
```

Now the variable “dataTable” is a table (two-dimensional matrix), whose rows represents genes, and whose columns represents probe IDs (column 1) and expression profiles (column 2 to column 13).

3. Separate probe IDs from expression profiles using the following code:

```
idList=Transpose[dataTable][[1]];
rawExpressionTable=Transpose[Drop[Transpose
[dataTable],{1}]];
```

Table 1
Summary of Microarray Studies on Circadian Rhythms

Authors	Year	Sample	DNA Chip	Design	Analysis method
Harmer et al. (8)	2000	<i>Arabidopsis</i>	HDO	12 time-points, 4-h interval, LL, $n = 2$	Cross correlation with cosine waves
Schaffer et al. (19)	2001	<i>Arabidopsis</i>	cDNA	4 time-points, 6-h interval, LD, $n = 1-4$ 1 time-point, DD, $n = 2$ 2 time-points, LL, $n = 1$	Two time-point comparison
Claridge-Chang et al. (9)	2001	<i>Drosophila</i> head	HDO	12 time-points, 4-h interval, LD followed by DD, $n = 3$	Fourier analysis
McDonald and Rosbach (20)	2001	<i>Drosophila</i> head	HDO	6 time-points, 4-h interval, DD, $n = 3-5$	Cross correlation with cosine waves
Grundschober et al. (21)	2001	Rat-1 fibroblasts	HDO	20 time-points, 4-h interval, DD, $n = 1$	Spectral analysis
Kit et al. (14)	2002	Rat liver Rat kidney	cDNA	2 time-points, 12-h interval, LD, $n = 1$	Two time-point comparison
Humphries et al. (15)	2002	Rat pineal gland	cDNA	2 time-points, 12-h interval, LD, $n = 3$	Two time-point comparison
Akhtar et al. (18)	2002	Mouse liver Mouse hypothalamus	cDNA	7 time-points, 4-h interval, DD, $n = 2$	Anchored comparison Moving window analysis
Duffield et al. (17)	2002	Rat-1 fibroblasts	cDNA	13 time-points, 4-h interval, DD, $n = 1$	Cosine wave fitting
Ueda et al. (4)	2002	<i>Drosophila</i> head	HDO	12 time-points, 4-h interval, LD and DD, $n = 1$	Cross correlation with cosine waves
Storch et al. (5)	2002	Mouse heart Mouse liver	HD)	12 time-points, 4-h interval, DD, $n = 1$	Autocorrelation analysis
Panda et al. (6)	2002	Mouse SCN Mouse liver	HDO	12 time-points, 4-h interval, DD, $n = 2$	Cosine wave fitting
Lin et al. (13)	2002	<i>Drosophila</i> head	HDO	6 time-points, 4-h interval, LD, $n = 2-3$, and DD, $n = 2$	Autocorrelation analysis
Ueda et al. (3)	2002	Mouse SCN Mouse liver	HDO	12 time-points, 4-h interval, LD and DD, $n = 1$	Cross correlation with cosine waves

Ceriani et al. (7)	2002	<i>Drosophila</i> head	HDO	12 time-points, 4-h interval, LD and DD, $n = 2$	Cosine wave fitting
Hirota et al. (16)	2002	Rat-1 fibroblasts	HDO	3 time-points, 0 h, 1 h, 4 h, $n = 1$	Time-point comparison
Nowrousian et al. (22)	2003	<i>Neurospora</i>	cDNA	5 time-points, 4-h interval, 1 cycle, DD, $n = 3$ and temprature entrainment	Time-point comparison Cosine wave fitting
Oishi et al. (10)	2003	Mouse liver	HDO	2 time-points, 12-h interval, 1 cycle, DD, $n = 1$	Two time-point comparison
Salter et al. (12)	2003	<i>Arabidopsis</i>	HDO	7 time-points, $n = 1$	Time-point comparison
Grechez-Cassiau et al. (11)	2004	Mouse liver	HDO	2 time-points, 12-h interval, 1 cycle, DD, $n = 2-3$	Two time-point comparison

Studies are listed by publication date.

HDO, high-density oligonucleotide microarray; cDNA, complementary DNA microarray; SCN, suprachlasmatic nucleus; LD, light-dark; DD, constant darkness.

Table 2
Profiles of Gene Expression Over 2 d at 4-h Intervals

1415670_at	313.6	332.7	313.1	425	599.7	463.8	429.2	324.6	554.4	461.2	575.6	349.5
1415671_at	680.4	799	805.5	1019.7	1031.7	1008.5	1006.5	707.5	756.8	1123.4	1195.1	675
1415672_at	1281.6	1484.1	872.7	1058.8	1184	1084	1227.2	931.4	1059.4	1214.8	1203	764.2
1415673_at	124.3	95.3	80.4	110.3	132.9	112	103.9	58	64.9	108.1	101.5	65.4
1415674_a_at	307.4	335.8	312.1	376.6	350.2	340.7	394.6	289	284.8	385	375.8	245.7
1415675_at	258.8	229.4	231.9	282.3	245.4	271.7	315.5	228.3	167.5	227.4	242.4	170.9
1415676_a_at	1094.3	1415.7	1330.2	1327.6	1242.9	1221.8	1722.2	1248.4	1092.6	1446.3	1311.5	1173.6
1415677_at	441.3	480.6	557.8	737.4	434.2	523.2	789.9	635	372.1	850.9	524.1	625
1415678_at	828.6	930.7	884	967.3	950	818.9	749.2	687	685	984.4	792.3	570.2
1415679_at	1274.7	1409.7	1202	1358.2	1286.6	1249.3	1500.2	993.2	1185.1	1428.4	1565.9	958.2

This table is created with Microsoft Excel. The first column shows the “Affymetrix Probe Set Ids” and the following columns indicate the expression level for each gene. The first 10 out of 22,690 rows are shown here. There is no header row to simplify Mathematica codes.

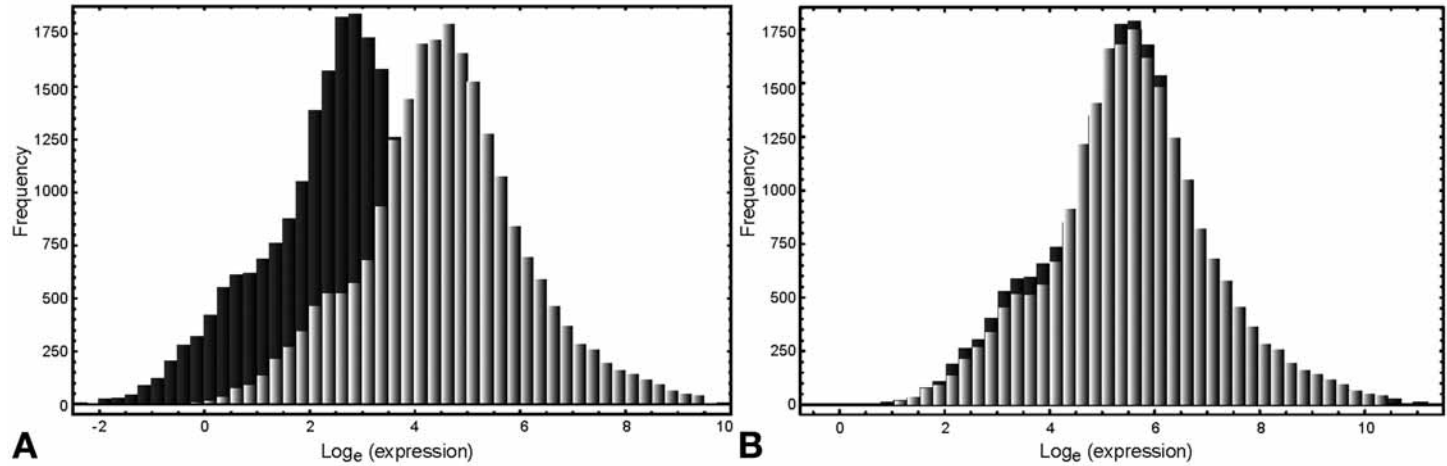


Fig. 1. Schematic representation of the normalization procedure. Gene expression data from two different chips are shown before (A) and after (B) normalization to illustrate how these procedures transform the data sets. The normalized distributions, shown in (B), are shifted and aligned at their centers. Gene expression comparisons between the two distributions can now be made without systematic experimental bias.

The first code exchanges rows and columns of `dataTable`, and then extracts the first column (probe IDs). The second code exchanges rows and columns of `dataTable`, and then drops the first column (probe IDs), and exchanges its rows and columns again. The produced “idList” is an array of probe IDs, and “rawExpressionTable” is a table (two-dimensional matrix), whose rows represent genes, and whose columns represent expression profiles.

3.3.2. Equalize Average or Median of Each Chip

1. Scale the level of expression of each probe so that the average expression level for each chip becomes 1000 (*see Note 1*) using the following Mathematica code:

```
normalizationFactors=1000/Mean[rawExpressionTable];
normalizedExpressionTable=rawExpressionTable.Diagonal
Matrix[normalizationFactors];
```

The first line calculates scaling factors and put them in a vector. The second line multiplies “rawExpressionTable” with a diagonal matrix of the scaling factors to produce normalized expression profiles “normalizedExpressionTable,” whose rows contains normalized expression profiles of each gene.

Alternatively, scale the expression levels for each probe so that the median of each chip becomes 1000 (*see Note 1*), using the following Mathematica code:

```
normalizationFactors=1000/Median[rawExpressionTable];
normalizedExpressionTable=rawExpressionTable.Diagonal
Matrix[normalizationFactors];
```

3.4. Evaluation of Circadian Expression

Several procedures exist by which to evaluate whether the expression of a gene is under circadian control. One of them is based on the assumption that the expression profile of a gene exhibiting circadian rhythmicity approximates a cosine wave with a period of 24 h (*see Note 2*). A significant correlation can therefore be found between a rhythmically expressed gene and a theoretical cosine wave cycling with an appropriate phase, as can be seen in the following:

1. Generate 60 cosine waves with the equation defined below (*see also Fig. 2*).

$$C_i = \cos(2\pi(\frac{1}{24}t - \frac{1}{60}i)) \quad (t = 0, 4, 8, \dots, 44) \quad (i = 0, 1, 2, \dots, 59)$$

The following properties apply:

- 24-h period.
- 48 h long (two cycles).
- Interval between adjacent phases equal to 0.4 h.

The above formula is expressed in Mathematica as the following:

```
cosines=Table[Cos[2Pi(t/24-i/60)], {i, 0, 59, 1}, {t, 0, 44, 4}];
```

2. Calculate the correlation coefficient between each expression profile and each of the 60 cosine waves (C_i). The highest correlation coefficient among them should

be selected as the representative value of circadian rhythmicity. We have termed this value *max correlation* (*maxCorr*). For a gene k , $maxCorr_k$ is defined as follows:

$$maxCorr_k = \max(\text{Correlation}(\text{expression\_profile}_k, C_i)) \quad (i = 0, 1, 2, \dots, 59).$$

A list of *maxCorrs* can be calculated by the following Mathematica code:

```
maxCorrs = {};
peakTimes = {};
For[g = 1, g <= Length[normalizedExpressionTable], g++,
  normalizedExpression = normalizedExpressionTable[[g]];
  corrs = Table[Correlation[normalizedExpression,
    cosines[[i]]]
    , {i, 1, Length[cosines]}];
  maxCorr = Max[corrs];
  peakTime = 0.4*(Position[corrs, maxCorr][[1, 1]] - 1);
  AppendTo[maxCorrs, maxCorr];
  AppendTo[peakTimes, peakTime];
];
```

Note that “peakTime” indicates the estimated *peak time* of normalized expression data, which is estimated by the peak time of the best-correlated cosine curve.

3.5. Statistical Significance: p Value

In this context, the p value can be defined as the probability that a random expression profile shows *max correlation* greater than a given value (**Fig. 3**). The p value is experimentally calculated by generating random expression profiles, calculating *maxCorr* for each random profile, and finally, counting the frequency of a random expression profile showing *maxCorr* greater than the chosen value. When a *maxCorr* value increases, the associated p value decreases; in other words, a smaller p value indicates that random expression profiles are more unlikely to show a defined *maxCorr*.

1. Generate 100,000 random expression profiles and calculate *maxCorr* for each of them, thereby creating the *maxCorr* distribution of random expression profiles. The Mathematica code to obtain this distribution is as follows:

```
randomCorrs={};
For[i=1,i<= 100000,i++,
  randomExpression=Table[Random[NormalDistribution[0,1]],
    {t,1,12}];
  corrs=Table[Correlation[randomExpression,cosines[[i]]]
    , {i,1,Length[cosines]}];
  randomCorr = Max[corrs];
  AppendTo[randomCorrs,randomCorr];
];
```

This process usually takes several hours on an up-to-date PC (such as Pentium4 3GHz; see **Note 3**).

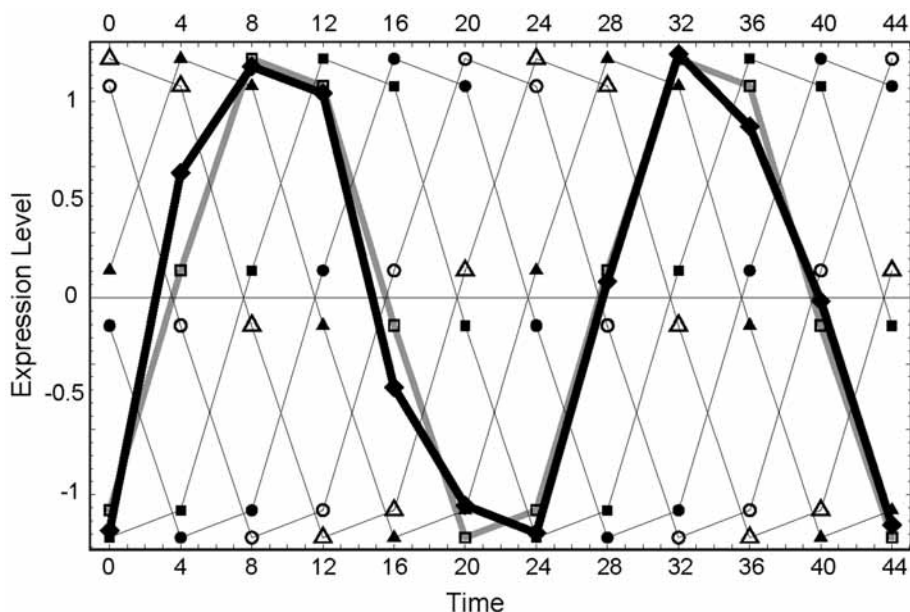


Fig. 2. Crosscorrelation between the expression profile of a gene and theoretical cosine waves. The experimental profile (black line) is compared with 60 cosine waves of fixed periodicity (e.g., 24 h), varying in phase from 0 to 24 h. A gray line indicates the best-correlated cosine wave. For convenience, only 6 out of the 60 cosine waves are shown here.

2. Save the result in a file:

```
Save["C:\\work\\randomCorrs.txt", randomCorrs];
```

3. Using the following Mathematica code it is possible to reload the random correlation data at any time, even after restarting Mathematica:

```
<<"C:\\work\\randomCorrs.txt";
```

4. Count the number of times that a random expression profile shows *maxCorrs* greater than a specified value. The following Mathematica code can be used to perform this (see **Note 4**):

```
CountGreater[maxCorr_, value_] := Length[
  Select[randomCorrs, (# > value)&]]
```

5. Calculate the *p* value with the following code:

```
sortRandomCorrs = Sort[randomCorrs];
pValues = Table[CountGreater[sortRandomCorrs,
  maxCorrs[[i]]/Length[sortRandomCorrs], {i, 1,
  Length[maxCorrs]}];
```

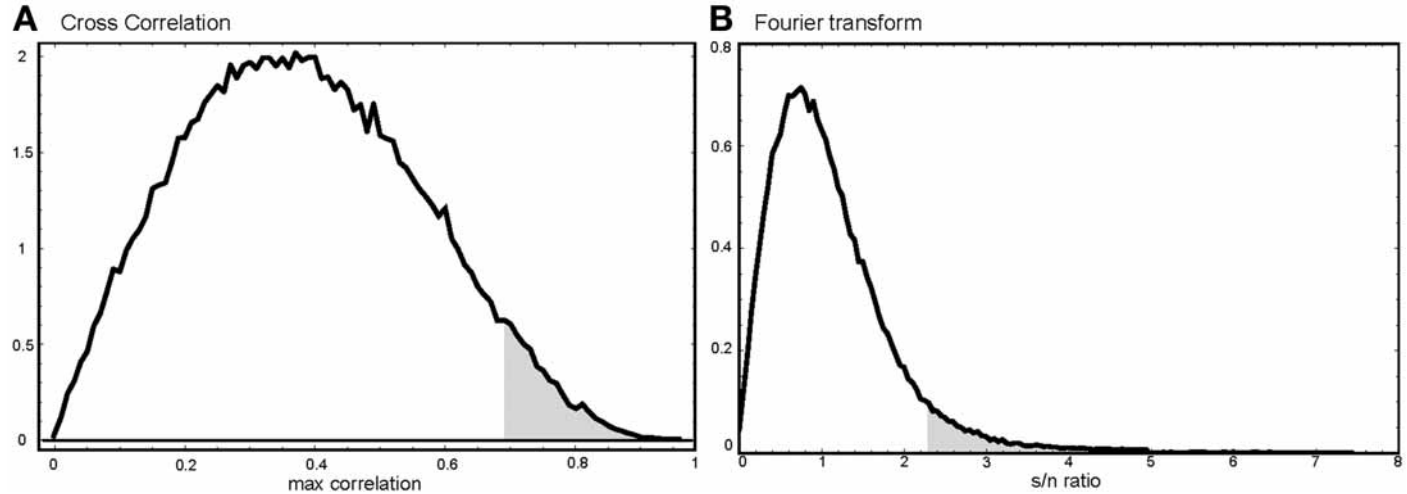


Fig. 3. *maxCorr* and *s/n ratio* distributions calculated from 100,000 random expression profiles. The curves indicate the probability of obtaining a particular value of *maxCorr* (A) or *s/n ratio* (B), when 100,000 random expression profiles are generated. The shaded area, compared with the total under the probability curve, indicates the *p* value associated with a particular value of *maxCorr* (= 0.7, A) or *s/n ratio* (= 2.3, B). In this graph the *p* values are about 0.05.

3.6. Probability of False-Positives: *fp* Value

500 “positive” genes out of 10,000 genes may be obtained by chance, when you call genes with $p \leq 0.05$ as “positive.” This means, assuming that the relevant research involves the expression of 10,000 genes on a microarray and that 1000 positive genes ($p \leq 0.05$) are obtained, 500 genes are expected to be false positives among the 1000 positive genes. The probability of false-positives will therefore be $500/1000 = 0.5$ (see **Notes 5** and **6**).

1. This probability is defined as the *fp* value (**Fig. 4**) and is calculated by the following Mathematica code:

```
sortMaxCorrs=Sort[maxCorrs];
sortRandomCorrs=Sort[randomCorrs];
randomCorrsSize=Length[randomCorrs];
maxCorrsSize=Length[maxCorrs];
fpValues=Table[
  (CountGreater[sortRandomCorrs,maxCorrs[[i]]]/
   randomCorrsSize)
  *(maxCorrsSize/CountGreater[sortMaxCorrs,maxCorrs[[i]]])
, {i,1,maxCorrsSize}];
```

2. As the *fp* values are calculated experimentally and not theoretically, they may not exhibit monotonous decreasing that parallels smaller p values. To correct for this, we use an additional process shown below.

```
pv=pValues;
fp=fpValues;
idxPV=Transpose[{Range[Length[pv]],pv}];
sortedIdxPV=Sort[idxPV, (#1[[2]]>#2[[2]])&];
minFP=1;
idxSmoothedFP=Table[
  idx=sortedIdxPV[[i]][[1]];
  {idx,minFP=Min[fp[[idx]],minFP]},
  {i,Length[sortedIdxPV]}
];
idxSmoothedFP=Sort[idxSmoothedFP, (#1[[1]] < #2[[1]])&];
fpValuesSM=Transpose[idxSmoothedFP][[2]];
```

3.7. Fourier Analysis

The previous section stated that the identification of rhythmically expressed genes is based on the maximum correlation coefficients between expression profiles and cosine waves. In this section, we describe an alternative approach by Fourier analysis. The Fourier transform decomposes an expression profile into a linear combination of sinusoids of different periods, and circadian rhythmicity is measured by comparing the amplitude of the 24-h period sinusoid with that of other period sinusoids. In this section, we describe these procedures. Codes described in **Subheadings 3.1.1.** and **3.1.2.** and the function

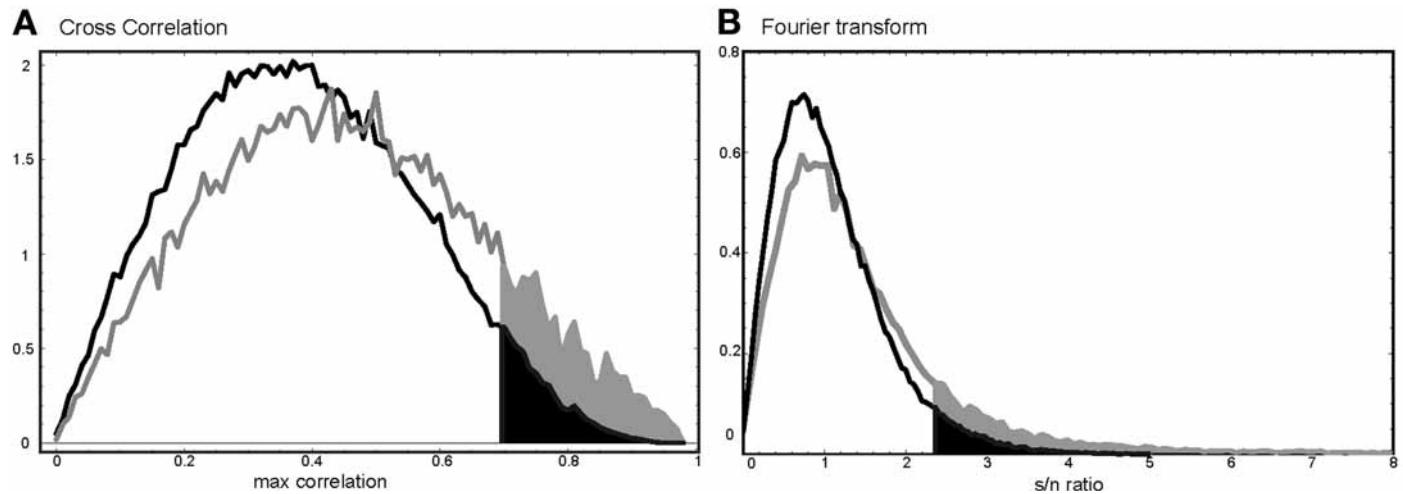


Fig. 4. Distributions of *maxCorr* and *s/n ratio* calculated from real and random expression profiles. The black curve indicates the distribution of *maxCorr* (A) or *s/n ratio* (B) from random expression profiles whereas the gray curve refers to the real expression profiles. The proportion between the black area and the total of the black and gray areas defines the *fp* value.

“CountGreater” are also used here and should be executed before implementation of the codes described in this section.

3.7.1. Discrete Fourier Transform

1. Before applying the Fourier transform, subtract the average expression level from the expression level of each gene.

```
subtractedExpressionTable =  
Table[normalizedExpressionTable[[i]]-Mean[normalized  
ExpressionTable[[i]]],{i,1,Length[normalized  
ExpressionTable]};
```

2. Mathematica can perform Fourier transform with just one function.

```
fourierTable=Table[Fourier[subtractedExpression  
Table[[i]]],  
{i,1,Length[subtractedExpressionTable]};
```

3. From a 12-sample-points time course, derive the amplitude of the 24-h period sinusoid from the third component of each “fourierTable[[i]]” ($i = 1, 2, \dots, n$) and calculate the *signal/noise (s/n) ratio* (Fig. 5) with the following Mathematica code:

```
snRatios=Table[  
amplitudes=Abs[fourierTable[[i]]];  
amplitudes[[3]]/Mean[Part[amplitudes,{1,2,4,5,6,7}]]  
,{i,1,Length[fourierTable]}  
];
```

4. *PeakTime* can also be calculated from the third component of each “fourierTable[[i]]” ($i = 1, 2, \dots, n$) by calculating its argument.

```
frPeakTimes=Table[  
components=fourierTable[[i]];  
Mod[24*Arg[components[[3]]]/(2Pi),24],  
{i,1,Length[fourierTable]}  
];
```

3.7.2. Statistical Significance

Using a similar approach as for the correlation coefficients, statistical significance and the probability of false-positives for *s/n* ratios can be assessed by calculating *p*- and *fp* values, respectively.

1. Generate random expression profiles and create a distribution of *s/n* ratios as follows:

```
randomSNRatios=Table[  
randomExpression=Table[Random[NormalDistribution  
[0,1]],{12}];  
randomFourierAbs=Abs[Fourier[randomExpression]];  
randomFourierAbs[[3]]/Mean[Part[randomFourierAbs,  
{1,2,4,5,6,7}]]  
,{100000}];
```

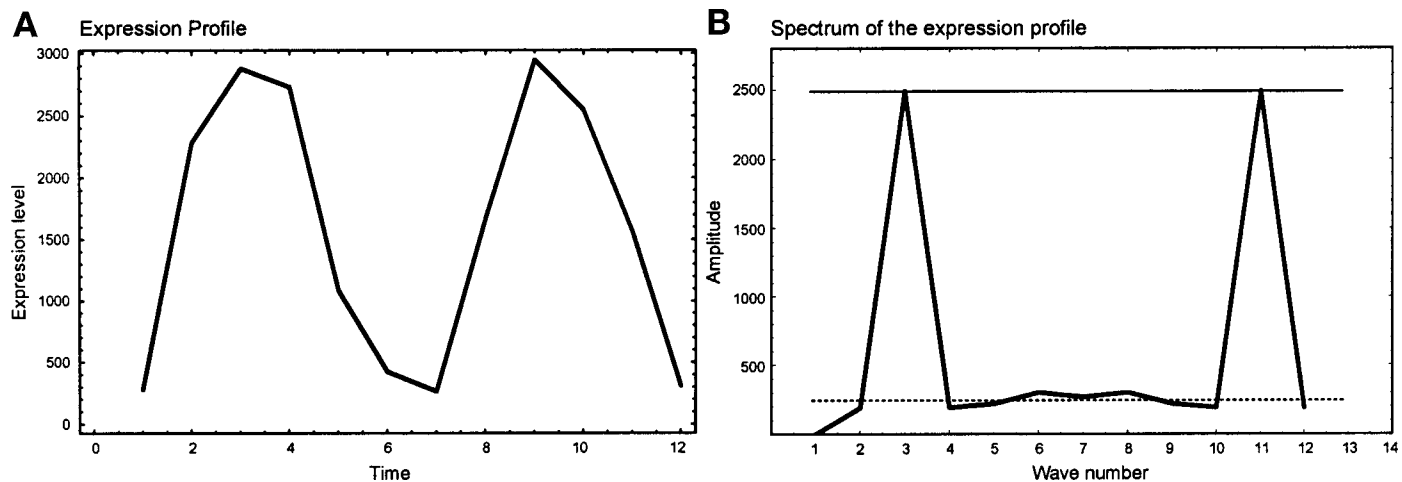


Fig. 5. (A) Expression profile of a gene and (B) its spectrum generated after Fourier transform. The solid line represents the amplitude of the 24-h-period sinusoid, whereas the dotted line represents the average amplitude of other period sinusoids. The *s/n ratio* is defined as the ratio of these two values (value of solid line/value of dotted line).

2. Use this distribution to calculate p values (**Fig. 3B**):

```
sortedRandomSNRratios=Sort[randomSNRratios];
snRatioSize=Length[snRatios];
randomSNRatioSize=Length[randomSNRratios];
frPValues=Table[CountGreater[sortedRandomSNRratios
  snRatios[[i]]]/randomSNRatioSize,{i,1,snRatioSize}];
```

3. Use it also to calculate fp value (**Fig. 4B**):

```
snRatioSize=Length[snRatios];
randomSNRatioSize=Length[randomSNRratios];
sortedSNRratios=Sort[snRatios];
sortedRandomSNRratios = Sort[randomSNRratios];
frFPValues=Table[
  (CountGreater[sortedRandomSNRratios,snRatios[[i]]]/
    randomSNRatioSize
  *(snRatioSize/CountGreater[sortedSNRratios,snRatios[[i]]])
  ,{i,1,Length[snRatios]}];
```

4. Use the following code to ensure that the fp values are monotonously decreasing along with the p values:

```
pv=frPValues;
fp=frFPValues;
idxPV=Transpose[{Range[Length[pv]],pv}];
sortedIdxPV=Sort[idxPV,({#1[[2]]>#2[[2]]})&];
minFP=1;
idxSmoothedFP=Table[
  idx=sortedIdxPV[[i]][[1]];
  {idx,minFP=Min[fp[[idx]],minFP]},
  {i,Length[sortedIdxPV]}
];
idxSmoothedFP=Sort[idxSmoothedFP,({#1[[1]] < #2[[1]]})&];
frFPValuesSM=Transpose[idxSmoothedFP][[2]];
```

5. Save the result in a file also containing additional statistics.

```
tableForFile=Table[
  {idList[[i]],
  N[avgs[[i]]],
  N[sdvs[[i]]],
  N[frPeakTimes[[i]]],
  snRatios[[i]],
  N[frPValues[[i]]],
  N[frFPValuesSM[[i]]]}, {i,1,Length[idList]}];
Export["C:\\work\\output_fr.txt",tableForFile,"TSV"];
```

3.8. Other Statistics

So far, we have demonstrated how to calculate the p value and fp value for the expression profile of each gene, which provides enough information to deter-

mine whether a gene exhibits rhythmic expression or not. This section describes how to calculate other useful statistics.

3.8.1. Average Expression

The average expression of a gene over a time course provides useful information for evaluating the general level of expression in the samples.

```
avgs=Mean[Transpose[normalizedExpressionTable]];
```

Generally, the expression profile of genes with a low average expression is unreliable, as experimental noise can obscure the true signal.

3.8.2. Standard Deviation of Expression

In this context, standard deviation refers to the size of the variation in the expression profile of a transcript; it is an estimate of the amplitude of expression of cycling genes.

```
sdvs=StandardDeviation[Transpose[normalizedExpressionTable]];
```

3.8.3. Average Peak Time

If you have two sets of samples—for instance, one under LD and the other under DD conditions—their average peak time might be useful. Defining “peakTimeLD” and “peakTimeDD” as peak time in LD and DD respectively, calculate the average peak time with the following code (*see Note 7*):

```
peakTimeAvgs=Table[(Mod[(peakTimeLD[[i]]+0.5*Mod
  [peakTimeDD[[i]] -peakTimeLD[[i]],24,-12]),24)]&,
  {i,1,Length[peakTimeLD]}];
```

3.9. Write Results to File

Entering analyzed data into a file that can be viewed and edited by spreadsheet software such as Microsoft Excel is useful for further analysis. In Mathematica, a tab-separated file in which each line consists of “id list,” “average of expression,” “standard deviation of expression,” “peak time,” “max correlation,” “*p* value,” and “*fp* value” can be written with the following code (*see Note 8*):

```
tableForFile=Table[
  {idList[[i]],
  N[avgs[[i]]],
  N[sdvs[[i]]],
  N[peakTimes[[i]]],
  maxCorrs[[i]],
  N[pValues[[i]]],
  N[fpValuesSM[[i]]]}, {i,1,Length[idList]}];
Export["C:\\work\\output.txt",tableForFile,"TSV"];
```

4. Notes

1. The number 1000 is arbitrary; it is possible to select a different number.
2. In other studies (3,4) different periods (20–28 h) are used to identify genes with a circadian expression pattern. The basic strategy described in **Subheading 3.4.** still applies and can be expanded to longer or shorter periods (e.g., 20–28 h).
3. A much faster Mathematica code to obtain the *maxCorr* distribution of random expression profiles is as follows:

```
randomExpressionTable=Table[Random[NormalDistribution
  [0,1]],{100000},{12}];
sinBase=Table[Sin[2*Pi(i/24)],{i,0,44,4}];
sinBase=sinBase / Sqrt[sinBase.sinBase];
cosBase=Table[Cos[2*Pi(i/24)],{i,0,44,4}];
cosBase=cosBase/ Sqrt[cosBase.cosBase];
f={cosBase,sinBase}.((#-Mean[#])/Sqrt[(#-Mean[#])
  (#-Mean[#])]) & /@randomExpressionTable;
randomCorrs=Sqrt[#. #]& /@ f;
```

This code uses the concept of Fourier transformation. Although this code runs much faster, it is advisable to save the result in a file.

4. A faster alternative to the previous code is the following:

```
CountGreater=Compile[{{l,_Real,1},{val,_Real}},
  ei=len=Length[l];          (* end index *)
  si=1;                      (* start index *)
  mi=Floor[(si+ei)/2];       (* middle index *)
  If[val>l[[len]],Return[0]];
  If[val<l[[1]],Return[len]];
  While[mi != si,
    If[l[[mi]]>val,
      ei=mi;
      mi=Floor[(si+ei)/2];
    ,
      si=mi;
      mi=Floor[(si+ei)/2];
    ];
  ];
  len-si
];
```

5. In our analyses we empirically use an *fp* value of 0.1, corresponding to 10% of false positives, as a threshold. You may increase this value to increase the sensitivity of identification, or may decrease it to increase the specificity of identification.
6. *fp* value is a conservative form of false discovery rate. Storey and Tibshirani have proposed a statistic known as *q* value (28) that corrects the tendency of the *fp* value to overestimate false positives. You can easily calculate the *q* values for your data set by feeding your list of *p* values assigned to each gene to the software made available by Storey et al. at their website (<http://faculty.washington.edu/~jstorey/qvalue/>).

7. A simple arithmetic average is inappropriate for calculating the average peak time. For example the average time between 23:00 and 1:00 should be 0:00, not 12:00.
8. In this example the data are recorded into an “output.txt” file. You can easily add annotation information to this file. Affymetrix provides annotation information for each target gene on their microarrays found on its website (27).

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Identification of Clock Genes Using Difference Gel Electrophoresis

Natasha A. Karp and Kathryn S. Lilley

Summary

Proteomics is the study of the complete set of proteins encoded by the genome. The study of the proteome involves the investigation of changes in protein abundance, localization, involvement in multiprotein complexes, and detection of different protein isoforms and posttranslational modifications under defined conditions, such as the circadian cycle. This type of approach complements comparative gene expression studies providing additional information with respect to posttranscriptional processing. One of the key techniques used to study the proteome is two-dimensional gel electrophoresis. This technique has the ability to separate complex protein mixtures with high resolution. A significant improvement in this technology has been development of difference gel electrophoresis. Here, proteins are first labeled with one of three spectrally resolvable fluorescent cyanine dyes before being separated in two dimensions according to their charge and size, respectively. Multiplexing can accurately and reproducibly quantify protein expression across multiple gels. A multiple-gel approach allows the detection of differentially expressed protein spots using statistical methods to compare expression across different experimental groups. The proteins can be subsequently identified by mass spectrometric methods. This approach now allows more complex experimental designs, such as the time course experiments essential to the study of circadian rhythms.

Key Words: Proteomics; 2D gel electrophoresis; fluorescent labeling of proteins; difference gel electrophoresis; mass spectrometry.

1. Introduction

In the past few years the circadian transcriptome has been studied in several model organisms (*1*). mRNA profiling, however, provides an incomplete characterization of the mechanisms underlying circadian regulation. If we are to fully understand how circadian time is generated and signaled to the organism, it is necessary to study the cycling proteome. Recent advances in the technology used to study the proteome are critical to characterizing the composition

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and functions of the protein that drive the core oscillation and its outputs, via transcription and temporally regulated degradation of clock-relevant factors. To achieve these goals, integrated data sets from a variety of protein expression studies, providing information on relative abundances, subcellular locations, protein complex formation, and the profiling of isoforms generated by either alternate mRNA splicing or posttranslational modifications, are required.

The proteome was originally defined, nearly a decade ago, as “all the proteins coded by the genome of an organism” (2). Nowadays the term “proteomics” is used to describe the discipline associated with the acquisition of these data sets. Linking elements of the proteome to function can be achieved either by looking for changes in the expression of either all or a subset of proteins, or by identifying binding partners for particular proteins and seeing how their interaction is affected by biological perturbation. Whatever the rationale of the investigation, or the number of proteins involved, the study of the proteome can be broken down into the following stages of analysis.

1.1. Separation of Proteins

Prior to the analysis of protein expression and abundance levels, proteins first have to be isolated into a “purified” state. Although there are a variety of chromatographic procedures for achieving this, two-dimensional (2D) gel electrophoretic separation has been the method of choice in the recent past. However, other new methodologies are now emerging, each methodology having complimentary strengths and weaknesses:

1. Analysis of comparative expression—once separated, it is then necessary to carry out some form of analysis to assess the relative abundance of the proteins present.
2. Identification of protein species—once a set of proteins showing differences in abundance between two or more states have been identified, digestion of the proteins to peptides and further analysis using mass spectrometric methodology can be used to determine their identities.
3. Confirmatory experiments—when a protein has been shown to be important in a given process by the above analysis, it may be necessary to perform further experiments to confirm its implied function or involvement in the process.

For proteomes that encompass the protein content of a given cell or tissue type, or that of a whole organism, there are two main methods that are first used to resolve the protein mixture, and then to visualize the individual components in such a way that their relative abundances can be quantified. The first method utilizes 2D polyacrylamide gel electrophoresis (2D-PAGE) followed by a variety of in-gel staining methods, whereas the second—more recent—technology couples liquid chromatographic separation to subsequent ultraviolet and/or mass spectrometric (MS) detection. This chapter focuses on 2D-PAGE

as the separation technique because this approach is better established and widely used in nonspecialist laboratories.

2D-PAGE has been routinely used over the past three decades to resolve and investigate several thousand proteins in a single sample. This has enabled identification of the major proteins in a tissue or subcellular fraction by MS methods. In addition, 2D-PAGE has been used to compare relative abundances of proteins in related samples, such as those from altered environments or from mutant and wild-type, thus allowing the response of classes of proteins to be determined. Problems with matching of spots from one gel to another, running variations, and the dynamic range of stains have limited quantitative studies. Visualization of spots on 2D-PAGE gels has traditionally involved silver staining, as it is more sensitive than conventional Coomassie staining methods. Silver staining is unsuitable for quantitative analysis; however, as it has a limited dynamic range, and the most sensitive of silver staining methods are also incompatible with protein identification methods based on mass spectrometry. More recently, the Sypro postelectrophoretic fluorescent stains (Invitrogen, Carlsbad, CA) have emerged as alternatives, offering a better dynamic range, and ease of use (3). Difference gel electrophoresis (DIGE), first described some time ago (4), circumvents issues with gel-to-gel variation and limited dynamic range and allows more accurate and sensitive quantitative proteomics studies. This technique relies on pre-electrophoretic labeling of samples with one of three spectrally resolvable fluorescent CyDyes (Cy2, Cy3, and Cy5), allowing multiplexing of samples into the same gel. There are currently two types of CyDye labeling chemistries available from GE Healthcare. The most established is the “minimal labeling” method. Here, the CyDyes are supplied as *N*-hydroxy succinimidyl esters, which react with primary amino groups. The stoichiometry of labeling is such that about 2% of available lysine residues are labeled. The CyDyes carry a positive charge and hence a labeling event does not alter the isoelectric point (*pI*) of the protein. In the second chemistry, uncharged CyDyes are supplied with a thiol-reactive maleimide group. These “saturation” dyes are utilized in such a way to bring about labeling of every cysteine residue within the protein. The saturation labeling is much more sensitive, as more fluorophore is introduced into each protein species (5). The use of these saturation dyes is not well established; therefore, this chapter focuses only on the minimal labeling of lysine residues.

For multiple gel studies such as a time course, samples can be labeled with either Cy3 or Cy5 minimal dyes, whereas Cy2 minimal dye is reserved for an internal standard sample. Up to three distinct labeled samples are run in one gel and viewed individually by scanning the gel at different wavelengths. Variation in spot volumes owing to gel-specific experimental factors—for example,

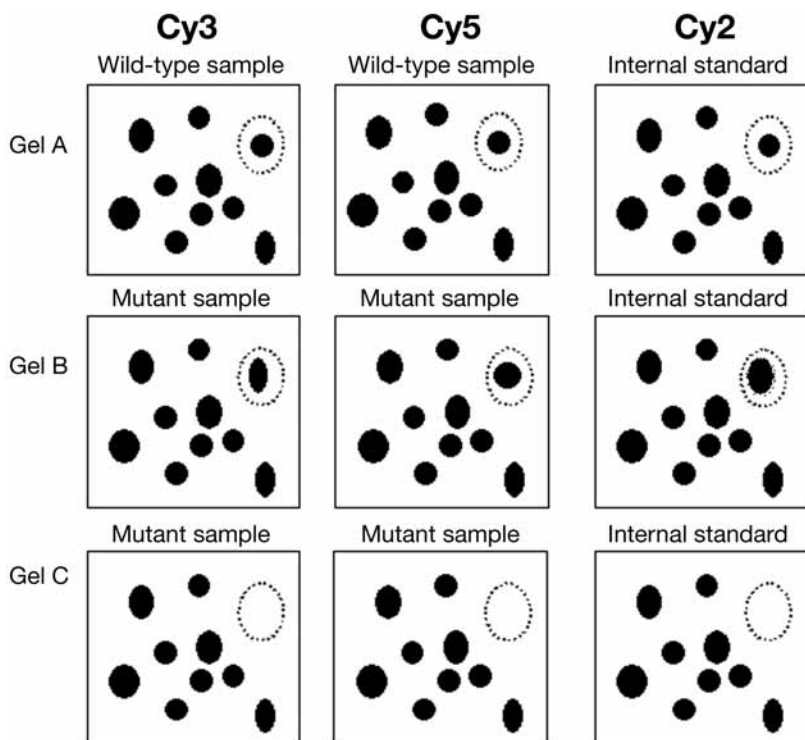


Fig. 1. Schematic diagram of a two-dimensional polyacrylamide gel with three spectrally resolvable samples resulting from the labeling with CyDyes, which highlights the importance of the internal standard in accounting for experimental variation. For the spot circled, if an internal standard were not included when comparing gel A to gel B it would be concluded that the protein expression had increased in the mutant samples. When using the internal standard to account for running success, it would be concluded that protein expression had actually decreased. Similarly, if gels A and C were compared without the internal standard it would be concluded that the protein was absent in the mutant samples where in fact the protein has not resolved on gel C. The inclusion of internal standard in the generation of standardized abundances can therefore take into account the experimental variation, allowing reproducible quantitation.

protein loss during sample entry into the immobilized pH gradient strip—will be the same for each sample within a single gel. Consequently, the relative amount of a protein in a gel in one sample compared with another will be unaffected (Fig. 1). In a multiple-gel experiment, the Cy2 is used to label a pooled sample consisting of equal amounts of each of the samples to be compared, and acts as

an internal standard. This ensures that all proteins present in the samples are represented, allowing both inter- and intragel matching. The spot volumes are normalized for dye discrepancy, arising from differences in laser intensities, fluorescence, and filter transmittance, using a method based on the assumption that the majority of protein spots have not changed in expression level (6). The spot volumes from the labeled samples are compared with the internal standard giving standardized abundances, which allows the variation in spot running success to be taken into consideration.

For the analysis, software developed for the DIGE system, such as DeCyder™ (GE Healthcare, Uppsala, Sweden) is typically used. This software has a codetection algorithm that simultaneously detects labeled protein spots from images that arise from the same gel and increases accuracy in the quantification of standardized abundance (6). The standardized abundances can then be compared across groups to detect changes in protein expression (*see* Fig. 2 for a sample time course profile obtained from a multiple-gel DIGE experiment). The technical improvements in this field have made possible more complex experimental designs in proteomics expression studies, such as a time course or a moving window approach. Given that proteins are separated by both *pI* and molecular weight (MW), certain posttranslational modifications that result in a change in either of these parameters are visible. Successive phosphorylation events, for example, lead to a “charge train” of spots as the phosphorylation event decreases the *pI* of the protein. Consequently, DIGE has the potential to identify changes that arise not only from changes in protein levels but also from posttranslation modifications (*see* Fig. 3 for an example). To date, the DIGE technology has been used with great success to study a variety of systems, allowing the detection of more subtle changes in protein expression than conventional methods in which separate samples are loaded onto each gel (7–12).

Regardless of the benefits of DIGE, the 2D-PAGE process itself has some limitations. For global expression analysis, every protein should be resolved as a discrete detectable spot; however, the following groups of proteins are often poorly represented: those with extreme *pI* or MW; hydrophobic proteins; lower abundance proteins. It has been calculated that somewhere in the region of 90% of the total protein of a typical cell is made up of only 10% of the 10,000 to 20,000 different species, and hence many low-abundance proteins may not be detectable (13). Improvements to the technique are ongoing, such as increasing resolution of protein species by the use of narrow-range immobilized pH gradient (IPG) strips. Moreover, prefractionation of samples has been demonstrated and greatly improves the chance of identification and assignment of function to low-abundance species (14,15).

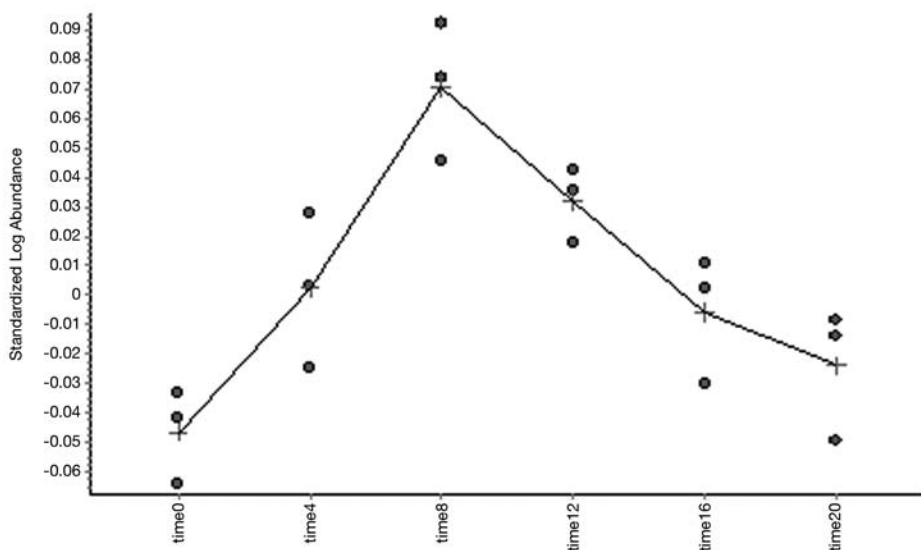


Fig. 2. Examples of changes in protein expression as seen as changes in the standardized log abundance obtained for a time-course multiple-gel difference gel electrophoresis experiment.

The stages required for the DIGE approach in the identification of proteins with expression changes are shown in [Fig. 4](#) as a flow diagram and are outlined in more detail in **Heading 3**.

2. Materials

2.1. Reagents

Unless otherwise stated, all solutions are made with distilled water as the diluent.

1. Amidosulfobetaine-14 (ASB-14) lysis buffer: 2% (w/v) ASB-14 (Calbiochem, San Diego, CA), 7 M urea, 2 M thiourea, 10–30 mM Tris-HCl, pH 8.0–9.0, magnesium acetate.
2. Stock CyDye solutions: 1 mM CyDye DIGE Fluors (GE Healthcare) in dimethyl formamide (DMF). Store in small aliquots (e.g., 2 μ L). Stable for 1 mo at -70°C .
3. Working CyDye solutions: 0.2 mM CyDye DIGE Fluors in DMF. Stable for 2 wk at -20°C .
4. Blocking solution: 10 mM lysine solution.
5. 2X Isoelectric buffer: 20 mg/mL dithiothreitol (DTT; add just before use), 2% IPG buffers (GE Healthcare), 2% ASB-14 (Calbiochem), 7 M urea, 2 M thiourea.

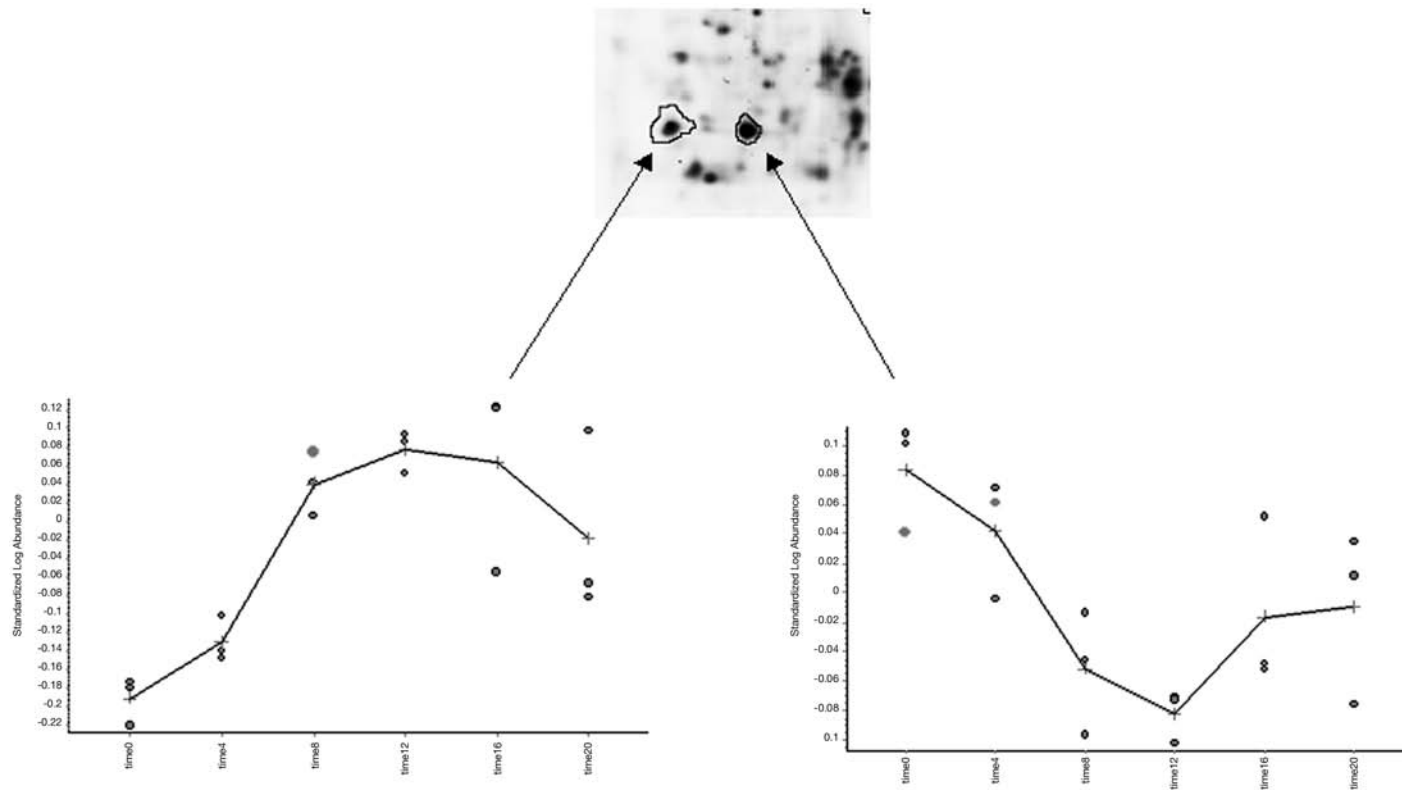


Fig. 3. Example of two spots identified as the same species by mass spectrometry; however, they have reciprocal profiles the time course. This suggests that rather than an absolute change in protein levels, the isoelectric point (pI) status of the protein is changing. The change in pI could be attributed to a phosphorylation event.

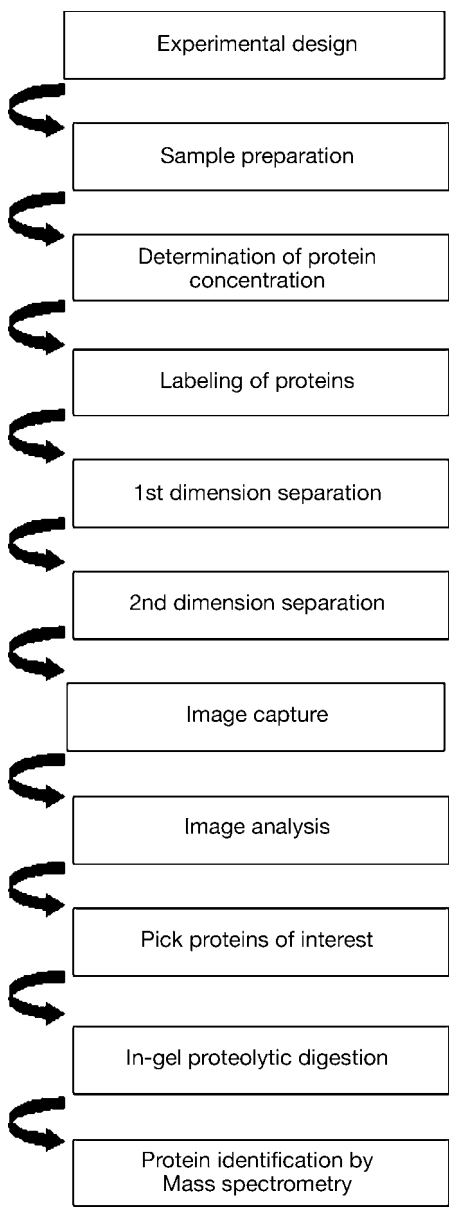


Fig. 4. Overview of the main stages involved in protein profiling by difference gel electrophoresis.

6. Rehydration buffer: 2 mg/mL DTT (add just before use), 2% IPG buffers (GE Healthcare), 2% ASB-14 (Calbiochem), 7 M urea, 2 M thiourea.
7. Equilibrium solution: 100 mM Tris-HCl, pH 6.8, 30% glycerol, 8 M urea, 1% sodium dodecyl sulfate (SDS), 0.2 mg/mL bromophenol blue, 5 mg/mL DTT (add fresh).
8. Overlay agarose: 1% agarose in SDS running buffer with 0.3% bromophenol blue. Store as 1-mL aliquots at -4°C .
9. SDS running buffer: 25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS.
10. Fixing solution: 45% methanol, 1% acetic acid.

2.2. Equipment

1. Drystrip cover fluid (GE Healthcare).
2. Low-fluorescence glass gel plates.
3. Lint-free tissues.
4. Fluorescence scanner, e.g., Typhoon 9400 (GE Healthcare).
5. Toothbrush.
6. Laminar flow cabinet.
7. Parafilm.
8. Protein concentration determination kit, e.g., BioRad DC (Bio-Rad Laboratories, Hercules, CA).
9. Kit to concentrate sample, e.g., PerfectFOCUSTM (Genotech, St. Louis, MO).
10. IPG strips (GE Healthcare).
11. Isoelectric focusing apparatus, e.g., IPGphorTM including strip holders (GE Healthcare).
12. Vertical electrophoresis apparatus, e.g., SE600 gels (GE Healthcare).
13. IPGphor strip holder cleaning solution (GE Healthcare).

3. Methods

3.1. Experimental Design

The multiple-gel approach allows many data points to be collected for each group to be compared. Spots of interest can be selected by looking for significant change across the groups—for example, with a univariate statistical test such as a Student's *t*-test or analysis of variance. These give a probability score (*p*) for each spot. This score indicates the probability that the groups are the same; consequently a low score is of interest, and $p < 0.01$ is typically used as a threshold for significant difference. In the study of the circadian cycle, for example, these tests would identify proteins with rhythmic expression. Alternatively, a curve fitting to the data can be used to identify the rhythm profile and its characteristics. It is recommended that each group be represented by at

least three data points. The number of replicates required depends on the amount of variation in the system being investigated, and on how small the changes in expression are that you wish to measure at a given confidence level. As a general rule, increasing the number of replicates will increase confidence in smaller changes in expression. It is advantageous to reduce biological variation to a minimum, as the reduction of within-group variation will increase the sensitivity of the experiment to changes between groups. This can be achieved by using homogenous genetic population and homogeneous experimental conditions.

The experimental design and the manner in which repeat data points are obtained are crucial to the conclusions that can be drawn. Biological replicates can be used with a large sample size, where biological replicates are obtained from two distinct sources but belong to the same group. In this case, protein spots highlighted can be said to be changing above biological noise. However, this approach might be unsuitable because of the amount of biological variation present and the quantity of material available from each sample. Alternatively, pooled samples can be used to reduce the biological variation. In this approach the system is more sensitive to change, but the spots highlighted can be said only to be changing above the average sample formed from the pool. In instances in which insufficient material requires pooling to achieve enough material to carry out an experiment, the use of many small pools is advisable. A third approach is to use technical replicates where one sample is available in each group but is run multiple times. In this case, the conclusion drawn is that the highlighted spot is changing in these specific samples and is above technical noise.

3.2. Sample Preparation

Protein extraction protocols will be very specific to the type of samples used. Generally a protein sample will be solubilized using a lysis buffer (*see Note 1*). DIGE, however, requires the use of a lysis buffer that is compatible with the labeling procedures (*see Note 2*). In the materials section a recipe for a recommended lysis buffer is given. In all cases it is advisable to check the pH of the samples before labeling, as it is imperative that the final pH of this solution is between pH 8.0 and pH 8.8 for efficient labeling to occur.

1. Test the sample by spotting 3 μL on a pH indicator strip.
2. If the pH is too low, adjust by careful addition of dilute ammonium hydroxide (50 mM) and retest the pH.
3. If the pH is too high adjust by careful addition of 50 mM acetic acid and retest the pH.
4. Store all samples in aliquots at -70°C until required for labeling.

The multigel pooled standard sample is formed by taking equal amounts of protein from each sample that contributes to the study.

3.3. Determining Protein Concentration

The protein concentration of all samples must be determined before labeling proceeds. There are numerous kits available for carrying out this procedure, but it is imperative that such kits are compatible for use with samples containing detergents, e.g., BioRad DC (BioRad) and PlusOne 2-D Quant kit (GE Healthcare).

3.4. Labeling

CyDyes described in this chapter are formulated as *N*-hydroxy succinimidyl esters, which react with primary amines. Labeling reactions are set up such that the stoichiometry of protein to fluor results in only 1 to 2% of the total number of lysine residues being labeled and is described as minimal labeling. The fluors also carry a net charge of +1, in order for the pI of the protein to be maintained when labeled. The three fluors are also mass-matched, such that a labeled protein will migrate to the same position.

3.4.1. Protein Concentration

For efficient labeling to take place, a protein concentration of 5 to 10 mg/mL is required in order to achieve low-volume labeling reactions. To concentrate protein samples there are several commercial kits, such as PlusOne 2-D Clean-Up Kit (GE Healthcare) and PerfectFOCUS™ (Genotech, USA). A standard laboratory protein precipitation-based concentration method is as follows:

1. Add 5 vol of cold 0.1 *M* ammonium acetate in methanol.
2. Leave at -20°C for 12 h or overnight.
3. Centrifuge at approx 1400g (~3000 rpm on a standard tabletop centrifuge) for 10 min at 4°C and remove the supernatant.
4. Wash the pellet in 80% 0.1 *M* ammonium acetate in methanol.
5. Centrifuge at 1400g (3000 rpm) for 10 min at 4°C and remove the supernatant.
6. Wash the pellet with 80% acetone.
7. Dry pellet for 15 min by leaving open tube in a laminar flow cabinet.
8. Redissolve the pellet in a smaller volume of the appropriate lysis buffer.
9. Remeasure the protein concentration.

3.4.2. Preparation of CyDye DIGE Fluors for Labeling

CyDye can be purchased as a powder; for long-term storage it should be reconstituted with DMF to a final concentration 1 nmol/ μL (see **Note 3**). This solution is stable at -70°C for 2 mo. The stock fluor solution should be stored in small aliquots to make freeze-thawing of the stock solution unnecessary. The fluors will need to be further diluted to produce the working fluor solution (400 pmol/ μL) required for the labeling protocol. The working fluor solution is stable for 2 wk at -20°C .

3.4.3. Labeling Procedure for Analytical Gels

1. Add the equivalent of 50 μg of the relevant protein sample to the appropriate 0.5-mL microfuge tube.
2. Normalize the reaction volumes to ensure an equivalent labeling efficiency across samples (*see Note 4*). *See Table 1* for a sample calculation of volumes required.
3. Dilute an aliquot of stock fluor solution with DMF to make a working CyDye solution of 400 pmol/ μL (i.e., 1 μL of stock fluor + 1.5 μL DMF).
4. To each tube add 1 μL of the appropriate working fluor solution to the normalized reaction volume and mix thoroughly by vortexing.
5. Briefly centrifuge the tubes to ensure that the reagents are at the bottom of the tube and leave on ice for 30 min in the dark.
6. Add 1 μL of 10 mM l-lysine to quench the reaction. Vortex and briefly spin and leave on ice in the dark for a further 10 min.
7. Pool differentially labeled samples to be run on the same gel into a single tube (*see Note 5*).
8. The labeled proteins are now stable for 3 mo at -70°C .

3.5. First-Dimension Separation: Isoelectric Focusing

First-dimension separation, isoelectric focusing (IEF), is based on the movement of proteins along a pH gradient under the influence of an applied voltage. Proteins will migrate to a position where they have no net charge. This position is consistent with the *pI* of the protein, which is determined by the primary sequence of the protein and posttranslational modification.

3.5.1. Preparation of Sample for IEF

Prior to the carrying out IEF of labeled proteins with IPG strips, the sample must be diluted with an appropriate buffer system for effective focusing (*see Note 6*).

1. Add an equal volume of the 2X isoelectric buffer and incubate on ice for 10 min.
2. Add rehydration buffer sufficient to increase the volume to that required for the strip length (*Table 2*).

3.5.2. Preventing Contamination Between Experiments

1. Clean the IPG strip holders (coffins) with IPGphor Strip Holder Cleaning Solution using a soft toothbrush.
2. Rinse thoroughly with hot water and distilled water.
3. Soak the coffins to be used with 1 mL of ASB-14 lysis buffer for 30 min.
4. After soaking, rinse with water and then dry thoroughly.

3.5.3. Loading Sample Onto Strip

1. Spin sample at 13,000 rpm on a standard microcentrifuge for 2 min and load the supernatant into the coffin base (*see Note 7*).

Table 1
Calculating Volumes Required for Labeling Procedure

	Sample A example		Sample B example		Pooled sample example	
Volume required for 50 µg	A	7.3 µL	B	7.0 µL	C	10.1 µL
Buffer added to normalize volumes	D	3.7 µL	E	4.0 µL	F	0.9 µL
Dye volume added	1 µL	1 µL Cy3	1 µL	1 µL Cy5	1 µL	1 µL Cy2
Lysine volume added	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
Total volume	A + D + 2	13 µL	B + E + 2	13 µL	C + F + 2	13 µL
Totalled of pooled protein	= volume from each labeling reaction = (A + D + 2) + (B + E + 2) + (C + F + 2)					39 µL
Add 2X isoelectric buffer	= add same volume again = (A+D+2) + (B+E+2) + (C+F+2)					39 µL
Add rehydration buffer	= make up the volume to that required to rehydrate the strip (Table 2) = G - [(A + D + 2) + (B + E + 2) + (C + F + 2)]					250 - (2 × 39) = 172 µL

An example calculation is shown for a 13-cm IPG strip. A, B, and C are the volumes required for 50 µg of A, B, and pooled sample, respectively. Volume D, E, and F are the volumes required to normalize the volumes to a consistent volume across the experiment for samples A, B, and pooled sample, respectively. Volume G is the volume required to rehydrate the strip.

Table 2
Rehydration Volume Depends on Size of IPG Strip Used

IPG strip length (cm)	Rehydration volume (μ L)
7	125
13	250
24	450

2. Thaw out the IPG strips to be used 5 min before use. Strips can be handled with tweezers by holding the blunt end.
3. Load each pooled group into different coffins. Remove the backing strip from the IPG strips and lay an IPG strip into each coffin, gel side down. When loading, orient the end of the strip marked with a positive toward the pointed end of the coffin. To keep a track of the samples use either the coffin number or the strip barcode number.
4. Overlay the IPG strip with Drystrip cover fluid (paraffin oil) using a disposable pipet and place the lid on top of the coffin (*see Note 7*). Try to ensure that no air bubbles are formed within the chamber upon placement of the lid onto the coffin.
5. Wipe off excess oil. Place the coffins onto the IPGphor with the pointed end electrode sitting on the anodic plate (+) and the blunt end electrode sitting on the cathodic plate (–). Ensure that the long edges of each coffin are parallel to the edges of the IPGphor.
6. Running parameters depend on strip length, pH range, and IEF apparatus used (*see apparatus manual*).

3.5.4. Preparation of Strips for Second Dimension

1. Remove the IPG strips from the IPGphor coffin and wipe away excess oil from the plastic backside of the strip.
2. Transfer the IPG strip to a sterile Petri dish with the plastic backside of the strip facing the inside edge of the Petri dish. The Petri dishes can be wrapped in Parafilm and stored at -20°C for 1 wk.
3. Add a minimum of 10 mL of equilibration buffer to each dish and incubate for no more than 15 min at room temperature on a rotator.

3.6. Second-Dimension Separation: SDS-PAGE

Second-dimension separation involves the use of SDS-PAGE to resolve proteins according to their denatured molecular weight. *See Fig. 5* for a sample of the gel image obtained.

The second dimension is a flexible system depending on available equipment and objectives of research. Typically 12% 16×14 cm SDS-PAGE gels are used, as 12% allows resolution for a wide range MW proteins (between 20

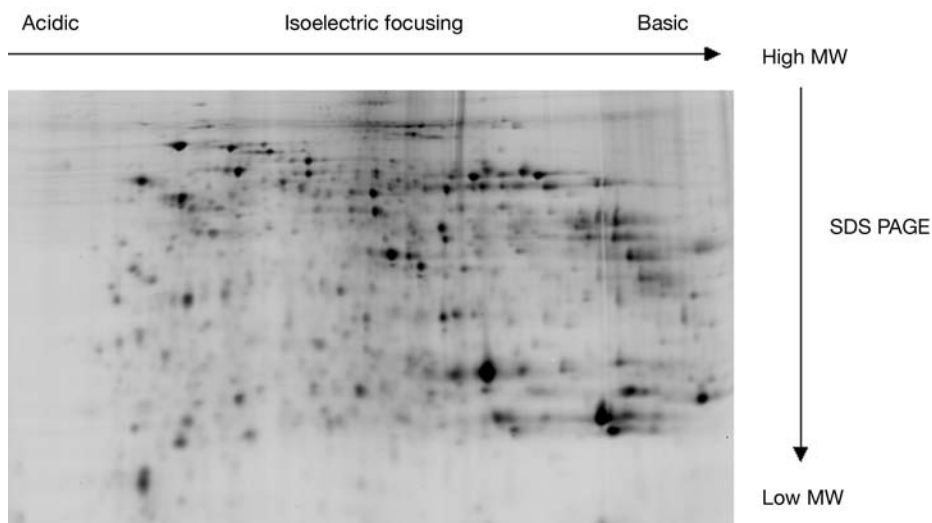


Fig. 5. Example of two-dimensional gel obtained as part of a multiple-gel circadian time-course difference gel electrophoresis experiment using soluble proteins extracted from mouse liver.

and 110 kDa). Larger gels (e.g., Ettan DALT gels 26 × 20 cm; GE Healthcare) are advantageous because of the increased resolution of the protein spots. Using 12% SDS-PAGE gels allows resolution of a wide range of MW proteins, but gradient gels may be used to separate an even wider MW range of proteins if required.

When utilizing DIGE, the following additional precautions are required:

1. Cast gels at least 9 h prior to use to enhance reproducibility of second-dimension separation by ensuring that all acrylamide has polymerized.
2. Filter acrylamide solution to remove dust particles that may lead to scanning problems and fibers from clothing that may add unwanted keratin into the system.
3. Prevent unacceptable levels of background fluorescence with imaging systems that scan through gel plates, by using low-fluorescence glass plates.
4. After pouring the acrylamide mixture, overlay the second dimension with ethanol to obtain a level surface to allow effective protein transfer between the strip and second dimension.
5. Prevent potential photobleaching of the fluorophores by running both dimensions in the dark.
6. At all times wear powder-free gloves and work in an environment that is as dust-free as possible. This is needed to reduce keratin contamination to maximize the success rate of protein identification by MS.

3.6.1. Fixing IPG Strip to Second Dimension

1. Pour off the ethanol from the second dimension and wash three times with SDS running buffer.
2. Rinse excess equilibration fluid from the strip using SDS running buffer and load onto the surface of the second dimension. Use a spatula to ensure that the strip is flush with the surface of the second dimension.
3. Melt the overlay agarose aliquots (one 1-mL aliquot for each gel) and keep molten at 55°C prior to use.
4. Drain off the running buffer from the top of the gel and pipet on a layer of molten agarose that covers the surface of the entire length of the strip and weighs it down onto the second dimension. The agarose contains bromophenol blue, which acts as a tracking dye to monitor the running of the second dimension.
5. Allow the agarose to set and remove any bubbles with a spatula.
6. Layer the top of the gel with SDS running buffer.
7. Run electrophoresis until the tracking dye has migrated and run off the bottom of the gel.

3.7. Image Capture

Image acquisition can be achieved using a variety of scanners or digital imagers, most of which are based on photomultiplier tubes or charged-coupled devices. Several such systems are commercially available that are compatible with CyDye DIGE fluors, such as the ProXPRESS™ (PerkinElmer Life Sciences, Wellesley, MA), and Typhoon 9400 (GE Healthcare). **Table 3** gives the excitation and emission parameters for all three fluors. When scanning DIGE gels, the following should be considered:

1. When saving data, avoid compression of the data, as this can affect the accuracy of recording and the amount of retrievable information upon transport into analysis packages.
2. For accurate quantitation, do not exceed the maximum pixel intensity of the instrument. At the maximum pixel intensity, saturation of the detector system is reached, resulting in inaccurate volume measurements, and in the case of charged-coupled device-based systems, risk of bleedover of signal from one pixel to its neighboring pixels.
3. The linear dynamic range of DIGE labeling is five orders of magnitude. Data quality will be lost if the dynamic range of the imaging system used is significantly less than the DIGE dynamic range. In extreme cases two different image intensity settings may be employed, resulting, in the case of the higher setting, in some spots with pixel intensities at saturation.
4. Save individual images as 16-bit TIFF (tagged image file format) images for import into most commercially available 2D gel image analysis packages. This is the most commonly accepted format for files to be exchanged between different software applications and platforms.

Table 3
CyDye DIGE Fluor Excitation and Emission Parameters

	Excitation maxima (nm)	Emission maxima (nm)
Cy2	480	530
Cy3	540	590
Cy5	620	680

5. The most commonly used analysis packages require images to be acquired with pixel resolution of 100 μm for accurate determination of image information.

3.8. Image Analysis

The image analysis process includes selecting the area to be analysed, spot detection, background subtraction, and gel-to-gel matching and analysis of the differences between groups. Typically comparative analysis results in spots with relative intensities or normalized spot volumes expressed as ratios (e.g., the standardized abundance). Analysis of data can be achieved using a variety of commercial packages. GE Healthcare supplies DeCyder™ software, which has been designed for use with DIGE and involves the use of one of labeled image as an internal standard, simplifying gel-to-gel analysis. Furthermore, the proprietary co-detection software increases accuracy, as the same spot area is compared for the images obtained from the same gel. Other software packages can also be used, such as Phoretix and Progenesis (Nonlinear Dynamics Ltd, Newcastle-Upon-Tyne, UK), MELANIE (Geneva Bioinformatics, Switzerland), AlphaMatch 2D (Alpha Innotech, San Leandro, CA), PDQuest (BioRad), and Z3 and Z4000 (Compugen, Ontario, Canada), to name but a few.

3.9. Excision of Protein Spots of Interest

DIGE labeling of proteins is compatible with in-gel digestion of proteins to peptides by the application of proteases, and subsequent identification by MS techniques, such as peptide mass fingerprinting.

To increase the success rate of protein sequencing, spots can be picked from a “preparative” gel where a larger amount of protein has been loaded. For the preparative gel, a sample from the pooled internal standard should be used to ensure that all spots that potentially need to be picked are present on the gel. Once first and second dimensions of the preparative gel have been run under identical conditions to those used for the analytic gels, the gel can then be fixed overnight by storing the gel in fixing solution. This results in precipitation of the proteins to prevent diffusion within the gel matrix.

After fixing the gel can be poststained to visualize the proteins. With DIGE minimal fluor labeling poststaining is required, because the majority of the protein on a gel is unlabeled and as such is not seen on the DIGE images. The fluor molecules add approx 500 Da to the proteins, causing the labeled and unlabeled proteins to migrate to different positions within the SDS-PAGE gel. This is most significant at low molecular weight. It is often necessary, therefore, to use a total protein stain to visualize and identify the unlabeled proteins in order to excise sufficient protein for in-gel trypsin digestion and identification by MS. Alternatively, an automated robot with appropriate fluorescence detection is used to cut the spots of interest for a DIGE gel, or one that can import spot coordinates from a scanned image, but these approaches may result in suboptimal amounts of protein being excised from gels. For manual spot excision, colloidal Coomassie brilliant blue G (30–100 ng detection limit) (16) is commonly used, but the gel could alternatively be stained with a more sensitive fluorescent stain, such as SYPRO Ruby stain (Invitrogen) (detection limit 0.25–1 ng (17), for use with an automated spot-picker.

3.10. In-Gel Proteolytic Digestion

The proteolytic digestion procedure is easily automated by robotics, which primarily reduces preparation time but also minimizes contamination by keratins from hair, skin, dust, and clothing. Excised spots must first be destained, depending on the visualization method used. Proteins within these spots are then reduced and alkylated to prevent interpeptide disulfide bridge formation, and finally digested into relatively short peptides using a robust protease. The protease most frequently employed is trypsin that cleaves the peptide bond at the C-terminal side of lysine and arginine residues. In the case of 2D DIGE, the minimal labeling results in only approx 2% of lysine residues being modified and therefore does not significantly reduce the number of available tryptic sites. Peptides generated are extracted in an appropriate solvent compatible with the mass spectrometric technique to be used.

3.11. Protein Identification From DIGE Gels by Mass Spectrometric Techniques

There are two different MS techniques that are typically employed for the identification of proteins by analysis of peptide fragments. These differ primarily in the method of ionization of peptide species. The first method, peptide mass fingerprinting, employs the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (18). The second method utilizes nanospray tandem mass spectrometry (nanospray/LC-MS/MS)

(19,20) and results in the acquisition of peptide sequence. These two techniques have complementary strengths and weaknesses as discussed under the following subheadings.

3.11.1. Peptide Mass Fingerprinting by MALDI-TOF MS

In this technique, peptides produced from in-gel digestion are coprecipitated with an organic matrix (typically α -cyano-4-cinnamic acid) on a metal sample plate. Ions are generated by the application of a laser (usually nitrogen). The mass/charge ratio of the resulting ions formed are simultaneously analyzed to produce peptide mass fingerprints, which are then matched against protein databases in order to identify the corresponding proteins (20–23). This high-throughput technique is relatively inexpensive. This method does not always result in protein identification, particularly when the correct protein sequence does not appear in a database or, more commonly in the case of complex samples, the spot chosen contains a mixture of proteins (*see Note 8*).

3.11.2. Nanospray Ionization MS

In cases in which peptide mass fingerprinting fails to give identifications, nanospray/LC-MS/MS is an alternative technology. In this technique, peptides are separated by reverse phase chromatography using a low-flow-rate high-performance liquid chromatograph that is coupled to a mass spectrometer containing mass analyzers in series (MS/MS). The ions are formed during the process of spraying peptides into the mass spectrometer from the high-performance liquid chromatograph outlet in the presence of an organic solvent at high-voltage differentials and increased temperatures. Individual ions (precursor ions) are then selected in the first mass analyzer and introduced into a collision cell within the mass spectrometer that contains an inert gas such as argon. Bombardment of the precursor ions within the collision cell results in fragmentation, typically at the peptide bond. The fragment ions are then analyzed by the second mass analyzer, which is generally a TOF detector. Sequence information from the fragmentation of each peptide taken as a precursor ion can then be interpreted. Generally, however, “uninterpreted” fragmentation data (MS/MS data) from all peptides generated from a single excised spot are submitted to powerful search engines such as MASCOT (Matrix Science Ltd., London, UK). Such programs compare databases of peptide sequences and their theoretical fragmentation patterns with a given MS/MS protein profile. The advantages of this method over peptide mass fingerprinting are that (1) mixtures of peptides can be identified and (2) if no sequence is obtained from a database search using uninterpreted fragmentation data, the peptide sequence can be deduced *de novo* and used in a BLAST search.

4. Notes

1. Protein extraction procedures must be optimized to give reproducible samples in order for accurate quantitation across groups and increase consistency in spot patterns to be achieved. The addition of proteins such as BSA and DNase may lead to irreproducible patterns. Wherever possible, avoid the use of these proteins during sample preparation and wear gloves at all times to prevent contamination with keratins. Contaminating proteins will reduce the success in the identification of proteins by MS. If sonication is required during protein extraction, it is essential to ensure that the sample does not heat up as there is a risk of carbamylation of primary amines within the proteins leading to changes in *pI* in the presence of urea.
2. If considering alternatives to the ASB-14 lysis buffer, the following components should be excluded:
 - a. Any compound containing primary amines.
 - b. Reducing agents:
 - >2 mg/mL DTT.
 - >1 mM TCEP.
 - β-Mercaptoethanol at any concentration.
 - c. Buffers:
 - >5 mM HEPES, CHES, PIPES.
 - Ampholine or IPG buffers at any concentration.
 - d. Detergents:
 - >1% TritonX-100, SDS, NP40.
 - e. Protease inhibitors:
 - Any preparation containing AEBSF.
 - >10 mM EDTA.

It is important to choose lysis buffers that do not contain large amounts of salts or ionic detergents, as these will interfere with IEF. It is advisable to add a protease inhibitor cocktail (e.g., Roche Diagnostics protease inhibitor cocktail tablets) to the lysis buffer at manufacturer's recommended concentrations. If an alternative lysis buffer is used where ASB-14 is substituted with another detergent or 7 M urea/2 M thiourea for 8 M urea, these alterations can be maintained in the 2X IEF and the rehydration buffers (**Subheading 3.5.1**). If a lysis buffer is used that contains 2% SDS, the sample must be diluted in such a way that the final percentage of SDS in sample when applied to the IPG strip is less than 0.2%, as greater amounts of SDS severely compromise the IEF of proteins.

3. DMF will degrade with time to form amine compounds; this will reduce efficiency of labeling. It is therefore recommended that DMF be replaced with a fresh bottle at least every 3 mo.
4. After normalization, the reaction volume should not exceed 20 μL. If it does, the sample needs to be concentrated as described in **Subheading 3.4.1**. This is important, as the recommended ratio of fluor to protein is 400 pmol/50 μg. If this

ratio is too low, labeling will be inefficient, whereas if the ratio is too high, there is a possibility that multiple labeling events will take place per polypeptide chain, resulting in smearing of spots on the 2D-PAGE gels. If larger amounts of proteins are to be labeled, add a correspondingly larger amount of dye to maintain the fluor:protein ratio (e.g., to 100 μL of protein add 2 μL of fluor working solution). Where larger volumes of fluor are used, the volume of blocking agent (lysine) also needs to be increased by an equivalent amount.

5. To reduce technically introduced bias, it is important to design the multiple gel experiment with a dye swap approach and randomize the samples across the gels (24). For example, if there are four samples in a group, two of those samples should be labeled with Cy3 and two with Cy5.
6. Ampholine tube gels are an alternative to IPG strips; however, the gradient can be less reliable and consequently the IPG strips are recommended for multiple-gel experiments. IPG strips are commercially available in a variety of different lengths and pH ranges, and the experimental aims will determine which is the most suitable. To obtain an overview of protein expression while maintaining the highest possible resolution, it is best to use a long IPG strip of a wide pH range. Alternatively, a long strip with a narrow pH range will focus the study and has the advantage of increasing the resolution such that more low-abundance proteins can be analyzed. IPG buffers contain carrier ampholytes. These molecules are capable of high buffering capacity around their pI and are included in the sample buffer to enhance protein solubility by minimizing protein aggregation, which would otherwise be caused by charge–charge interactions.
7. The oil prevents water loss and carbon dioxide dissolving from the air at the alkaline part of the gradient altering the pH gradient.
8. If during MS identification a protein spot is shown to be a composite of different protein species, the expression change data must be discarded, as it cannot be determined in these studies from which species the change is arising. If this is a frequent problem, approaches to increase the resolution of the gel are required, such as zoom in stripes (see **Note 6**).

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IV

GENE EXPRESSION: RNA

Isolation of Total RNA From *Neurospora* Mycelium

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Summary

In filamentous fungi, including the model organism *Neurospora crassa*, plentiful biological tissue from which RNA can be extracted may be obtained by allowing fungal spores to germinate and form a mycelium in liquid culture. The mycelium constitutes a mosaic of multinuclear, tubular filaments known as hyphae or mycelia. In general, when exposed to air, fungal hyphae quickly start to develop spores, which are often colorful. However, when submerged in liquid under rapid agitation large amounts of vegetatively growing mycelium can be obtained, which can be easily harvested by means of filtration. To preserve the physiological state of the culture, the mycelium is snap-frozen, and then to free its contents, the mycelium is ground under liquid nitrogen to break all hyphal structures. Here a method to extract high-quality total RNA from *Neurospora* mycelium using TRIzol[®] reagent is described.

Key Words: Circadian; filamentous fungus; bread mold; hyphae; mycelium; mycelial disk; liquid nitrogen; RNA; TRIzol.

1. Introduction

Isolation of good-quality RNA is an essential step in all gene expression studies. Controlling ribonuclease activity during the extraction procedure is key to obtaining undegraded total RNA preparations. Simultaneous cell lysis and inactivation of endogenous RNases has proved to be the most effective way of extracting good-quality, undegraded RNA from eukaryotic tissue. Guanidinium chloride and guanidinium thiocyanate are strong protein denaturants and effective inhibitors of ribonucleases (1–4). Since Cox in 1968 first described the use of guanidinium chloride as an RNase inhibitor in an RNA isolation protocol (2), guanidinium extractions have replaced phenol extractions as the preferred method for RNA purification. Reports of the combined use of guanidinium and phenol some 20 yr later (5,6) formed the basis of the commercialized and widely used TRIzol[®] reagent (Invitrogen), a monophasic solution of guanidine isothiocyanate and phenol. TRIzol reagent will break

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down and dissolve cell components within homogenized biological material, while the integrity of RNA is protected. The addition of chloroform will split the solution into aqueous and organic phases and segregates the RNA from protein and DNA. Subsequently, high-quality total RNA can be recovered by alcohol precipitation (7).

In the filamentous fungus *Neurospora crassa*, commonly known as the pink (or orange) bread mold, total RNA can be easily extracted from its mycelium (the white fluffy part of the mold), a network of tubular filaments, known as hyphae or mycelia. Mycelia may grow vegetatively or may differentiate into aerial hyphae, on top of which the conspicuously orange macroconidia (asexual spores) are formed (8,9). As described in Chapter 3, the rhythmic production of conidia forms the basis of the classical (race tube) assay to monitor the *Neurospora* clock. To monitor the clock at the molecular level, mycelium in its vegetative stage is used. Small pieces of mycelium, so-called “mycelial disks,” are grown submerged in liquid growth medium under rapid agitation, which prevents the development of aerial hyphae and subsequent macroconidial formation (10–12). Cultures may be subjected to different experimental conditions (e.g., free-run, light pulses), after which gene expression can be frozen in time by snap-freezing the mycelium. Using TRIzol reagent, total RNA is then extracted from frozen mycelium, which is ground to a fine powder under liquid nitrogen.

2. Materials

1. 50X Vogel's salts (see **Note 1**): Per 1 L, 150 g $\text{Na}_3\text{citrate}\cdot 5\text{H}_2\text{O}$, 250 g KH_2PO_4 , 100 g NH_4NO_3 , 10 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 5 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ (predissolved in 20 mL H_2O ; see **Note 2**), 5 mL trace elements (see **item 2**), 2 to 5 mL chloroform (see **Note 3**). Store at room temperature in the dark.
2. Trace elements: in 100 mL distilled H_2O , 5.0 g citric acid $\cdot \text{H}_2\text{O}$, 5.0 g $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 1.0 g $\text{Fe}(\text{NH}_4)_2\text{SO}_4\cdot 6\text{H}_2\text{O}$, 250 mg $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 50 mg $\text{MnSO}_4\cdot \text{H}_2\text{O}$, 50 mg H_3BO_3 (anhydrous), 50 mg $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$, 1 mL chloroform (see **Note 3**). Store at room temperature.
3. 1000X Biotin stock: 0.5 mg/mL in 50% ethanol. Store at 4°C in foil-covered bottle.
4. Minimal sucrose medium (see also **items 1 and 3**): 2% sucrose, 1X Vogel's salts, 1X biotin, 1.5% agar. Boil to dissolve the agar, aliquot into “slants,” and autoclave. Slants are cotton wool-plugged 150-mm test tubes containing approx 5 mL medium, slanted at a steep angle when agar is setting after autoclaving. Autoclaved slants can be stored at 4°C for months (in a plastic bag to prevent drying out and contamination).
5. Vogel's minimal medium (see also **items 1 and 3**): 2% glucose, 1X Vogel's salts, 1X biotin. Do not autoclave, but filter-sterilize through a 0.45- μm bottle-top filter, to prevent caramelization of the glucose. This is usually freshly prepared, but minimal medium can be stored at room temperature or at 4°C.

6. Petri dishes or cell culture dishes (Corning).
7. Large number of identical small 100-mL Erlenmeyer flasks.
8. Set of cork borers (within the range of 4–17 mm).
9. Two identical orbital (platform) shakers.
10. Temperature- and light-controlled incubators.
11. Darkroom facilities, including standard “safe” red light.
12. Büchner funnel, large Büchner flask, and vacuum pump or facility.
13. Whatman 3MM paper.
14. Liquid N₂ and small or medium cryogenic dewar.
15. Mortar and pestle (several sets).
16. Small (4 mm) and medium (10 mm) spatula.
17. Medium or large forceps or tongs.
18. Dry ice.
19. TRIzol Reagent (Invitrogen). **Caution:** Toxic—contains phenol. Store at 4°C.
20. Chloroform/IAA (isoamyl alcohol) 24:1.
21. RNase-free MilliQ-quality water.

3. Methods

A schematic overview of the RNA extraction method described in this chapter is presented in [Fig. 1](#). The method is divided into three major phases. The first phase of the protocol describes the preparation of small, equal amounts of fresh *Neurospora* mycelium, so-called mycelial disks (*see Subheading 3.1.*). The second phase is the circadian experiment to be conducted (*see Subheading 3.2.*). The final phase in the protocol describes the actual extraction of total RNA from *Neurospora* mycelium, using TRIzol reagent (*see Subheading 3.3.*).

3.1. Generation of Mycelial Disks (*see also Fig. 1, steps 1–3*)

To obtain small pieces of vegetatively growing mycelium of equal size, a floating “mat of mycelium” (known as a “hyphal mat” or “mycelial mat”) is grown in standing liquid culture, from which small disks can be cut for experimental purposes ([10,11](#)).

3.1.1. Preparation of Mycelial Mat

Two days prior to the intended start of the experiment:

1. Make sure to have one or two fresh slants (3–10 d old) for each *Neurospora* strain to be used (*see Note 4*).
2. Add 30 mL of Vogel’s minimal medium to a sterile Petri dish or cell culture dish (*see Note 5*). Depending on the scale of the experiment use one to three dishes for each *Neurospora* strain.
3. Add 1 to 2 mL Vogel’s minimal medium to each slant, replace cotton wool plug, and vortex vigorously.
4. Take off spore suspension with a sterile filtered pipet tip and transfer to a 1.5-mL Eppendorf tube.

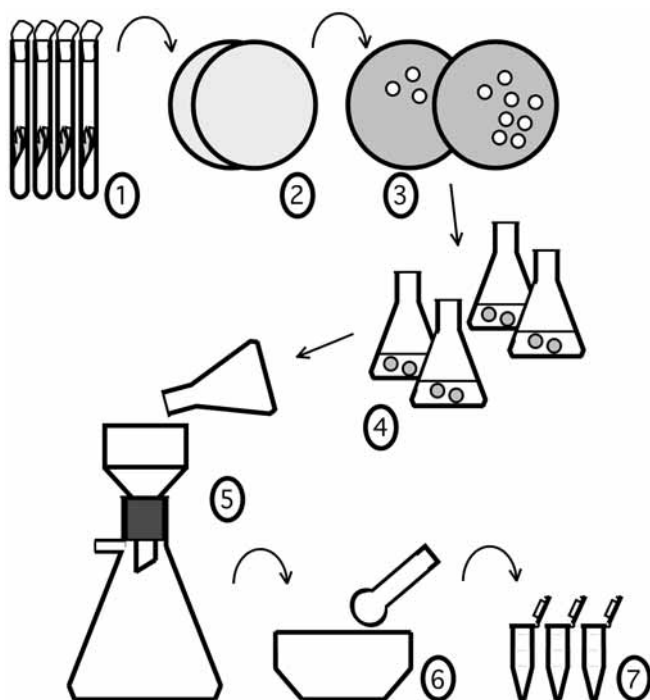


Fig. 1. RNA isolation from *Neurospora*. Schematic overview of the processes involved in extracting total RNA from *Neurospora* mycelium. 1. Fresh slants; 2. Mycelial mats; 3. Mycelial disks; 4. The circadian experiment; 5. Harvest; 6. Homogenization of mycelium; 7. Extraction of total RNA. Conidia are harvested from fresh slants and used to inoculate liquid medium to produce mycelial mats, from which mycelial disks are cut. These segments of vegetatively growing mycelium are subjected to experimental procedures and are subsequently harvested and snap-frozen. RNA is then extracted from frozen, ground-up mycelium.

5. Measure the optical density (OD_{530}) from a dilution of the spore suspension (e.g., use 2.5 μ L in 1 mL H_2O). $OD_{530} = 1$ equals approx 3×10^6 spores/mL.
6. Vortex the spore suspension vigorously and transfer approx 1×10^8 spores into the liquid in each cell culture dish and pipet slowly up and down to distribute the spores evenly (*see also Note 6*).
7. Leave cultures on the lab bench or incubate at 25°C or 30°C (static incubation under constant light; *see also Note 6*). After 12 to 18 h a mycelial mat will form, floating on the liquid. To get a good mycelial mat of even thickness, care should be taken not to disturb the dishes when the mat is still thin and fragile.
8. If necessary, vary the growth conditions in order to obtain a thick and rigid nonsporulating mycelial mat on the intended starting day of the experiment (*see Note 6*).

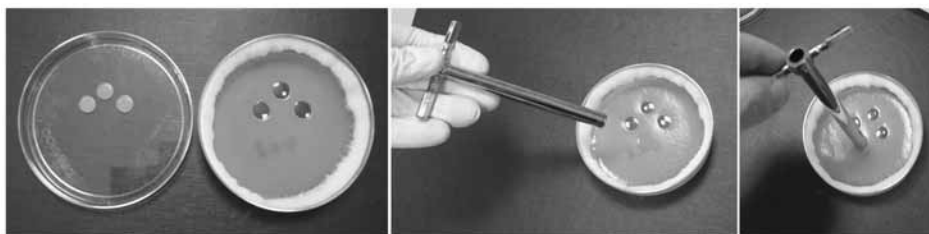


Fig. 2. Cutting mycelial disks. **Left:** Mycelial disks have been cut from a mycelial mat and transferred to a fresh dish, ready for inoculation of liquid cultures. **Middle and right:** Another mycelial disk is cut using a flamed cork borer. Pictures by C. Heintzen, University of Manchester, UK.

3.1.2. Cutting of Mycelial Disks

On the starting day of the experiment:

1. Check to make sure the mycelial mat is thick, quite rigid, and not overgrown or sporulating. Only then proceed to the next step (*see Note 7*).
2. Make sure flasks for inoculation have been prepared (*see Subheading 3.2.1.*).
3. Cut small pieces of mycelium of equal size, so-called mycelial disks, from the mycelial mat using a flamed cork borer (**Fig. 2**) (*see also Note 8*). Avoid cutting disks in the peripheral areas of the mycelial mat where aerial hyphae are present or areas where fungal spores may have developed (*see Note 7*).
4. Transfer mycelial disks to a fresh dish, containing a small volume of Vogel's minimal medium, using a pair of flamed pointed forceps (**Fig. 2**).

3.2. Circadian Experiment (*see also Fig. 1 steps 4–5*)

Irrespective of the experiment objectives, several steps toward obtaining mycelium from which total RNA may be extracted are identical, and are described below (*see Subheadings 3.2.1. and 3.2.2.*). Subsequently, a “classical” circadian free-run experiment is described, whereby mycelium for RNA extraction is harvested in the dark at 12 sequential time-points covering two circadian cycles (*see Subheading 3.2.3.*).

3.2.1. Inoculation and Incubation of Liquid Cultures

1. Autoclave foil-covered, identical, small Erlenmeyer flasks.
2. Prepare Vogel's minimal medium and filter-sterilize.
3. Using a sterile 50-mL Falcon tube add aseptically 50 mL of sterile Vogel's minimal medium to each flask.
4. Inoculate each flask with one or two mycelial disks (prepared as described in **Subheading 3.1.**; *see also Note 8*) using a pair of flamed pointed forceps. There is no real need to flame the neck of each flask or to keep flaming the forceps. Just work cleanly, quickly, and near a flame.

5. Place flasks on an orbital shaker under constant agitation at 125 rpm and incubate at 25°C in constant light for at least 4 to 6 h to synchronize the cultures (see **Note 9**), before changing any growth conditions to experimental conditions.

3.2.2. Harvest of Liquid Cultures (see **Note 10**)

1. Harvest cultures (under red light) onto 3MM Whatman paper by filtration through a Büchner funnel under vacuum (see **Note 11**).
2. Using a gloved finger, “rub and roll” the dried mycelial disk(s) from the filter paper (see **Note 12**).
3. Depending on the amount of mycelium, place mycelium into a 1.5-mL screw-cap Eppendorf tube or 15-mL Falcon tube and snap-freeze in liquid N₂ (see **Note 13**).
4. In general, when harvesting mycelium, work quickly (see **Note 14**).
5. Mycelium can be stored frozen at –80°C indefinitely until RNA extraction is undertaken.

3.2.3 Circadian Time Course Experiment

As an example, a circadian time course experiment is described in which clock gene expression is followed after lights off every 4 h over 2 circadian days (see **Note 15**). Instead of harvesting mycelium every 4 h over a 48-h period, cultures are staggered into the dark at 12-h intervals (**12**).

1. On day 1 of the experiment prepare at least 12 small flasks containing 50 mL Vogel’s minimal medium for each *Neurospora* strain.
2. Inoculate each flask with one 5-mm mycelial disk.
3. Incubate in constant light at 25°C under constant agitation (125 rpm); start of the experiment: **Day 1 15.00 h**. (Time is given as an example; see also **Table 1**).
4. After 6 h transfer 3 cultures for each *Neurospora* strain (labeled as indicated; see **Table 1**) to constant darkness at 25°C under constant agitation (125 rpm).
5. Continue to transfer cultures to constant darkness every 12 h (as indicated; see **Table 1**).
6. Harvest cultures (under red light) in three consecutive sessions: 4, 8, and 12 h after the last transfer (as indicated; see **Table 1**). In this way, all cultures will have been grown for 48 h; however, the timing of lights-off has been varied (**12**).

3.3. Extraction of Total RNA Using TRIzol (see also **Fig. 1**, steps 6–7)

3.3.1. Homogenization of Mycelium

In the initial step of the extraction protocol the mycelium is broken up and all hyphal structures are disrupted. The cell contents are thus released, total RNA but also endogenous RNases included. To prevent ribonuclease activity it is essential to keep the mycelium frozen at all times, until the TRIzol reagent is in contact with the homogenized mycelium. Therefore, it is recommended at

Table 1
Labeling, Transfer, and Harvest of Time Course Cultures

Transfer to DD	Harvest day 3 13.00 h	Harvest day 3 17.00 h	Harvest day 3 21.00 h
Day 1 21.00 h	DD40	DD44	DD48
Day 2 09.00 h	DD28	DD32	DD36
Day 2 21.00 h	DD16	DD20	DD24
Day 3 09.00 h	DD4	DD8	DD12

Times are given as an example.
DD, constant darkness.

certain steps to also freeze the tools with which the mycelium is handled (as indicated below).

1. Wear suitable protective clothing, eye protection, and gloves when working with liquid N₂ (*see also* **Note 16**). Pour some liquid N₂ (*see* **Note 17**) into a clean mortar and place a pestle into it to precool both. There is no need for the mortar and pestle to be autoclaved prior to use.
2. Again, pour some liquid N₂ into the mortar and place 100 to 200 mg of frozen mycelium into it. Grind the mycelium under liquid N₂ to a fine powder (*see* **Note 18**). Keep adding liquid N₂ as needed to keep mycelium frozen during the grinding process.
3. Precool a 2-mL labeled Eppendorf tube by dipping it into the liquid N₂ using a large forceps or tongs, empty it, and leave aside. Meanwhile, make sure the mycelium is still frozen. Keep adding liquid N₂ if needed (if working quickly this should not be necessary).
4. Then quickly cool a 10-mm spatula by dipping it into the liquid N₂ for about 5 s. Again, make sure the mycelium is still frozen.
5. Transfer 50 to 100 mg of powdered frozen mycelium into the precooled 2-mL Eppendorf tube using the precooled spatula (*see* **Note 19**). Work quickly.
6. Place tube on dry ice to keep mycelium frozen (*see also* **Note 20**) until all samples have been ground.
7. Clean up the mortar, pestle, and spatula (*see* **Note 21**) or use another clean set. Repeat all previous steps for all other samples.
8. If preferred, ground mycelium can be stored at –80°C for years for future extraction of RNA (or DNA or protein extraction).

3.3.2. *RNA Isolation Using TRIzol Reagent*

Protocol essentially according to manufacturer’s recommendations (7). Once TRIzol has been in contact with the mycelium (**step 2** below), the chance of

RNA degradation is much reduced; hence there is no need to work on ice. Unless otherwise stated all steps can be conducted at room temperature.

1. Wear gloves and work in a fume hood when working with TRIzol.
2. At room temperature add 1 mL of TRIzol to each tube containing the powdered mycelium. Let the mycelium defrost while vigorously shaking and vortexing the tube.
3. Homogenize fully by vortexing each tube continuously for 60 s.
4. Leave for 5 min to allow for complete dissociation of all RNA–protein complexes. Then spin for 10 min at high speed to remove all insoluble material.
5. Carefully transfer the supernatant to a fresh 1.5-mL Eppendorf tube to which 0.2 mL chloroform/IAA has been added.
6. Make sure all tubes are securely closed, then shake each tube violently for 15 s by hand and briefly vortex for 2 s. Phase separation of aqueous and organic phases has occurred but is not yet complete. The liquid should have a “strawberry milkshake” appearance at this stage. Leave for 3 min.
7. Centrifuge for 15 min at high speed to establish full phase separation into a red, organic lower phase, a thick, white interphase, and a clear, aqueous upper phase, which contains the RNA.
8. Carefully transfer the upper phase to a fresh 1.5-mL Eppendorf tube (*see Note 22*).
9. Add 0.5 mL of isopropanol to precipitate the RNA. Mix by inverting the tubes 8 to 10 times and leave for 10 min.
10. Centrifuge for 10 min at high speed and carefully remove the supernatant.
11. Wash the RNA pellet with 1 mL of 70% ethanol. Disturb the pellet with a yellow tip and vortex.
12. Centrifuge for 5 min at 7500g. Gentle pelleting of the RNA is essential at this stage, as otherwise the RNA becomes very difficult to dissolve.
13. Remove most of the supernatant with blue tip (1000- μ L tip), taking great care not to suck up the RNA pellet (which is not very well stuck the tube). Centrifuge for another 30 s at 7500g to collect all the liquid in the bottom of the tube. Then carefully remove all liquid using a yellow tip (200- μ L tip).
14. Air-dry the pellet for 10 to 15 min at room temperature. Take care, as overdrying the pellet makes redissolving very difficult, if not impossible.
15. Add 100 μ L of RNase-free H₂O and leave the pellet overnight at 4°C.
16. Dissolve the RNA fully for 10 to 60 min at 65°C. Check for completion of this process by pipetting the solution up and down (*see also Notes 23 and 24*). Store RNA at –80°C.
17. RNA quantity and quality are determined by spectrophotometric analysis (*see Note 25*). RNA integrity may be determined using formaldehyde agarose gel electrophoresis (*13*) (*see also Note 24*) or RNA can be used directly in Northern analysis, as described in Chapter 23.

4. Notes

1. In 1956 Vogel (14) described a formula for a 50X strength salt solution, now commonly known as 50X Vogel's, which is still used today in the majority of *Neurospora* minimal growth media. For 50X Vogel's salts solution, dissolve with vigorous stirring in 750 mL H₂O the chemicals in the given order. It is essential to dissolve each ingredient completely before adding the next chemical. For some chemicals this can take many hours. Failing to do so can create insoluble precipitates. Vigorous stirring using a large stirring bar may speed up the process. Remember, it is better to leave the solution stirring overnight than rushing the preparation and allowing precipitates to form. When all chemicals are dissolved, adjust volume to 1 L, pH 5.8 (no adjustment in pH should be necessary). Finally, add the chloroform as preservative (see Note 3).
2. Predissolving the CaCl₂ in distilled water helps to prevent the formation of insoluble precipitates, which will almost inevitably appear when solid CaCl₂ is used. Addition of the CaCl₂ solution to the salt stock solution must be carried out slowly, allowing cloudiness to disappear after every few drops.
3. Addition of chloroform to the 50X Vogel's salts and trace elements is an essential step. Failing this, airborne fungal spores will quickly form myriad fungal colonies on its surface, as these stock solutions are not sterilized.
4. To prepare fresh *Neurospora* slants, inoculate minimal sucrose medium slants from frozen stock slants (15). Incubate for 2 to 3 d at 30°C until a large amount of light orange spores have developed. Slants can then be stored on the lab bench at room temperature until use. Exposure to the light will intensify the color of the spores to bright orange, will also color the aerial hyphae, and will increase the conidial yield in young cultures (9). Spores should be collected from fresh slants to obtain consistent results. Spores may be taken from frozen stock slants, but the germination and the initial growth may be inconsistent, and is therefore not recommended. Spores should not be used when slants are older than 10 d, as *Neurospora* conidia lose viability quickly after 10 d and the chance of picking up mutants increases significantly.
5. Consistency in obtaining good-quality mycelial mats is greatly enhanced by the use of cell culture dishes instead of standard Petri dishes. The use of a Corning cell culture dish (100 × 20 mm style, treated polystyrene, nonpyrogenic, sterile) is recommended (M. Elvin, personal communication).
6. The number of spores to be added to a cell culture dish is given only as a rough guide, as the way a mycelial mat grows is also very much strain-dependent. It is advisable, for instance, to inoculate several dishes with a different amount of spores for each *Neurospora* strain to be used. To avoid disappointment on the intended starting day of the experiment, check the cultures regularly and, if necessary, vary the growth conditions. If the mycelial mat grows too slowly transfer the dish to a warmer incubator, or use more spores next time. Practice makes perfect!

7. To obtain consistent results it is important to get clean mycelial disks of equal size and texture. If the mat is too thin, postpone the experiment. If the mat is overgrown and heavily sporulating, cancel the experiment and set up new cell culture dishes. When sporulating mycelial disks are used, the spores will germinate and form new separate mycelia in the liquid culture during the experiment. When overgrown mycelial disks (disks with aerial hyphae) are used, the disks are likely to float in the liquid culture and/or from large amounts of aerial hyphae during the experiment. Both situations involve developmental stages other than vegetatively growing mycelium and should thus be avoided to obtain consistent results.
8. The size of the mycelial disk to be used—i.e., the size of the cork borer to be used for cutting—depends greatly on the length of the circadian experiment and the percentage of glucose used in the liquid growth medium. Mycelial growth is also strain-dependent. Usually disks of 5 to 10 mm are convenient. As a general rule of thumb, use only one small mycelial disk (5 mm) when the culture is growing for up to 48 h before harvest; use more or larger disks when the culture is growing for less than 24 h (*see also* **Note 10**).
9. A preincubation of all cultures prevents variation in gene expression that may occur due to cutting and handling the mycelium. Transfer of cultures to the dark after a prolonged period in the light (in the laboratory and during preincubation) set the clock to defined time (**10,11,16,17**).
10. The “mycelial balls” to be harvested (when growing, mycelial disks become ball-shaped) should still be fully submerged (no aerial hyphae, as this involves a developmental switch with obvious changes in gene expression), yet large enough to obtain sufficient amount of biomass for intended RNA, DNA, and/or protein extraction.
11. There is no need for the filter paper to be sterile. Use a fresh piece of filter paper for each harvest. Mycelium can also be harvested without the use of a vacuum. Collect mycelium through a piece of funnel-shaped Whatman paper. Squeeze out any remaining liquid by pressing hard onto a fresh piece of filter paper using a gloved hand.
12. Do not roll the mycelial disks too tightly, but roll them just enough to be able to fit the rolled-up tissue in a tube. It is much easier to grind a thin, crisp flake of *Neurospora* than a solid, frozen block of *Neurospora* mycelium (*see also* **Note 18**). When harvesting small mycelial disks, watch carefully where the disks “hit the filter paper,” as vacuum-dried mycelium becomes very thin and may be difficult to find under red light.
13. When labeling tubes for storage of mycelium, remember not to use a red marker pen when harvesting is to be done in the dark under red light.
14. When harvesting large numbers of samples, work quickly. Remember, in an ideal world, the gene expression of all samples should be frozen in time at exactly the same second. Time is an important factor in a circadian experiment, especially when using light pulses, as clock gene expression can be induced very rapidly (**18,19**).
15. Northern analysis of *Neurospora frq* RNA from total RNA extracted from time course samples as described here, is given as an example in Chapter 23 and results have been published (**19**).

16. The use of suitable heavy-duty gloves to handle liquid N_2 is not practical when grinding mycelium using small or medium mortars and pestles and handling small tubes and spatulas, and is therefore not recommended. Using a double surgical glove on the hand holding the mortar does help. Using a cotton or a tiger-grip glove covered by a surgical glove on that hand is even more comfortable, yet allows sufficient sensation. Be aware, however, that this is inadequate to protect against cryogenic burns.
17. Pouring small amounts of liquid N_2 from a medium-sized dewar into a mortar is a bit of an art, especially when the container is quite full. A small glass (or metal) beaker can also be used to ladle the liquid N_2 . Take care, prevent cryogenic burns!
18. Frozen mycelium often comes in large, hard lumps. The easiest way to start the grinding process is to carefully, but forcefully, crush and beat the mycelium into small bits. Having enough liquid N_2 in the mortar helps to prevent the mycelium bits from flying out. Then forcefully grind the mycelium to a fine powder. A good rule of thumb is: when the mycelium appears to be a fine powder, it probably can be ground even finer, so add liquid N_2 again and grind one more time.
19. There is no real need to weigh the amount of mycelium; three to four “spatulas-full” is a good amount (approximately one-third of the volume in a 2-mL Eppendorf tube). Do not use too much, as this will make vortexing at later stages difficult. Furthermore, the use of a large amount of mycelium will not improve the RNA yield. If large quantities of RNA are needed it is recommended to divide the ground mycelium into multiple tubes. RNA can be isolated from even very small amounts (<20 mg) of frozen mycelium. If preferred, small amounts of ground mycelium can also be transferred into a tube by pouring a “mycelium/liquid N_2 mixture” into the tube and letting all N_2 escape before closing the tube lid (*see also* **Note 20**).
20. Liquid N_2 can also be used (instead of dry ice) to temporarily store the ground samples. However, care should be taken that tubes do not pop open in liquid N_2 , resulting in loss of material. When ground mycelium has been put into the tube, leave the tube open for a little while for all N_2 to escape before closing the lid (but do not let the sample defrost).
21. The mortar and pestle can be cleaned for immediate reuse by wiping the excess of ground mycelium out of the mortar with a dry soft tissue. Then use IMS and a soft tissue to clean the mortar, pestle, and spatula. Finally wipe dry with a tissue.
22. As the extraction protocol described yields large quantities of total RNA (up to 1.5 mg RNA from 100 mg *Neurospora* mycelium), there is no need to come anywhere near the interphase when taking off the RNA-containing aqueous upper phase.
23. After overnight incubation the RNA seems to be dissolved, but this is often not the case. The RNA pellet is just not visible. When pipetting, a gel-like, clear “ball of RNA” often prevents sucking up the full amount of liquid. Continue to incubate at 65°C and check the pellet every 10 min by pipetting again. It has been noted that simply dissolving the RNA pellet at 65°C without overnight incubation does not seem to work very efficiently.

24. Remember, that from this stage onward RNA is susceptible to RNase contamination originating from the experimenter and the laboratory. Wear gloves. Single-use plasticware is recommended. Plastics for multiple use, such as gel electrophoresis tanks, should be soaked in 0.5 M NaOH for at least 30 to 60 min (no adverse effects are observed when soaking for longer periods, e.g., hours or overnight) and rinsed thoroughly with Milli-Q H₂O before use.
25. Take 1 μ L RNA in 500 μ L H₂O and measure OD₂₆₀ and OD₂₈₀. Typically, RNA concentrations between 8 and 12 μ g/ μ L are obtained, with a 260/280 ratio of 1.76 to 1.82.

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RNA Extraction From *Drosophila* Heads

Patrick Emery

Summary

In *Drosophila*, input, pacemaker, and output genes are expressed circadianly. mRNA oscillations contribute largely to these rhythms. Determining RNA levels of circadian genes is thus frequently necessary to understand their regulation, or the effect of mutations and genetic manipulations on the function of the circadian pacemaker. RNA extraction is the prelude to several techniques aimed at measuring RNA levels. The procedure presented in this chapter is a rapid method to obtain a clean preparation of total RNA from fly heads that can be used for RNase protection, Northern blots, and real-time polymerase chain reaction (*see* Chapters 23–25).

Key Words: mRNA extraction; circadian mRNA oscillations; RNase protection; Northern blots; real-time PCR.

1. Introduction

In most organisms, the transcriptional regulation of key pacemaker genes plays a central role in the generation and maintenance of circadian rhythms (*1*). In *Drosophila*, the expression of circadian genes is particularly high in heads. As heads can easily be separated from the rest of the fly, they are most frequently used for studying circadian mRNA oscillations. However, it is important to keep in mind that most of the signal obtained with whole heads comes from the eyes—a peripheral oscillator—and not from the brain cells controlling circadian behavior (*ref. 2*; *see Note 1*).

2. Materials

1. Incubator with light and temperature control.
2. More than 50 flies per time-point, 3 to 7 d old.
3. Dry ice.
4. One medium-sized and one small funnel, chilled at -80°C .
5. Plastic mesh or brass sieves (nos. 25 and 40), chilled at -80°C .

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6. Pellet pestle motor and RNase-free pellet pestles (Kontes).
7. TRIzol (Invitrogen), stored at 4°C.
8. Chloroform.
9. Isopropanol.
10. 75% Ethanol.

3. Methods

3.1. *Light and Temperature Treatment and Fly Collection*

Usually, flies are subjected to a 12-h light:12-h dark regime at a constant temperature of 25°C. Synchronization is achieved with 2 full days and flies can therefore be collected on the third day under the desired conditions (e.g., constant darkness). Temperature cycling can also be used as a synchronizer, with, for example, 25°C for the day and 20°C for the night. At the time of collection, flies are transferred with a funnel to 15-mL tubes chilled on dry ice. Flies should be collected in the dark or under red light. They can be stored at -80°C indefinitely.

3.2. *RNA Extraction From Whole Heads*

1. Flies and their heads must be kept as much as possible on dry ice until extraction actually begins; RNase free conditions are required.
2. Decapitate the flies by vigorously vortexing the 15-mL tubes twice during 15 s.
3. If the number of heads per time-point is small, heads are counted on a plastic mesh placed over dry ice and transferred into microcentrifuge tubes with a small brush. If the number of heads is large, brass sieves can be used to separate the heads from the bodies and other small body parts such as legs and antennae. The top sieve (no. 25) will let the heads go to the bottom sieve (no. 40), which will separate the small fly body parts from the heads. The heads can then be transferred to microcentrifuge tubes with a small funnel.
4. Homogenize the heads with a pellet pestle in 800 μ L TRIzol (*see Note 2*).
5. Incubate the homogenate at room temperature for 5 min.
6. Add 160 μ L of chloroform, shake vigorously for 15 s, and incubate for 3 min at room temperature.
7. Microcentrifuge (12,000g) for 15 min at 4°C.
8. Transfer the aqueous phase to a fresh microcentrifuge tube and precipitate with 400 μ L isopropanol.
9. After 10 min at room temperature, spin down the RNA with a 15-min microcentrifugation (12,000g) at 4°C.
10. Discard the supernatant, rinse the pellet with 1 mL 75% ethanol, and microcentrifuge again for 5 min.
11. Discard the supernatant and air-dry the RNA pellet for 15 min (*see Note 3*).
12. Resuspend the pellet in water or any buffer of choice. A 10-min incubation at 55°C helps resuspension (*see Note 4*).

4. Notes

1. Most of the RNAs extracted from whole heads are coming from the eyes, a peripheral oscillator (2). The neurons controlling circadian behavior contribute to only a small fraction of the extracted RNA. To concentrate the contribution of these neurons, eyeless mutant flies (e.g., *eyes-absent*) can be used as a source of RNA, or brains can be dissected (2). However, as many other neurons and glial cells express circadian RNAs (3–5), even in brains the RNA signal comes from a mixture of different tissues. *In situ* mRNA hybridization is the only direct method to study mRNA oscillations in the *Drosophila* pacemaker neurons, the ventral lateral neurons (see Chapter 40).
2. For more than 80 mg of heads, it is recommended to scale up the amount of TRIzol used.
3. This protocol can be adapted to RNA extraction from bodies. Eight bodies will yield approximately the same amount of RNA as 50 heads.
4. An additional DNase treatment is useful if the RNA is going to be used for RNase protection or quantitative PCR. RQ1 RNase-free DNase (*Promega*) can be used, for example.

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Extraction of Plant RNA

Michael G. Salter and Helen E. Conlon

Summary

This protocol details an RNA preparation for medium-scale, high-purity RNA production from higher plants. It uses hot acid phenol with standard sodium acetate ethanol precipitation and is suitable for producing RNA for both Northern blotting and enzyme-based downstream applications such as RT-PCR and microarray studies.

Key Words: *Arabidopsis thaliana*; total RNA extraction; acid phenol method; efficient; limited tissue.

1. Introduction

The protocol described here is a manual one that can be performed in any laboratory with basic equipment suitable for molecular biology and is based on the work of Verwoerd et al. (1). The reagents are relatively inexpensive and large quantities of high-purity RNA can be obtained. It is important to note before embarking on such protocols that there are a number of proprietary kits available for RNA extraction from any number of organisms, including plants. These kits can be rapid and easy to use for many applications and so are often the methods of choice for RNA extraction. In our experience, however, we have found that the use of these kits did not result in RNA from seedlings or adult plants that was pure enough for use in the preparatory reactions for microarray analysis (2). As a result we found that, despite the recommendation of Affymetrix to the contrary, a further acid phenol chloroform extraction was required to enable efficient *in vitro* transcription reactions to take place. It is usual to use DEPC-treated water to make the extraction buffer and for resuspending the RNA at the end of the protocol. We tend to use nuclease-free water purchased from one of the common suppliers, such as Sigma or Ambion, both because there is a guarantee of purity and because it is more convenient. This protocol is an amalgam of a number of protocols developed over time.

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The preparation of total RNA from plant cells requires caution during the early phases. Plants, like all organisms, contain large amounts of nucleases and so it is important to either remove or neutralize these as quickly as possible once the tissue to be used is disrupted. It is also important to remember that hands and any other parts of exposed skin contain nucleases, so it is essential to wear gloves, laboratory coats, and so on and to take every precaution to prevent avoidable contamination of the sample both before it enters the extraction buffer following tissue disruption and once the purified RNA has been resuspended in water. This includes making sure that no one who is not wearing gloves touches anything that you will use; paranoia is the key to avoiding the “disappearing before your eyes” syndrome common in RNA work. The protocol detailed here uses hot acid phenol to neutralize the RNase activity rapidly and efficiently. Some early protocols and current mammalian protocols utilize guanidinium salts to neutralize the RNases and for the disruption of cells. Unfortunately, these salts can result in the contamination of the RNA with polysaccharides (3), a constant and recurring problem with many plant nucleic acid preparations and so are less appropriate in plant applications.

Acid phenol:chloroform (pH 4.5, Ambion) is used because at acidic pH, the DNA from the sample will partition within the organic phase or at the interface between the two phases (4,5). The required RNA will remain in the aqueous phase, although some DNA contamination will remain, and so an additional step will be required for downstream applications using reverse transcription polymerase chain reaction (RT-PCR)-based assays. For applications such as Northern blotting where larger quantities of RNA are used and there is no amplification step, the small quantities of contaminating DNA should not affect the validity of the results.

Following the phenol extraction a second phenol chloroform extraction is used. The use of chloroform helps reduce the loss of messenger RNA that has become bound to proteins within the interphase between the phenol and extraction buffer. The chloroform helps solubilize the RNA from the proteins and also assists in the denaturation of the protein (6–8).

Precipitation of RNA can be performed using lithium chloride, ammonium acetate, potassium acetate, and sodium acetate in conjunction with ethanol. In this protocol there is only a sodium acetate precipitation. This is because lithium chloride is toxic and can interfere with downstream reactions such as RT-PCR and in vitro transcription reactions. In addition to this, lithium chloride will not precipitate RNAs lower than 300 bp and a concentration of at least 200 $\mu\text{g/mL}$ concentration is required for efficient precipitation. If the downstream application will use a sodium dodecyl sulfate buffer, then it is important to avoid precipitation with potassium acetate.

2. Materials

1. Extraction buffer: 100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.5% w/v sodium dodecyl sulfate (*see Note 1*).
2. 3 M Sodium acetate, pH 5.2 (*see Note 2*).
3. DEPC water: add 0.1% (v/v) DEPC to the water and incubate overnight. Autoclave twice the next day and keep sealed until use.
4. Equilibrated acid phenol, pH 6.9.
5. Acid phenol:chloroform isoamyl alcohol (IAA) 5:1, pH 4.5 (Ambion).
6. Chloroform IAA 24:1.
7. Ethanol 100% molecular biology grade.
8. 70% Ethanol, 30% DEPC-treated water (v/v).
9. Swing-out rotor centrifuge capable of 3500g and able to take 50-mL tubes.
10. Sorval-type centrifuge with SS34 rotor and appropriate tubes.
11. Pestle and mortar set appropriate to the scale of the preparation.
12. Metal spatula.
13. Liquid nitrogen in an appropriate vessel.
14. 50-mL RNase-free polypropylene centrifuge tubes.
15. Centrifuge tubes suitable for use in a Sorval SS34 rotor and treated by either autoclaving twice or using proprietary RNase removal systems so as to be RNase-free.
16. DNase 1 or “DNA free” (Ambion).
17. Single- or dual-beam UV spectrophotometer with 200- μ L quartz cuvet.

3. Methods

3.1. Preparation

1. Set up mortars and pestles, one set for each sample.
2. Heat the water bath to 65°C.
3. Put 50-mL tubes in a rack, one for each sample labeled appropriately, and add 5 mL of acid phenol plus 5 mL of extraction buffer. Place the rack in the water bath and mix occasionally until the solutions are at 65°C (*see Note 3*).
4. Prechill the mortar and pestle with liquid nitrogen. Make sure both are down to the temperature of the liquid nitrogen—this is when the nitrogen stops bubbling violently in the mortar, leaving a pool.

3.2. Extraction

1. Harvest 1 g of tissue and drop it immediately into the pool of liquid nitrogen in the mortar (*see Note 4*).
2. Grind the tissue to a fine powder, adding more liquid nitrogen as it evaporates (*see Note 5*).
3. Chill an appropriate-sized spatula in the liquid nitrogen and use it to scrape the powder into the tube for that sample in the water bath. Vortex immediately and return to the water bath.

4. Incubate the sample in the water bath for 30 min, vortexing every 5 min.
5. Remove the tubes from the water bath and centrifuge at 3000g in a swing-out rotor centrifuge at room temperature for 15 min.
6. Transfer the aqueous phase plus the interphase to a new tube and add 5 mL acid phenol:chloroform IAA, 5:1, pH 4.5. Vortex and centrifuge at 3000g in a swing-out rotor centrifuge at room temperature for 15 min (*see Note 6*).
7. Transfer only the aqueous phase to a new tube, taking care not to disturb the interphase. You should leave a little of the aqueous phase behind to avoid contamination.
8. Add 5 mL of chloroform IAA 24:1 to the aqueous phase. Vortex and centrifuge at 3000g in a swing-out rotor centrifuge at room temperature for 15 min.
9. Repeat **step 8** and then remove the aqueous phase and put into tubes suitable for use in a Sorval SS34 rotor.
10. Add 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of molecular biology grade 100% ethanol. Store in -80°C freezer for at least 1 h, but overnight is preferable.
11. Centrifuge in a Sorval centrifuge in a prechilled SS34 rotor at 4°C for 30 min at 10,000g.
12. Remove the aqueous phase, taking care not to disturb the pellet (*see Note 7*). Wash the pellet with 70% ethanol and centrifuge in a Sorval centrifuge in a prechilled SS34 rotor at 4°C for 30 min at 10,000g.
13. Repeat **step 12**.
14. Remove all traces of 70% ethanol from the pellet, using a small-volume pipet, and dry the pellet on the bench or using a vacuum dryer if available (*see Note 8*).
15. Resuspend the pellet in either DEPC-treated water or proprietary RNase-free water (*see Note 9*).
16. Quantitation of RNA can be performed using a spectrophotometer reading at 260 and 280 nm. Dilute 1 μL of the sample with 199 μL of DEPC-treated water. This is then placed in a 200- μL quartz cuvet and read at 260 nm and 280 nm. The concentration of RNA in the sample is the 260 nm reading $\times 40 \times$ the dilution factor (in this instance, 200-fold). The purity with regard to protein contamination is judged by the ratio of the 260-nm reading to the 280-nm reading. The reading 260:280 should be 1.8 to 2:0 (*see Note 10*).
17. Treat with DNase 1 according to manufacturer's instructions (*see Note 11*).

4. Notes

1. It is important to make up the buffer using DEPC-treated water and autoclave twice before use. As with all such buffers, it is inadvisable to keep them for extended periods or use them multiple times, thereby risking contamination, particularly by nucleases.
2. Sodium acetate, pH 5.2, is one of those awkward solutions to make. It requires pH adjustment with glacial acetic acid and its buffering capacity is such that it requires large volumes of acetic acid to adjust pH correctly. For 500 mL you will need around 150 to 200 mL of glacial acetic acid.

3. Important safety note: phenol is extremely dangerous and safety notes supplied with the product should be read. A supply of polyethylene glycol 300 should be kept close to the working area (for decontamination purposes in case of splashes to your person). Safety glasses, gloves, and laboratory coats should be worn when conducting this procedure. As with all hazardous chemicals you should read the material safety data sheet before commencing work.
4. This is the most important part of the procedure. Both tissue selection and grinding are crucial to the RNA yield recovered. Plants should, if possible, be taken to the bench for harvesting. If this is not possible, take both liquid nitrogen and tubes to the place of harvesting. The tubes are then prechilled using the nitrogen ready to accept the sample. For tissue harvesting, selection of fresh and actively growing leaf tissue is preferable, depending of course on the tissue specificity of that particular experiment; the tissue should be weighed before placing in the tube as quickly as possible. The tube should then be sealed and placed immediately in the liquid nitrogen. If possible it is best to have some liquid nitrogen in the tube when you drop the tissue in and then let the nitrogen evaporate while cooling the tissue before you seal the lid and drop the tube into the reservoir of liquid nitrogen.
5. The tissue must be ground to a fine powder. The tissue will have a very pale green appearance. If at any time it begins to go dark green this is an indication that the sample is thawing out and you risk potential nuclease activity. It is important to make sure all the tissue is ground up to powder; any residues in the form of small pieces of plant will become a problem later in the preparation when attempting to separate the organic and aqueous phases. Small pieces of leaf tend to be buoyant and float in the aqueous phase.
6. At this stage removing both the interphase and the aqueous phase will allow the recovery of RNA that is protein bound and retained in the interphase. The inclusion of the chloroform in the second extraction will release the bound RNA. An alternative way would be to remove the phenol, of course, and add the phenol:chloroform to the aqueous phase. For multiple tubes you need to balance the tubes before centrifuging using the extraction buffer.
7. It is helpful to mark the centrifuge tube at the top on the outside when placing the tubes in the centrifuge. In this way you know which side of the tube to expect the pellet to be situated. Usually it is easier to angle the tube with the pellet on the top and remove the liquid from below.
8. Drying the sample is much easier if all 70% ethanol is removed before commencing the drying. Tubes can be left on the bench, in a 37°C oven, or in a vacuum dryer to effectively dry off the ethanol. Any ethanol left in the tubes will inhibit downstream reactions, so it is important to ensure no ethanol remains before the final resuspension. This is often best achieved by smelling the top of the tube for ethanol traces. It is preferable not to overdry the sample, as this makes resuspension more difficult. For this reason it is better to regularly check for evidence of ethanol contamination, resuspending the RNA as soon as ethanol is no longer present.

9. At this point the quantity of water for resuspension is important. It is possible to resuspend RNA in a greater than 5- $\mu\text{g}/\mu\text{L}$ concentration, although it risks waste if you quantify 1 μL at such concentrations. You should expect between 500 μg and 2 mg of total RNA from this preparation using 1 g of tissue, so resuspension in 1 mL of water is likely to be appropriate.
10. Analysis of final purity and concentration can be measured using a spectrophotometer however new techniques and equipment are more effective if available. Quantitation is more accurate and consistent using a Turner Dynamics TD360 fluorimeter. Each assay is more expensive, however, because it requires a small amount of SYBR Green. For analysis of sample purity when using either RT-PCR or microarray applications downstream of the preparation it is advisable to analyse the sample using an Agilent bioanalyzer.
11. Treatment with DNase 1 is suitable for the removal of DNA contamination following the preparation. Following treatment the sample should be acid phenol:chloroform extracted and ethanol precipitated as in **steps 12–15**. Ambion DNA-free has the benefit that it can be used and then the supplied inactivation reagent negates the need for the further phenol:chloroform extraction that is required with DNase 1 treatment alone.

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RNA Extraction From Mammalian Tissues

Stuart N. Peirson and Jason N. Butler

Summary

Purification of intact RNA is the primary step of many molecular biology techniques, including Northern blotting, RNase protection, quantitative polymerase chain reaction, and microarray assays. RNA extraction is typically conducted using either a phenol–chloroform or a solid phase method. This article concentrates primarily on the former approach, which is highly versatile, and is easily adapted to different tissues ranging from whole organs down to submillimeter biopsy punches.

The major problem with RNA extraction is the ubiquitous nature of RNases, enzymes that rapidly degrade RNA. RNases may be difficult to eliminate, although with care and appropriate countermeasures, reproducible extraction of high-quality RNA from important biological samples should be attainable.

This article focuses on the isolation of RNA from the tissue collection step, homogenization all the way through to the quantification of the purified nucleic acid, providing guidelines for the prevention of the problems associated with RNase contamination.

Key Words: RNases; quantification; homogenization; microfluidics.

1. Introduction

Isolation of RNA is the initial step in a wide variety of molecular biological techniques, including Northern blotting, (ribonuclease) RNase protection assays, reverse transcription polymerase chain reaction, and more recently, microarrays and quantitative PCR (qPCR). A typical mammalian cell contains around 10^{-5} μg of RNA, of which ribosomal RNA (rRNA) comprises some 80 to 85%, smaller species such as transfer RNA make up around 10 to 15%, and mRNA contributes a mere 1 to 5% (*1*).

Extracting high-quality RNA is fraught with potential pitfalls owing to the labile nature of this nucleic acid, coupled with the ubiquitous presence and robust biochemical nature of RNases that will rapidly degrade RNA. This problem is compounded as RNases occur naturally in all cells and are released fol-

lowing cell lysis—an essential step of RNA extraction. The presence of additional hydroxyl groups on the 2' and 3' positions of the ribose residues in RNA result in a nucleic acid that is much more reactive than DNA, and is especially prone to hydrolysis of the diester bonds linking phosphate and ribose residues by RNases. Many RNases possess intrachain disulfide bonds, rendering them particularly resistant to denaturation. Both prolonged boiling and denaturing agents may be ineffective, as RNases are able to rapidly refold, and moreover, unlike DNases, RNases do not require divalent cations for activity, so chelating agents, such as EDTA, are ineffective ([1](#)). RNases are also present on the skin, providing another source of potential contamination, and may be widespread throughout the laboratory environment. There is no simple method of inactivating RNases, so constant vigilance is required.

A number of approaches to RNA extraction from mammalian cells are available, although the most commonly used approaches are either a variation of the Chomczynski and Sacchi phenol–chloroform technique ([2](#)) or are based on a solid phase approach using glass fiber filters. For purposes of this article the phenol–chloroform protocol is primarily considered. The main reason for this bias is the versatility of this approach for different tissues, particularly the single-step method, along with the widespread use of this approach with both qPCR and microarray techniques. Coupled with this, as most solid phase methods are available only in kit form and are supplied with protocols optimized for their use, any coverage of these protocols would be repetitious and, given variations in commercially available kits, potentially misleading (*see* **Note 1**).

We have found the phenol–chloroform technique to be practical for a wide variety of mammalian tissues, ranging in size from biopsy punches up to large tissue samples. Another advantage is that the scaling of the extraction can easily be modified to cope with differing tissue volumes.

Considerations of tissue composition are an important aspect of RNA extraction, and it is recommended that whenever a new tissue is examined, a pilot extraction be conducted to ensure that no unforeseen problems will compromise the RNA extraction from valuable experimental samples. Tissue-specific problems include the high-lipid content of myelinated central nervous system tissue, high RNase content of enzyme-rich tissues such as spleen and liver, and the presence of potential downstream inhibitors, such as melanin, which may inhibit DNA polymerase activity ([3](#)). The level of homogenization is also dependent on the tissue composition, with tough tissues such as muscle requiring considerably more homogenization than tissues such as liver. Finally, the actual RNA and DNA content of tissues also varies considerably, affecting RNA yields and the extent of DNA contamination. Successful RNA extraction is dependent on exercising care to minimize degradation by exogenous RNases, coupled with speed to rapidly inactivate endogenous RNases. The effects of

endogenous RNase degradation can also be limited by, where possible, avoiding freeze–thawing and maintaining samples at low temperature to minimize RNase activity.

RNA degradation may never present problems for many researchers, but may become apparent when quantitative approaches to gene expression are undertaken. For quantitative assays RNA quantity, as well as quality, is essential to ensure comparable RNA loading. If twice as much RNA is assayed then the expression level will be found to be twice as high; equally, if comparable concentrations of RNA are analyzed and one sample contains only 50% intact RNA, the expression level obtained will be proportionally lower.

RNA degradation can have devastating effects, and whole experimental tissue groups can be rendered unusable. The aim of this chapter is to try to provide advice on how to minimize RNA degradation and ensure that comparable RNA yields are obtained for subsequent analysis.

2. Materials

1. Laboratory gloves.
2. RNase-free microcentrifuge tubes.
3. RNase decontamination solution.
4. Diethylpyrocarbonate (DEPC)-treated or other nuclease-free water (*see Note 2*).
5. Experimental tissue samples.
6. Homogenization system.
7. Monophasic lysis reagent.
8. RNA precipitating solution: 1.2 M NaCl, 0.8 M disodium citrate; no pH adjustment required.
9. Spectrophotometer, set up for gel electrophoresis or lab-on-a-chip system.

3. Methods

An overview of RNA extraction is shown in **Fig. 1**, demonstrating the main steps in the progression from fresh tissue to RNA ready for experimentation. The methods described below broadly follow this format.

3.1. Tissue Collection

1. Sterilize and clean with an RNase decontamination solution any tools used for the dissection of the experimental tissue (*see Note 3*) to ensure that no exogenous RNases are introduced.
2. To preserve RNA integrity, snap-freeze the tissue very rapidly after dissection. Alternatively, store the tissue in an RNA-preservation medium such as Ambion's RNAlater. (For further information, *see* RNAlater protocol available from Ambion website: www.ambion.com/techlib/prot/bp_7020.pdf.) The latter is a high-salt buffer that precipitates proteins, and as long as tissue samples are small enough to enable penetration, this offers a simple method of preventing any further enzymatic reactions from occurring.

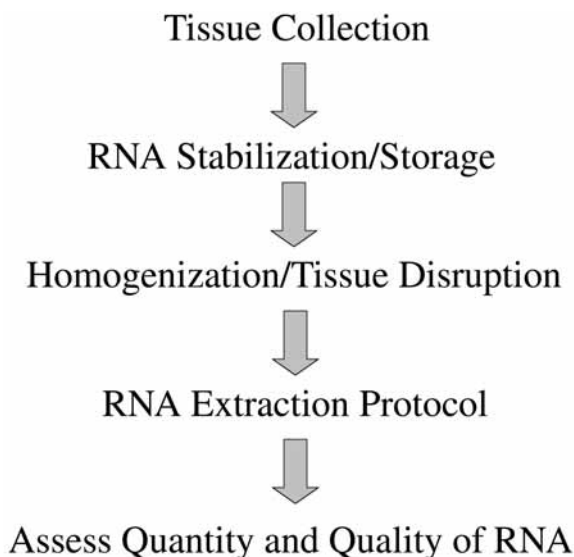


Fig. 1. Flowchart of RNA extraction, from tissue to purified total RNA.

3.1.1. Samples From Environment-Sensitive Tissues

RNAlater is of particular use with scenarios where gene expression may change rapidly, providing a stable medium for later tissue dissection (*see* www.ambion.com/techlib/prot/bp_7020.pdf).

1. Place the dissected tissue in RNAlater in darkness.
2. Store in a light-tight container at 4°C if dissection is to be carried out a few days later. For prolonged storage keep the sample at –20 or –80°C.

In circadian biology, assaying changes in gene expression following light pulsing provides an obvious example. **Figure 2** shows qPCR data from *c-fos* induction in the mouse retina collected in this manner (data courtesy of D. Elfant, unpublished observations). Despite storage at 4°C prior to dissection, the characteristic rapid light induction associated with this immediate-early gene is unaffected by dissection in the light (4).

3.1.2. Hypothalamic Tissue Punches

Extraction of small samples such as hypothalamic tissue punches are also feasible using a phenol–chloroform approach, although pooling of samples may be necessary to obtain enough RNA.

1. Rapidly remove the brain from anesthetized animals and snap-freeze in isopentane at –60°C on dried ice for 20 s.
2. Cut a 1-mm coronal section at the level of the optic chiasm.

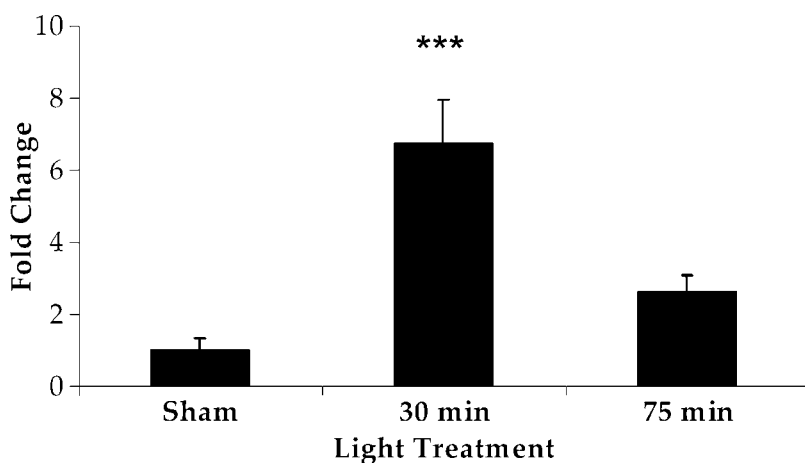


Fig. 2. Collection of light-sensitive tissues, such as retinae, may be facilitated by an RNA stabilization medium, such as RNAlater (Ambion). This enables tissue to be collected in darkness, and dissected at a later date in light, without the problems associated with freeze–thawing. This figure shows *c-fos* induction in the murine retina, where whole eyes were collected in darkness with an infrared viewer, and dissected out under light 48 h later.

3. Take a suprachiasmatic nuclei (SCN) punch using a flat-tipped 25-G needle (diameter approx 1 mm) and store the tissue on dried ice until phenol–chloroform extraction.

Figure 3 shows the SCN of the hypothalamus. **Figure 3A** shows staining of the SCN for *mPer2* mRNA as assayed by *in situ* hybridization (images courtesy of Marta Muñoz), illustrating the region of the SCN sampled. **Figure 3B** shows *mPer2* expression data from six pooled SCN assayed by quantitative real-time PCR using SYBR Green I, as described previously (5). Although this represents quite a gross approach to tissue sampling in comparison with more refined techniques such as laser capture microdissection, it illustrates that even small tissue samples may provide enough RNA for reliable quantitative analysis.

3.2. Laboratory Preparation

The laboratory environment offers many potential sources of RNase contamination, and a certain level of paranoia may be beneficial when extracting RNA on a routine basis.

1. Prior to RNA extraction, clean the benchtop and pipets with RNase decontamination solution.
2. Use only RNase-free tips and tubes. Also make sure that any distilled water used is nuclease-free (e.g., DEPC-treated; see **Note 2**).

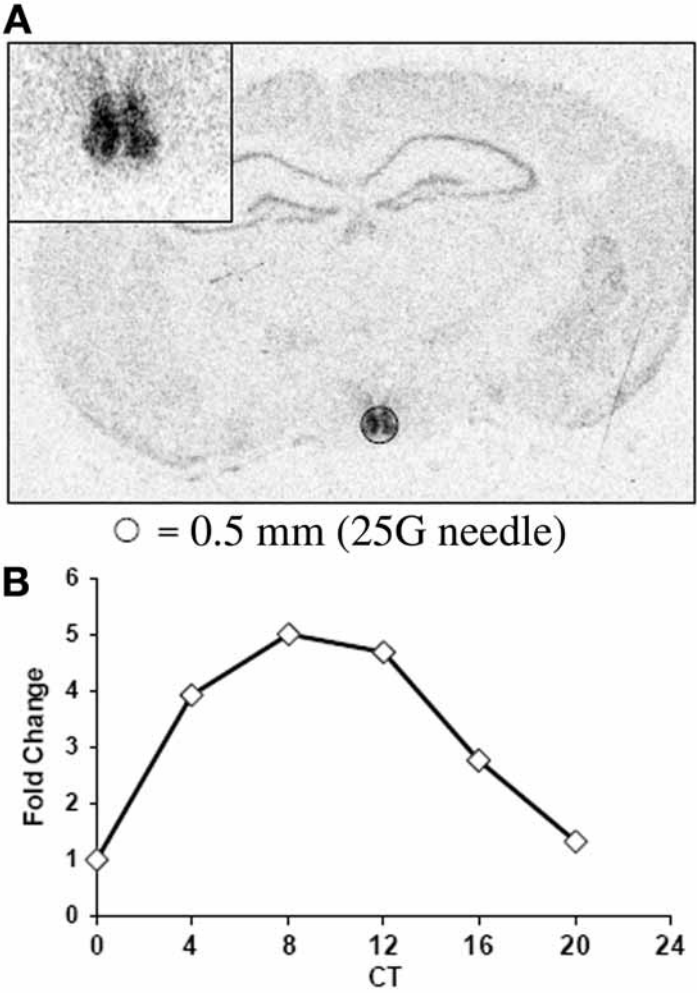


Fig. 3. RNA extraction using a phenol–chloroform methodology is possible even with small tissue samples. (A) Coronal section showing staining of *mPer2* by *in situ* hybridization, illustrating region of sampling and high *mPer2* expression. (B) Quantitative real-time polymerase chain reaction data from six pooled suprachiasmatic nuclei punches, illustrating clear circadian rhythm in *mPer2* expression. RNA was extracted in 0.5 mL monophasic lysis reagent.

- 3. Wear laboratory gloves at all times, and change them whenever the RNase-free working area is re-entered.
- 4. Exercise particular care with new lab users to ensure that they are aware of the ubiquitous nature of RNases, and are familiar with the necessary precautions.

3.3. Homogenization

A variety of approaches are available for tissue homogenization, and their use is largely dependent on the tissue used. Fragile tissue such as retina can easily be disrupted by repeated aspiration through a narrow-gage syringe needle. Use of a chilled pestle and mortar is the traditional approach to tissue homogenization, but this is time-consuming, particularly when processing a large number of samples. Use of a micropestle may speed up this process (again chilled), but may provide poor disruption of tougher tissues (e.g., muscle or connective tissue), and as with any pestle approach requires repeated cleaning of the pestle between samples to prevent crosscontamination.

More advanced mechanical homogenization is offered by powered pestle homogenizers or rotor–stator systems, but despite effective homogenization such systems still require cleaning between samples to prevent crosscontamination. Alternative options include the Qiasredder (Qiagen) or the FastPrep® (Q-biogene) systems, both of which involve disposable elements containing a homogenizing matrix, enabling an increased throughput. The Qiasredder consists of a spin-column format, requiring no additional instrumentation, with equivalent yields to rotor–stator homogenization (*see Note 1*). The FastPrep system requires an additional instrument, which applies vertical angular motion to up to 12 tubes simultaneously at a speed of up to 6 m/s. Based on a closed-tube system, the homogenization is extremely effective even on tough tissues, and results in very good RNA yields (*see Note 4*).

3.4. Phenol–Chloroform Extraction

As mentioned previously, the Chomczynski and Sacchi method is the basis of many commonly used RNA extraction protocols (2). A variation of this method is the single-step extraction described by Chomczynski (6) enabling a simultaneous extraction of RNA, DNA, and protein. This method uses a monophasic lysis reagent, containing phenol, guanidium isothiocyanate, and solubilizing agents such as glycerol (*see Note 5*). Such reagents are widely available, and are sold in different formulations under a number of tradenames, including TRIzol (Invitrogen), TriReagent (Sigma-Aldrich), ToTALLY RNA (Ambion), and FastPrep Pro (Q-biogene).

The single-step extraction method is widely used, and in our laboratory we have found this approach to be highly suited for qPCR applications of a wide range of mammalian tissues, including retina, retinal pigment epithelium, heart, liver, and brain.

3.4.1. General Extraction Protocol

1. Homogenize tissue in ice-cold lysis reagent to yield a cellular homogenate. The volume of lysis reagent required is dependent on the tissue size, but for samples

- of around a few millimeters in size, we have found 0.5 mL to generally provide good results (*see* **Note 6**). Centrifuge the homogenized tissue at 12,000g at 4°C for 10 min and transfer the resulting cleared cellular homogenate to a fresh tube.
2. Incubate the cleared cellular homogenate at room temperature for 5 min to allow dissociation of nucleoprotein complexes.
 3. Add 100 μ L of chloroform (or 200 μ L per milliliter of lysis reagent) to the sample, and mix well by vigorously shaking or vortexing for 15 s. Allow the samples to stand at room temperature for 5 min.
 4. Separate the mixture by centrifugation at 12,000g at 4°C for 15 min. The mixture will separate into three phases, consisting of a red organic phase containing protein, an organic interphase containing DNA, and a colorless upper aqueous phase containing RNA. Transfer the upper aqueous phase to a fresh tube. With a starting volume of 0.5 mL of lysis reagent, this aqueous phase may be expected to be around 100 to 200 μ L. Be careful when aspirating near the interphase, as DNA can be easily transferred along with RNA, potentially requiring additional DNase treatment.
 5. The RNA is precipitated from the aqueous phase by the addition of 250 μ L of isopropanol (or 0.5 mL per milliliter of lysis reagent). Mix well and let stand for 5 min at room temperature.
 6. Centrifuge at 12,000g at 4°C for 15 min to precipitate the RNA as a pellet. Remove the supernatant and wash the pellet with 75% ethanol. Remove as much of the ethanol as possible with a disposable pipet tip, and air-dry for 5 to 10 min. Do not allow the pellet to dry completely.
 7. Resuspend the pellet in 20 to 50 μ L of nuclease-free water (*see* **Note 7**). Store the resulting RNA at -70°C .

3.4.2. Possible Modifications

Modifications of the above protocol have been suggested to improve the RNA yields and prevent carryover of polysaccharides and proteoglycans that may prevent RNA resuspension as well as inhibit downstream reactions such as reverse transcription polymerase chain reaction and Northern blotting (7–9).

To prevent carryover of polysaccharides and proteoglycans a modified precipitation protocol may be used. Rather than precipitating in just isopropanol, by using equal volumes (125 μ L each if using volumes described above) of isopropanol and RNA precipitating solution, these problems may be circumvented (10).

3.4.3. Microvolume Extractions

Extraction of RNA from small tissue volumes poses an additional set of problems. Many suppliers provide kits optimized for small volume extractions, including RNAqueous-Micro (Ambion) and RNAeasy (Qiagen). However, as shown in **Fig. 3**, extraction from needle biopsy-sized samples is possible with the monophasic lysis reagent as described above.

3.5. Quantification of RNA

Quantification of the final product is an essential step for many applications to ensure similar RNA loading across subsequent assays. Four methods are commonly used for assessing the quantity and quality of the RNA extracted:

1. UV spectroscopy.
2. Fluorescent dyes (RiboGreen, Molecular Probes).
3. Denaturing gel electrophoresis.
4. Microfluidic approaches.

3.5.1. UV Spectroscopy

Nucleic acids strongly absorb light in the UV region of the spectrum, and UV spectroscopy has, as such, become the traditional approach to assessing the concentration and purity of RNA. The concentration of nucleic acid is calculated using the Beer-Lambert law, predicting a linear change in absorbance with concentration. An A_{260} reading of 1 is equivalent to 40 $\mu\text{g/mL}$ of single-stranded RNA.

RNA purity may be assessed by the A_{260}/A_{280} ratio, with high-purity RNA possessing a ratio between 1.8 and 2.0. It should be noted that the A_{260}/A_{280} ratio is also dependent on both pH and ionic strength. As the A_{280} decreases with pH (whereas the A_{260} is unaffected), this may lead to a lower A_{260}/A_{280} ratio when using acidic diluent. Using a buffered solution with a slightly alkaline pH (such as TE, pH 8.0) will therefore provide more accurate and reproducible readings (11).

Spectroscopy has several failings, which many users do not stop to consider. First, this technique offers no measure of RNA quality. Many users confuse purity with quality, but spectroscopy offers no indication of whether the RNA is intact or degraded. Second, spectroscopy is accurate over only a limited dynamic range of concentrations (1).

3.5.2. Fluorescent Dyes

Several fluorescent dyes are available that demonstrate a large increase in fluorescence when bound to nucleic acids. By use of a fluorometer, these dyes enable comparisons to be made against known concentrations of RNA. Information regarding these dyes and the necessary protocols are available from suppliers (see **Note 8**). Although they are insensitive to non-nucleic acid contaminants, again these approaches offer little in the way of quality assurance.

3.5.3. Gel Electrophoresis

The integrity of total RNA may be confirmed by gel electrophoresis, enabling the 18S and 28S rRNA species to be visualized. Although a nondenaturing gel may be used, most forms of RNA possess extensive secondary structure that

prevents them from migrating strictly according to size (in a similar way that supercoiling may affect plasmid migration size). Precise quantification of RNA is difficult with electrophoresis, although the ability to detect the integrity of rRNA offers a distinct advantage over spectroscopy.

A problem with denaturing gel electrophoresis is that it necessitates the use of formaldehyde, which is toxic by inhalation and is also classified as a carcinogen. Use of denaturing gels therefore requires a separate electrophoresis setup to limit formaldehyde exposure, as well as to prevent RNase contamination. For these reasons, coupled with increased availability of lab-on-a-chip systems, denaturing gel electrophoresis has become a less frequent approach to RNA analysis.

For simple detection of 18S and 28S bands (at around 1.9 and 5 kb in size, respectively), nondenaturing agarose gel electrophoresis may suffice. In this case a 1% agarose gel loaded with around 1 μ g of total RNA should enable detection of the two larger rRNA bands, indicative of intact RNA.

3.5.4. Lab-on-a-Chip

The availability of lab-on-a-chip systems, exemplified by the Agilent 2100 bioanalyzer, enables a new approach to RNA quantification, and most important, a single method of enabling concentration and RNA integrity to be measured simultaneously (*see Note 9*). The bioanalyzer utilizes a combination of microfluidics, capillary electrophoresis, and fluorescent dyes, recording the fluorescence of RNA as it migrates through the channels of the chip. The microfluidic approach offers a major advantage in that it requires much smaller volumes of RNA. The output is usually represented as an electropherogram, within which the 18S and 28S ribosomal RNA species are clearly visible as large peaks (**Fig. 4A**). By calculating the area under the curve of these major rRNA species, the integrity of the RNA may be calculated, with degraded RNA producing a shift toward lower molecular weights (**Fig. 4B**).

4. Notes

1. Additional information on solid phase kits may be found on the following supplier websites: Ambion (www.ambion.com); Amersham (www5.amershambiosciences.com); Invitrogen (www.invitrogen.com); Qiagen (www.qiagen.com); and Sigma-Aldrich (www.sigmaaldrich.com). In addition, the Ambion website offers an excellent library of RNA related resources: www.ambion.com/techlib/basics/rnaisol/index.html.
2. DEPC is a highly reactive alkylating agent, used to eliminate RNases from solutions, glassware, and plasticware. DEPC is a potent protein denaturant and a suspected carcinogen. As such, wear appropriate gloves and lab coat and use only in a fume hood. Although reverse-osmosis systems are typically free of RNases, microbial growth may result in contamination, particularly in centralized sys-

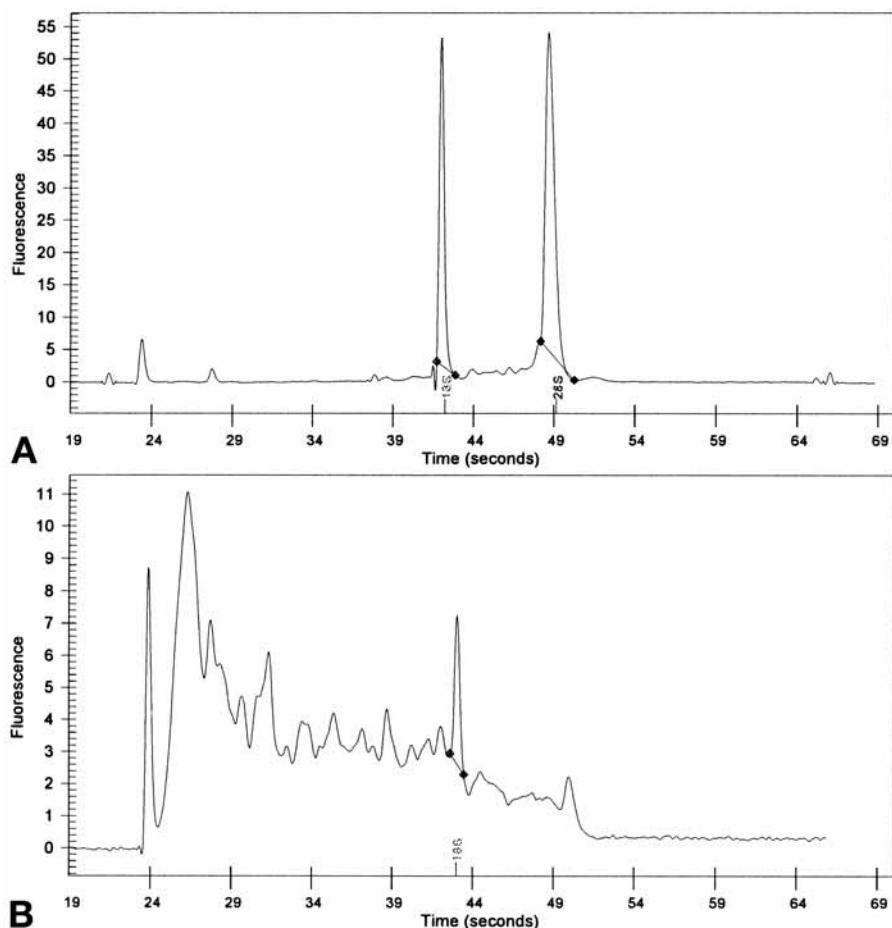


Fig. 4. Analysis of RNA quality using the Agilent Bioanalyzer 2100, in which 1 μ L of total RNA was run, enabling the 18S and 28 bands to be visualized as an electropherogram. The 28S/18S ratio should be around 2 for high-quality RNA, with a flat baseline (**A**). RNA degradation is visible as a decrease in the two ribosomal RNA peaks with a corresponding increase in smaller RNA degradation products, resulting in a noisier baseline (**B**).

tems. To treat H₂O, add 0.1% DEPC at 37°C for 1 h, or overnight at room temperature. The DEPC is then inactivated by autoclaving on a liquid cycle (*1*).

3. RNAZap® is available from Ambion, RNAZap™ Sigma-Aldrich, and most molecular biology suppliers.
4. For details, see the Q-biogene (www.qbiogene.com/fastprep/index.shtml) or Qiagen websites.

5. **Important safety note:** Guanidium salts are chaotropic agents that destroy the three-dimensional structure of proteins (hence inhibiting RNases) and are therefore dangerous. Phenol is toxic and extremely corrosive. Handle with care, and wear gloves, lab coat, and eye protection at all times.
6. With small tissue samples (<5 mm), 0.5 mL of lysis reagent works well. For larger samples, increase the volume of chloroform and isopropanol proportionally. For RNA-rich tissues such as liver, 1 mL of lysis reagent is recommended.
7. RNAsecure™ (Ambion) performs well for resuspension of RNA pellets. For further information, see the Ambion website: www.ambion.com/catalog/CatNum.php?7005.
8. See, for example, RiboGreen from Molecular Probes (www.probes.com/).
9. For details on the RNA LabChip® and 2100 Bioanalyzer, see the Agilent website: www.chem.agilent.com/Scripts/PCol.asp?IPage=50.

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Northern Analysis of Sense and Antisense *frequency* RNA in *Neurospora crassa*

Cas Kramer and Susan K. Crosthwaite

Summary

In Northern analysis the presence of specific RNA transcripts is detected and their quantity can be estimated. RNA is separated using denaturing agarose gel electrophoresis and is subsequently transferred and fixed to a solid support, such as a nitrocellulose filter. When labeled probes are hybridized to these immobilized RNA molecules, their presence can be visualized by autoradiography. Here we describe Northern hybridization using radioactively labeled riboprobes to show circadian expression of endogenous sense and antisense *frequency* RNA in the filamentous fungus *Neurospora crassa*.

Key Words: Circadian; filamentous fungus; bread mold; mycelium; RNA; sense; antisense; endogenous; TRIzol; blotting; nitrocellulose; riboprobe; RNA probe.

1. Introduction

In the late 1970s, early methods to detect specific messenger RNAs (1–3) became commonly known as Northern blotting, Northern hybridization or Northern analysis, comically referring to the method to detect DNA described by Sir Ed Southern in 1975 (4). Almost three decades later, despite emerging powerful polymerase chain reaction (PCR)-based and microarray techniques, Northern analysis is still a preferred method for the detection of specific RNA transcripts and the analysis of mRNA levels. Like DNA molecules, RNA transcripts will advance toward the anode in an electric field. In Northern analysis total RNA is first size-fractionated using agarose gel electrophoresis under continuous denaturing conditions, using formaldehyde, for instance, as the denaturant. After electrophoresis, RNA is transferred to a solid support by means of passive capillary blotting or active transfer, such as electroblotting. In early methods treated cellulose was used as the solid support (1,3), whereas nowadays a positively charged nylon membrane or a nitrocellulose filter are preferred. The RNA molecules can be irreversibly crosslinked to the filter or

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membrane by baking or by UV treatment. Immobilized RNA can be visualized after hybridization to a DNA probe or RNA probe (riboprobe). In vitro-transcribed RNA probes using bacteriophage RNA polymerases were first used in the mid-1980s (5,6) and are up to 10-fold more sensitive than random primed labeled DNA probes (7). In vitro transcription of riboprobes not only made Northern analysis a more powerful detection method, but it also opened the way for detailed investigation of strand-specific RNA transcripts. In eukaryotes, the presence of endogenous antisense RNA appears to be more widespread than originally anticipated (8,9) and the mode of action by which antisense RNA and duplex RNA can regulate gene expression is receiving increased attention (10–12). In the filamentous fungus *Neurospora crassa* RNA is transcribed from both DNA strands of its *frequency* (*frq*) gene locus (13,14). The presence of these endogenous, complementary, sense and antisense *frq* RNA transcripts can be revealed by reverse transcription PCR and Northern analysis (14). The latter method is described in this chapter.

2. Materials

1. RNase-free glassware and laboratory ware (see **Note 1**).
2. RNase-free MilliQ-quality water or diethypyrocarbonate (DEPC)-treated water (see **Note 2**).
3. Electrophoresis equipment (set of two identical tanks).
4. 10X MOPS: 440 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), 100 mM Na-acetate, 20 mM EDTA, adjusted to pH 7.0 with NaOH. Store in the dark at room temperature (see **Note 3**).
5. RNase-free formaldehyde and deionized formamide. **Caution:** These are toxic.
6. Ethidium bromide (EtBr) RNA-stock: 0.1 mg/mL EtBr in H₂O. **Caution:** This is mutagenic.
7. DNA loading buffer: 40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol.
8. RNA loading buffer (see also **items 4 and 7**): 450 μ L deionized formamide (37% stock solution), 45 μ L formaldehyde, 90 μ L DNA loading buffer, 150 μ L 10X MOPS. Make fresh.
9. 20X Saline sodium citrate (SSC): 3 M NaCl, 0.3 M Na-citrate, pH 7.0.
10. Glass trays (Pyrex oven dishes).
11. Nitrocellulose: Nitropure (Micron Separation, Westboro, MA).
12. Whatman 3MM paper
13. UV crosslinker
14. 50X Denhardt's: 1% Ficoll (type 400), 1% polyvinylpyrrolidone, 1% nuclease-free bovine serum albumin. Stir the solution for a few hours and filter (0.45 μ m). Store in aliquots at -20°C .
15. Northern hybridization buffer (see also **item 14**): 50% formamide, 10X Denhardt's, 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1% Na-pyrophosphate, 0.1 mg/mL denatured salmon or herring sperm DNA. Prepare buffer in fume hood. Store at -20°C .

16. Denhardt's hybridization buffer (*see also* **item 14**): 5X Denhardt's, 6X SSC, 0.5% sodium dodecyl sulfate (SDS), 0.1 mg/mL denatured salmon sperm DNA. Store at -20°C .
17. Hybridization oven with large hybridization bottles (~ 8 cm diameter).
18. MAXIsript[®] kit (Ambion).
19. Set of NTP solutions.
20. α - ^{32}P -labeled UTP (800 Ci/mmol).
21. Random Primed DNA Labeling Kit (Roche).
22. α - ^{32}P -labeled dCTP (3000 Ci/mmol).
23. Chromatography spin columns (e.g., Micro Bio-Spin P-30; BioRad).
24. Appropriate laboratory facilities for ^{32}P -labeled radioisotope work.

3. Methods

3.1. Gel Electrophoresis

Here we describe the preparation and running of two identical RNA formaldehyde gels that are subsequently blotted separately (*see* **Subheading 3.2.**) and then probed for sense and antisense *frq* RNA (*see* **Subheading 3.3.**). When a smaller number of samples is used, one agarose gel will suffice. RNA can then be transferred to one membrane, which can be cut in half prior to hybridization.

3.1.1. Preparing Agarose Gels

1. Ensure that gel electrophoresis equipment is RNase-free by soaking gel tanks, casting trays, and combs in 0.5 M NaOH (*see* **Note 1**). Rinse thoroughly with MilliQ H_2O .
2. Dry combs and trays with a clean tissue and prepare two casts.
3. To prepare two 1% agarose formaldehyde gels (*see* **Note 4**), add to an RNase-free flask 170 mL MilliQ H_2O , 20 mL 10X MOPS, and 2 g agarose. Bring the solution to a boil, make sure all agarose has dissolved, and let it cool down to 60°C in a water bath.
4. In a fume hood add 10 mL formaldehyde and 2 μL EtBr RNA stock to this solution (*see* **Note 4**). Gently swirl the solution to mix. **Caution:** Formaldehyde vapor is toxic.
5. In a fume hood divide the solution over two gels of identical thickness (*see* **Note 5**).
6. Let the gels set for at least 1 h before loading the RNA (*see also* **Note 6**).

3.1.2. Preparing RNA

The method by which total RNA is extracted from biological tissue can strongly influence the results of Northern hybridization. Here we describe the use of total RNA extracted using TRIzol[®] Reagent (Invitrogen) as described in Chapter 19, which in our hands gave rise to good, consistent hybridization results.

1. To obtain 40 μg total RNA per well, take up 80 μg RNA in a volume of 20 μL (adjust with H_2O). Add 52 μL RNA loading buffer and mix.
2. For “cold controls” (see **Subheading 3.3.1.3.**) take several samples containing between 1 and 100 pg control RNA/ μL . Take 10 μL of each sample and add 26 μL RNA loading buffer and mix.
3. Denature RNA and cold controls for 10 to 15 min at 65°C and place immediately on ice.
4. Collect all liquid to the bottom of the tubes by centrifugation and place tubes back on ice.

3.1.3. Loading and Running of Gels

1. Prior to loading the gel, plan a well-loading sequence on paper, using an identical sequence for the samples, but varying positions for empty wells and/or cold controls to make both gels unique (see **Note 7**).
2. Place the gels in separate electrophoresis gel tanks in 1X MOPS running buffer.
3. Load 35 μL of each chilled RNA sample onto each gel.
4. Carefully load sets of cold controls on each gel, avoiding overspills at all costs.
5. Load an aliquot of RNA ladder (e.g., 0.24–9.5 kb; Invitrogen) onto gel (optional).
6. Run at room temperature at 30 V for 16 h (see **Note 8**).
7. After electrophoresis, do not take a picture of the gels (see **Note 4**), but continue with the blotting process.

3.2. Blotting

The principle of transferring a nucleic acid from a gel to a membrane, commonly known as blotting, is identical whether DNA or RNA is used. Here we describe a standard method for upward capillary blotting (15) of RNA to a nitrocellulose filter.

3.2.1. Preparation

1. During the blotting process the RNA is susceptible to degradation until the RNA has been fixed to the nitrocellulose (in **Subheading 3.2.3., step 5**). Therefore, make sure glass trays, glass plates, supports, and a glass test tube are RNase-free (see also **Note 1**). Wear gloves.
2. After electrophoresis, transfer the gels to a glass tray and soak in 10X SSC with gentle shaking for at least 1 h, replacing the 10X SSC buffer twice. Increasing the time for this step to several hours seems to improve the overall quality of the blot.

While the gels are soaking, do the groundwork for setting up two Northern blots (see **Subheading 3.2.2.**).

3. Cut 16 pieces of Whatman paper of gel size (100 $\times$ 125 mm).
4. Cut two pieces of Nitropure membrane (Micron Separation) of 105 $\times$ 130 mm, i.e., slightly larger than the gel.
5. Cut four pieces of Whatman paper the size of the support tray.

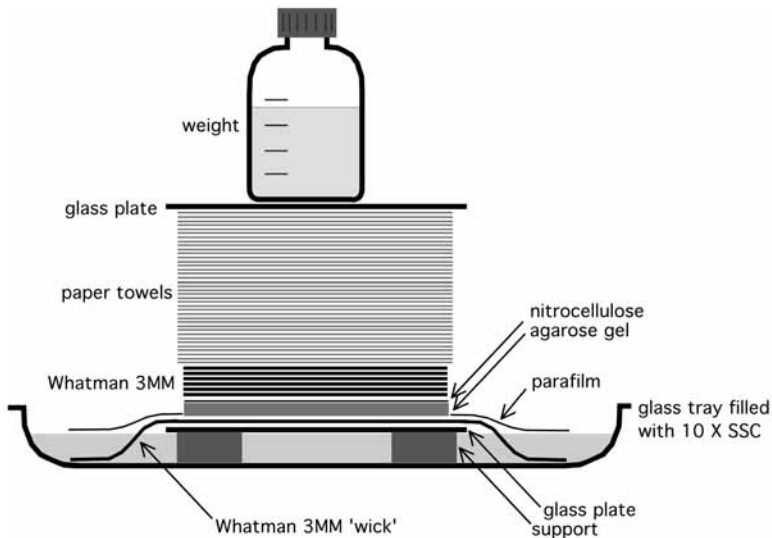


Fig. 1. Upward capillary transfer of RNA. The 10X SSC blotting buffer is drawn through the paper wick, the RNA agarose gel, and the nitrocellulose membrane into the stack of filter paper and paper towels, transferring the RNA transcripts out of the gel onto the nitrocellulose membrane.

6. Cut or fold a large amount of paper towels to gel size, enough to make two stacks of approx 10 cm.

3.2.2. Setting Up a Northern Blot (see [Fig. 1](#))

Steps 1–9 describe setting up one Northern blot. This will have to be done for both gels as prepared and described in **Subheading 3.1**.

1. Pour approx 0.5 L of 10X SSC in a glass tray and place a glass plate on supports (see **Note 9**) in the middle of the tray.
2. Pre-wet the nitrocellulose membrane in MilliQ H₂O for 5 min and then in 2X SSC for another 5 min.
3. Wet the two sheets of tray size-Whatman paper throughout in 10X SSC and drape over the support in such a way that the Whatman filter paper is overhanging like a wick into the buffer on all four sides. Remove any air bubbles between the filter paper and the support by rolling a glass 150-mm test tube (or an RNase-free solid glass rod or glass pipet) over the wet paper.
4. Using the test tube pour approx 5 to 10 mL of 10X SSC to flood the Whatman paper and then place the gel on the filter paper on the support, taking care not to trap any air bubbles under the gel. Gently roll and press the gel with the test tube to ensure that no air bubbles are trapped.

5. Flood the gel with a little 10X SSC and carefully place the pre-wetted nitrocellulose onto the gel, taking care not to trap any air bubbles. Try to put the membrane straight down in one go. Gently roll the test tube over the membrane to ensure good contact and to remove all air bubbles.
6. Wet thoroughly a stack of four pieces of gel-size Whatman paper in 10X SSC. Flood the nitrocellulose with a little 10X SSC and place the sheets of wet filter paper on top of the membrane. Again, roll the test tube over the paper to remove any air bubbles.
7. Add another approx 0.5 L of 10X SSC to the glass tray to raise the level to just under the top of the support.
8. Place strips of Parafilm tightly against the gel on all four sides on the overhanging tray size-Whatman paper. This step prevents filter paper and tissues short-circuiting the upward capillary flow through the gel and membrane.
9. Place another four pieces of dry gel-size Whatman paper on top of the wet filter paper, followed by a stack of paper towels, a glass plate, and a bottle filled with 300 to 400 mL of liquid as a weight.

3.2.3. RNA Transfer and Immobilization

1. Leave blot setup overnight as described above, allowing 12 to 20 h for RNA to be completely transferred (*see Note 10*). Make sure the stack of paper towels will not tilt, which will ensure even transfer across the membrane.
2. Dismantle, date, and label each blot with a unique identifier, and mark with a pencil the position of the wells. Also pencil-mark the side of the membrane that has faced the gel (RNA-side).
3. Wet the membrane in 3X SSC and briefly and gently “wash” the membrane—i.e., carefully remove any pieces of agarose that are stuck to the membrane by means of stroking with a gloved finger.
4. Place the membrane on a clean piece of Whatman paper and air-dry at room temperature.
5. UV-crosslink the RNA to the dry membrane (RNA-side up) with a dose of 120 mJ.
6. Take a picture of each blot (RNA-side down) using a UV transilluminator to check RNA integrity and blotting efficiency. Exposure of blots to UV light should be minimized.

3.3. Northern Hybridization

3.3.1. Preparing Sense and Antisense frq Riboprobes and Cold Controls

Here we describe the generation of RNA transcripts *in vitro* transcribed using a PCR amplification product as template (*see also Note 11*). The generation of the PCR template is described in **Subheading 3.3.1.1**. Riboprobes are produced when radioactively labeled nucleotides are incorporated during the transcription reaction (described in **Subheading 3.3.1.2**). When no radioisotopes are present in the transcription reaction the RNA transcripts produced

can be used as cold controls (described in **Subheading 3.3.1.3.**). The in vitro transcription protocol used is essentially as described in the MAXIscript® kit instruction manual (Ambion) (7).

3.3.1.1. PCR TEMPLATE

1. Perform a PCR reaction under standard conditions (16) using M13 forward and reverse primers and 25 ng pKAJ104 plasmid DNA. The vector pKAJ104 is a pBluescript-derivative that contains a 2.2kb *EcoRI* fragment from within the *N. crassa* FRQ open reading reading frame (17,18).
2. Run a small amount of PCR product on a standard agarose gel (15) to verify that a single amplification product of approx 2.5 kb has been produced. This PCR product (PCR104) contains the *N. crassa frq* sequences flanked by T3 and T7 promoter sequences.
3. Clean up the remainder of the PCR product through a QIAquick PCR spin column (Qiagen).
4. Estimate the DNA concentration by spectrophotometric analysis or agarose gel electrophoresis (15).

3.3.1.2. RIBOPROBES

In vitro transcription:

1. Thaw MAXIscript reagents on ice and briefly vortex the 10X transcription buffer.
2. Add at room temperature the following reagents in the given order to two 1.5-mL Eppendorf tubes—one tube for the reaction to produce sense RNA transcripts (using T3 RNA polymerase), the other to produce antisense RNA transcripts (using T7 RNA polymerase). Take appropriate precautions when working with ³²P-labeled radioisotopes. Mix the solutions by slowly pipetting up and down when adding the last two reagents.

PCR104 (~0.5 µg)	in 8.5 µL
10X transcription buffer	2 µL
10 mM ATP / CTP / GTP	1 µL each
0.2 mM “cold” UTP	2 µL (see Note 12)
[α- ³² P]UTP (800 Ci/mmol)	2.5 µL
T3 or T7 RNA polymerase	2 µL

3. Incubate reaction for 1 to 1.5 h at 37°C.
4. Add 1 µL of RNase-free DNase I (2 U/µL), mix with the pipet tip, and incubate for a further 15 min at 37°C, to degrade the template DNA.

Removal of free nucleotides:

5. Resuspend the contents of a Micro Bio-Spin P-30 chromatography column (Bio-Rad). Remove air bubbles by flicking the tube. Snap off the tip of the column and place column in a 2.0-mL Eppendorf tube. Take off the top cap and centrifuge at 1000g for 2 min.
6. Place column in a fresh 1.5-mL Eppendorf tube.

7. Add 50 μL of RNase-free H_2O to the 21 μL reaction volume from **step 4** and carefully pipet the sample onto the center of the column.
8. Centrifuge at 1000g for 4 min.
9. The solution collected in the tube is the cleaned RNA probe, ready for use in Northern hybridization (*see Subheading 3.3.3.*). Transfer 1 μL of riboprobe into a fresh tube and count the incorporation in a scintillation counter (cpm/ μL) by means of Cerenkov counting.

3.3.1.3. COLD CONTROLS

To produce sense and antisense *frq* cold controls, in vitro transcription reactions are performed, essentially as described for riboprobes (*see Subheading 3.3.1.2.*), using nonlabeled NTPs only.

1. Add at room temperature the following reagents:

PCR104 (~2 μg)	in 30 μL
10X transcription buffer	5 μL
10 mM ATP/CTP/GTP/UTP	2.5 μL each
T3 or T7 RNA polymerase	5 μL
2. Incubate reaction for 1 h at 37°C.
3. Add 2 μL of RNase-free DNase I (2 U/ μL), mix with the pipet tip, and incubate for a further 15 min at 37°C.
4. To remove free nucleotides clean up through a QIAquick PCR spin column (Qiagen) or an RNeasy spin column (Qiagen).
5. Estimate the RNA concentration by spectrophotometric analysis or gel electrophoresis.
6. Using RNase-free H_2O , make serial dilutions to obtain RNA concentrations ranging from 1 to 100 pg/ μL . Store at -80°C.

3.3.2. Preparing 18S RNA Probe

To be able to normalize the *frq*-specific hybridization signals to an internal standard, a DNA probe from the *N. crassa* 18S ribosomal RNA gene is prepared, using random primed labeling (Random Primed DNA Labeling Kit; Roche), essentially as described in **ref. 19**.

1. Take up 50 ng PCR product (containing a fragment of the *N. crassa* 18SrRNA gene) in 11.5 μL in a screw-top Eppendorf tube.
2. Boil for 10 min to denature the template DNA, and place immediately on ice.
3. Briefly spin down to collect all liquid to the bottom of the tube and replace on ice.
4. Add the following reagents at room temperature:

Reaction mix (containing hexanucleotides mix)	2 μL
10 mM dATP/dGTP/dTTP	1 μL each
[α - ^{32}P]-dCTP (3000 Ci/mmol)	2.5 μL
Klenow fragment DNA polymerase I	1 μL
5. Incubate the reaction for 3 to 4 h at 37°C.
6. Stop the labeling reaction by adding 2 μL 0.2 M EDTA and incubating for 10 min at 65°C.

7. Increase the volume by adding 50 μL H_2O and remove nonincorporated isotope by cleaning the probe using a Micro Bio-Spin P-30 chromatography column (BioRad) as described under **Subheading 3.3.1.2**.
8. Transfer 1 μL of cleaned-up DNA probe into a tube and count the incorporation of label in a scintillation counter (cpm/ μL). The remainder of the DNA probe is ready for use in Northern hybridization (*see* **Subheading 3.3.3.**) or can be stored at -20°C for up to 2 wk.

3.3.3. Prehybridization, Hybridization, and Washes

3.3.3.1. USING *frq* SENSE AND ANTISENSE RIBOPROBES (*SEE* **NOTE 13**)

1. Preheat hybridization oven and Northern hybridization buffer to 65°C .
2. Prehybridize two blots separately in two large hybridization bottles for 1 to 2 h at 65°C in 10 mL Northern hybridization buffer.
3. For each riboprobe calculate the amount of buffer needed to obtain a probe concentration of 2×10^6 cpm per mL hybridization buffer (*see* **NOTE 14**).
4. Accordingly, adjust volumes in each hybridization bottle to between 5 and 15 mL.
5. Add approx 900 μL Northern hybridization buffer (65°C) to approx 70 μL of each riboprobe (as obtained in **Subheading 3.3.1.2., step 9**).
6. Carefully pipet riboprobes into the buffer within the hybridization bottles (*see* **NOTE 15**) and gently swirl to mix.
7. Hybridize for 16 to 20 h at 65°C .
8. After hybridization remove probe-containing buffer from blot (*see* **NOTE 16**).
9. Firstly, rinse blots in 2X SSC, 0.1% SDS to remove excess probe, then wash five times for 20 to 30 min in approx 100 mL liquid each, as follows:
 - a. Two washes using 2X SSC, 0.1% SDS at room temperature.
 - b. Three washes using 0.1X SSC, 0.1% SDS at 65°C .
10. Take blots out of the hybridization bottles and wrap in cling film while still damp (*see* **NOTE 17**).
11. Expose RNA side of blots to a PhosphoImager screen (Bio-Rad) or X-ray film for 2 h to overnight.

3.3.3.2. USING 18S RIBOSOMAL RNA PROBE

After Northern hybridization signals (using sense and antisense *frq* riboprobes) have been scanned, the blots can be reused immediately for hybridization with the 18S ribosomal RNA probe (*see* **NOTE 18**). Alternatively, Northern blots can be stored in the dark at room temperature for many months prior to ribosomal hybridization. Northern hybridization using a randomly labeled DNA probe is essentially as described above for hybridization with riboprobes (*see* **Subheading 3.3.3.1.**). Minor differences are stated below.

1. Use Denhardt's hybridization buffer for prehybridization and hybridization.
2. Boil the probe in a small volume of hybridization buffer (e.g., 500 μL) prior to use, to make probe single-stranded.

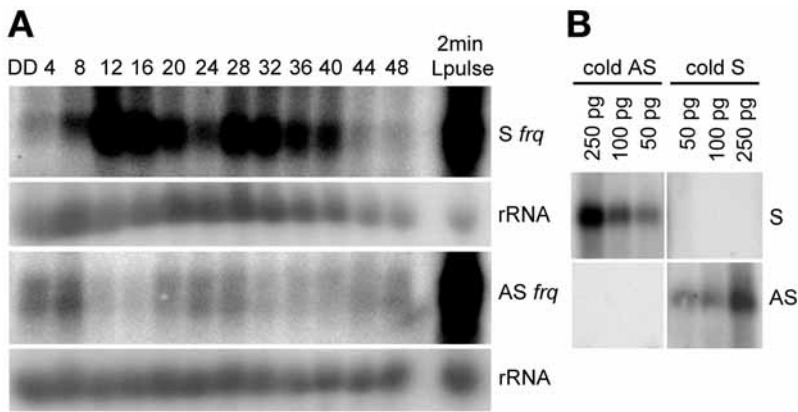


Fig 2. Sense and antisense *frq* Northern hybridization signals. Riboprobes in vitro transcribed (using T7/T3) from a PCR product of the 2.2 kb *Eco*RI fragment from within the FRQ ORF were used to assay the expression of *Neurospora crassa* sense and antisense *frq* transcripts, respectively. First published in [ref. 14](#). (A) Example of circadian expression of sense *frq* mRNA transcripts (*S frq*) and antisense *frq* transcripts (*AS frq*) in constant darkness (DD) and after a light pulse. RNA used was extracted from mycelial samples harvested in the dark every 4 h for 48 h and 30 min after a 2-min exposure to saturating light. Also refer to Chapter 19. Levels of ribosomal RNA (rRNA), visualized after hybridization with a *N. crassa* 18S rRNA probe, are used for normalization. (B) Example of cold controls routinely included on Northern gels to verify the integrity and sensitivity of the sense and antisense riboprobes.

3. Prehybridization, hybridization, and final washes are performed at 60°C.
4. Good results can be obtained using a probe concentration between 0.2×10^6 and 2×10^6 cpm per mL hybridization buffer (*see* **Note 18**).
5. Hybridization buffer (containing probe) can be reused up to three or four times without significant loss of signal intensities. There is no need to boil the probe before use in this case. If preferred, used probe can be stored at -20°C for reuse within 1 to 2 wk.

3.3.4. Hybridization Signal

The hybridization signals can be visualized by developing the X-ray film or by scanning the PhosphoImaging screen using the software package QuantityOne (BioRad). Examples of Northern hybridization results can be found in [Fig. 2](#). Using QuantityOne the intensity of individual hybridization signals can be quantified. As the sense/antisense *frq* signal and the 18S ribosomal RNA signal are both coming from the same RNA sample on the same blot, the levels of ribosomal RNA can be used to normalize the *N. crassa frq*-specific signals.

4. Notes

1. Always use RNase-free glass- and plasticware. Wear gloves at all times. Single-use plasticware, such as pipets and Falcon tubes, are recommended. Plastics for multiple use should be soaked in 0.5 M NaOH for at least 30 to 60 min (no adverse effects are observed when soaking for longer periods, such as hours or overnight) and rinsed thoroughly with RNase-free MilliQ H₂O before use. Bake glassware for at least 4 h at 200°C. There is no need to bake glassware after each use; just rinse with RNase-free MilliQ H₂O (*see also* **Note 2**). If time constraints demand, glass- and plasticware can also be sprayed and wiped with RNase-ZAP (Ambion) and rinsed with MilliQ H₂O.
2. In our hands there was no need to use DEPC-treated water (**15**). Under different lab conditions the use of DEPC may be necessary. RNA was dissolved in RNase-free H₂O (Qiagen) or MilliQ H₂O, which had been autoclaved and stored in Duran bottles that were used solely for that purpose. For all buffers, MilliQ H₂O was used and autoclaved as stated. There is no need to autoclave the gel running buffer, blotting buffers or buffers used for post-hybridization washes.
3. There is no need to autoclave 10X MOPS, but, if preferred, the buffer may be autoclaved. Autoclaving and exposure to light yellows the buffer. Straw-colored buffer can be used without any problems. When yellow color darkens, do not use. There is no need to autoclave 1X MOPS gel running buffer.
4. To optimize the transfer of RNA during the blotting process, use low-percentage agarose gels (<1.3%), a diluted EtBr stock (as EtBr can adversely affect the blotting efficiency of RNA), and, if possible, avoid UV exposure of RNA prior to blotting (i.e., do not take gel pictures, as RNA may crosslink to the gel, reducing the transfer efficiency).
5. It is recommended to prepare two gels of identical thickness to allow the subsequent, separate blotting process (*see* **Subheading 3.2.**) to be as similar as possible. This can be easily established by using clean 50-mL Falcon tubes or a baked 100-mL measuring cylinder to aliquot 98 mL (allowing for evaporation and inaccuracies) into each gel cast.
6. If preferred, gels can be transferred into the 1X MOPS running buffer in the electrophoresis gel tank within the hour. Take care when handling, as formaldehyde agarose gels are more fragile than same percentage TBE/TAE agarose gels.
7. To prevent confusion at later stages make the gels different by varying the positions for empty wells and/or cold controls. Blots from the gels will be probed with sense and antisense riboprobes from the same gene, so keep the sequence of the samples identical for easier and more visual-friendly comparisons. Make sure the highest concentration of cold control is always loaded away from the samples, and never loaded into a well adjacent to sample RNA. If possible, leave one or several empty wells between samples and cold controls.
8. In our experience overnight runs work best. Do not run faster than 40 V. To obtain reproducible results without taking a picture of the Northern gels (*see* **Note 4**) we successfully used “480–500 Volthours” as rule of thumb—e.g., 16 h at 30 V equals 12 h at 40 V and 24 h at 20 V.

9. As support either the gel casting tray turned upside down or a glass plate supported on four caps from 50-mL Falcon tubes can be used.
10. The time needed for complete transfer of RNA depends on the size of the transcript of interest and on the percentage of agarose used. Shorter periods are mentioned (4.5 to 6 h [15]), but overnight transfer is convenient.
11. It is also possible to use plasmid DNA as template for the in vitro transcription reaction. To prevent run-on transcription of vector, DNA should be cut with an appropriate restriction enzyme (3' overhangs should be avoided [15]). However, as one can never be sure of a full 100% digestion, it is safer to use a PCR template, in order to obtain "pure" sense and antisense riboprobes.
12. The in vitro transcription reaction can be optimized by adding "cold" UTP to the reaction mix, resulting in more full-length transcripts. Although not always essential for a good hybridization result, the use of a full-length probe generally results in less background (7). The amount of cold UTP to be added should be tested empirically. This can be achieved by running 1 μ L diluted riboprobe ($\sim 2 \times 10^4$ cpm), from samples that contained increasing amount of cold UTP, on a 4% denaturing polyacrylamide gel (15). In our hands, a final concentration of 10 to 20 μ M cold UTP gave satisfying results for the sense and antisense *frq* riboprobes described here.
13. At the hybridization stage it is critical to conscientiously note which blot will be exposed to which riboprobe (sense or antisense). Write it down. Remember that each riboprobe may give a similar (if not identical) hybridization result.
14. Lower probe concentrations, as low as 1×10^6 cpm per mL hybridization buffer, may give satisfying results. However, as antisense *frq* levels are very low in the dark (14), this may not be optimal.
15. Do not pipet the approx 1 mL of riboprobe directly onto the blot, as exposure to very high concentration of labeled probe may cause unequal hybridization results.
16. Reuse of riboprobes is possible once or twice within 3 to 4 d. The riboprobe can be used directly on another prehybridized blot or can be stored at -20°C . Incorporated radioactive nucleotides will degrade the RNA probe, resulting in reduced sensitivity/poor results after several days.
17. Double-wrapping of blots is recommended to prolong lifespan.
18. As ribosomal messages are abundant the hybridization signal obtained with a ribosomal probe is strong. Therefore, there is no need for blot stripping or waiting for the decay of *frq* hybridization background signal at the position of the ribosomal band. Also, lower probe concentrations, as low as 2×10^5 cpm per mL hybridization buffer, give rise to reliable and good quantifiable Northern hybridization signals.

Acknowledgments

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RNase Protection Assay

Patrick Emery

Summary

The RNase protection assay is a standard approach to determine mRNA levels of a gene of interest in different tissues, developmental stages, or times of the day. Splicing or promoter variants can be studied with specific probes. It is widely used in chronobiology to study the temporal profile of expression of circadian genes and the effects of genetic manipulation on these oscillations. Methods to generate the riboprobes and to perform the RNase protection assay itself are described in this chapter.

Key Words: Circadian mRNA oscillations; RNase protection; alternative splicing; alternative promoters.

1. Introduction

The RNase protection assay is a highly sensitive and reliable method for measuring RNA levels (**Fig. 1**), commonly used to measure circadian fluctuations of mRNA levels (see, for example, **refs. 1–4**). With exon-specific probes, it is also possible to study alternative splicings or the use of different promoters. Usually, the antisense riboprobes are about 100 to 200 nucleotides long. They can be placed at the junction between an intron and an exon, so that any trace of contaminating genomic DNA will result in a protected product of longer length than that obtained with the mRNA of interest. The riboprobes are radiolabeled during in vitro synthesis and hybridized to mRNA samples (*see Fig. 1*). The probe/mRNA hybrid is protected from RNase degradation, whereas the free probe is digested. A denaturing gel is then used to measure the amount of probe that was protected. It is the direct reflection of the amount of the RNA of interest that was present in the sample.

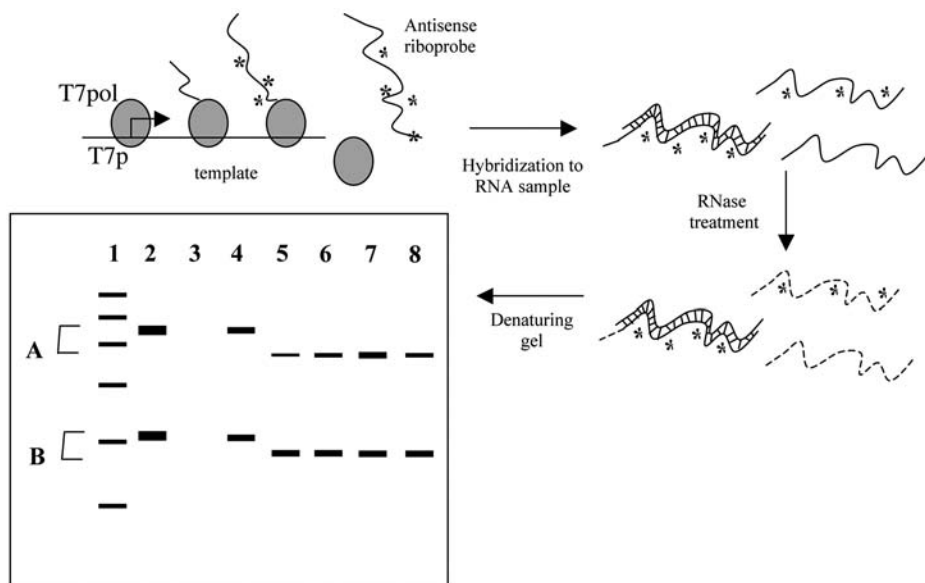


Fig. 1. Major steps of the RNase protection assay. Synthesis of the antisense riboprobe with an RNA polymerase (here T7 polymerase, T7pol), using as a template a linearized plasmid with a T7 promoter (T7p). Hybridization between the riboprobe and the RNA samples. *RNase digestion*: double-stranded RNAs are resistant to digestion. Denaturing polyacrylamide electrophoresis: Lane 1: radiolabeled molecular marker. Lane 2: untreated RNA probes. Lane 3: RNA probes without RNA samples, RNase treated (negative control; see **Note 1**). Lane 4: RNA probes hybridized to the template plasmids and treated with RNases (positive control; see **Note 1**). Lanes 5–8: RNA probes hybridized to RNA samples collected at different zeitgeber times, hybridized to two probes (A and B; see **Note 2**) and RNase-treated. (A) Signals obtained with a probe directed against a gene under circadian regulation. (B) Signals obtained with a probe directed against a gene expressed at constant levels, which can be used to normalize the A signal of each sample (see **Note 3**). When hybridized to the RNA, the 5'-end of the probes are cleaved by the RNases, because this region contains plasmid-derived sequences that will not hybridize to the targeted RNA. Thus, they will run faster on the gel than the full-length probes.

2. Materials

Solutions must be prepared RNase-free. Diethylpyrocarbonate-treated water should be used, and solutions autoclaved.

1. Geiger counter.
2. Diethylpyrocarbonate-treated water.
3. 5X Transcription buffer (supplied with the RNA polymerase).

4. 0.2 *M* Dithiotreitol (DTT).
5. Cold ribonucleotide mix: 4 *mM* GTP, 4 *mM* CTP, 4 *mM* ATP, 0.2 *mM* UTP.
6. Radiolabeled $\alpha^{32}\text{P}$ -dUTP (10 mCi/mL).
7. RNase inhibitor.
8. Linear template for the antisense riboprobe under the control of a T7, T3, or SP6 RNA polymerase promoter (1 $\mu\text{g}/\mu\text{L}$ if the template is a plasmid such as pBluescript).
9. T7, T3, or SP6 polymerase.
10. DNase I RNase-free.
11. TE buffer: 10 *mM* Tris-HCl, 1 *mM* EDTA, pH 8.0.
12. Yeast transfer RNA (tRNA; 20 mg/mL).
13. Phenol pH 7.5/chloroform (1:1) solution.
14. 4 *M* Ammonium acetate.
15. Ethanol.
16. Loading buffer: 90% formamide, 1 *mM* EDTA, pH 8.0, tinted with xylene cyanol and bromophenol blue.
17. Polyacrylamide gel electrophoresis material: glass plates, spacer and combs, power supply, 40% acrylamide solution (38:2 acryl:bisacrylamide ratio), 10% APS solution, TEMED, urea, 10X TBE buffer (108 g/L Tris-base, 55 g/L boric acid, 20 *mM* EDTA).
18. Radiolabeled RNA or DNA ladder.
19. Safe-Lock microcentrifuge tubes.
20. Elution buffer: TE buffer with 0.5 *M* ammonium acetate, 0.1% sodium dodecyl sulfate (SDS), 200 $\mu\text{g}/\text{mL}$ tRNA.
21. Shaker for microcentrifuge tubes.
22. 4 *M* NaCl.
23. Formamide.
24. 5X Hybridization buffer: 200 *mM* PIPES, pH 6.4, 2 *M* NaCl, 5 *mM* EDTA, pH 8.0.
25. Scintillation liquid and counter.
26. Yeast total RNA (20 mg/mL).
27. RNase buffer: 10 *mM* Tris-HCl, pH 7.5, 0.3 *M* NaCl, 5 *mM* EDTA.
28. RNase A (10 mg/mL).
29. RNase T1 (100,000 U/mL, Roche).
30. 10% SDS solution.
31. Proteinase K (20 mg/mL).

3. Methods

Great care must be taken to avoid RNase contamination. As radioactive material is used, protective clothing, gloves, and eyeglasses should be worn at all time. Plexiglas shielding should be used, and any radioactive material properly disposed of. A Geiger counter should be used to verify that no radioactivity has been spilled.

3.1. Preparation of Riboprobe (see also Note 4)

1. Mix in the following order:
 - a. 2.5 μL water.
 - b. 4 μL 5X Transcription buffer.
 - c. 1 μL of 0.2 M DTT.
 - d. 2 μL of cold ribonucleotide mix.
 - e. 7.5 μL of $\alpha^{32}\text{P}$ -dUTP (10 mCi/mL).
 - f. 1 μL RNase inhibitor (20 U/ μL).
 - g. 1 μL of 1 $\mu\text{g}/\mu\text{L}$ DNA template (if the template is a plasmid).
 - h. 1 μL T3, T7, or SP6 polymerase (20 U/ μL).
2. Incubate 30 min at 37°C.
3. Add 1.5 μL DNase I RNase-free (10 U/ μL).
4. Incubate 15 min at 37°C.
5. Add 30 μL TE, 2 μL yeast tRNA (20 mg/mL).
6. Extract twice with 1 vol phenol/chloroform.
7. Add 50 μL of 4 M ammonium acetate and 300 μL ethanol.
8. Precipitate for 2 h at -20°C, or 30 min on dry ice.
9. Microcentrifuge at 12,000g for 15 min. Remove the supernatant, which contains most of the free radiolabeled nucleotides
10. Rinse with 75% ethanol and centrifuge again for 5 min.
11. Discard the supernatant and speed-vac or air-dry the pellet.
12. Resuspend in 20 μL of loading buffer.
13. Run on a prewarmed 6% polyacrylamide gel containing 8 M urea and 1X TBE.
14. Remove one of the glass plates, wrap the gel in plastic wrap, and briefly autoradiograph it (usually 30 s are sufficient).
15. Excise the band corresponding to the riboprobe (see Note 5) and autoradiograph the gel again to verify that the band was properly excised.
16. Place the gel fragment, cut in small pieces, in a Safe-Lock microcentrifuge tube, and add 250 μL of elution buffer.
17. Place the tube on a shaker for 2 h at room temperature.
18. Remove the liquid, which should now contain most of the riboprobe. Place it into a fresh tube and microcentrifuge it for 10 s to remove any gel debris.
19. Place the liquid in a fresh tube, add 7.5 μL of 4 M NaCl and 600 μL ethanol, and precipitate for 2 h at -20°C or 30 min on ice.
20. Microcentrifuge the probe, rinse the pellet with 75% ethanol, and speed-vac or air-dry. Resuspend the probe in 20 μL of formamide/1X hybridization buffer.
21. Count 1 μL of probe with a scintillation counter and adjust its concentration to approx 500,000 cpm/ μL .
22. The probe can be stored for 2 wk at -80°C.

3.2. RNase Protection (see Note 6)

1. Place 15 μg of total RNA samples (or 1 μg polyA+) in microcentrifuge tubes (Safe-Lock). Add 35 (or 49) μg total yeast RNA and dry with a speed-vac.

2. Resuspend in 10 μ L 5X hybridization buffer and add 40 μ L formamide.
3. Add 2 μ L of riboprobe(s).
4. Denature for 5 min at 95°C.
5. Immediately transfer the tubes to a 50°C water bath and incubate overnight.
6. Add 350 μ L RNase buffer, 2 μ L RNase A (10 mg/mL), and 3.4 μ L RNase T1 (1 U/ μ L).
7. Incubate 30 min at 30°C.
8. Add 20 μ L 10% SDS and 2.5 μ L proteinase K (20 mg/mL).
9. Incubate 15 min at 37°C.
10. Extract twice with phenol/chloroform. For each extraction, vortex 1 min.
11. Add 1 μ L tRNA (10 mg/mL) and 1 mL ethanol.
12. Precipitate for 2 h at -20°C or 30 min on dry ice.
13. Microcentrifuge for 15 min, rinse the pellet with 75% ethanol, and resuspend the pellet in 10 μ L loading buffer.
14. Load half the sample on a denaturing 6% polyacrylamide gel (1X TBE and 8 M urea).
15. Dry the gel and autoradiograph it. Usually, an overnight exposure is adequate with X-O-MAT AR films (Kodak).

4. Notes

1. For positive control, mix a trace of probe template (1 ng) to 50 μ g yeast RNA. For negative control, just add 50 μ g yeast RNA and then proceed with the RNase protection. This will ensure that no signal comes from nonspecific hybridization to yeast RNA. If nonspecific signal is present, increase the annealing temperature to 60°C.
2. Several probes yielding protected bands of different sizes can be mixed together. However, they should be tested separately first to verify that there is no shorter-than-expected signal interfering with the signal of the shorter probe. Indeed, it is not rare to have weak partial digestion products visible under the fully protected signal.
3. For normalization, a probe directed against an RNA known for its constant expression is used (e.g., RP49 in *Drosophila*).
4. The amount and specific activity of probes may have to be adjusted to the abundance of the RNA. It is possible to increase the concentration of cold UTP to the synthesis reaction to reduce the specific activity of the probe. To increase the specific activity, a second radiolabeled nucleotide or a radionucleotide of higher specific activity can be used.
5. If a strong smear is visible under the probe after gel purification, it means that the probe has been degraded during the preparation. It is recommended to restart the preparation of the probe.
6. RNase One can be used instead of RNase A and T1. However, more reproducible and cleaner results have been obtained with the RNase A/T1 mix in our hands.

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Quantitative Polymerase Chain Reaction

Stuart N. Peirson and Jason N. Butler

Summary

Quantitative PCR (qPCR) has entered widespread use with the increasing availability of real-time PCR. By the incorporation of fluorescent dyes in the reaction mixture, increases in amplification products can be monitored throughout the reaction, enabling measurements to be taken in the exponential phase of the reaction, before the reaction plateau. Whatever the platform or chemistry involved, the starting point of a real-time assay is a tissue-specific RNA and the end point of a real-time reaction is an amplification plot. As such, rather than focusing on specific platforms or chemistries, herein we address the basic principles that underlie sample preparation, experimental design, use of internal controls, assay considerations, and approaches to data analysis.

The advent of real-time PCR has enabled high-throughput analysis of multiple transcripts from small tissue samples, with an unparalleled dynamic range and sensitivity. However, to new users, this technique may seem to require extensive optimization and troubleshooting to obtain reliable data; this is further compounded by the mass of technical variations present throughout the literature. The aim of this article is to provide the necessary basics to get a quantitative real-time PCR assay up and running, and to address some of the problems that may arise and how these may be resolved.

Key Words: RT-PCR; real-time PCR; amplification efficiency; gene expression; mRNA; cDNA.

1. Introduction

Attempts to make the polymerase chain reaction (PCR) quantitative have been made ever since the technique entered routine use (1). End point approaches are plagued by the problem of the plateau that occurs when reaction components become limiting and accumulating PCR products compete for polymerase binding (2). As a consequence of this plateau effect, a similar end point concentration may occur when samples contain quite different initial concentrations of nucleic acid (Fig. 1).

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Edited by: E. Rosato © Humana Press Inc., Totowa, NJ

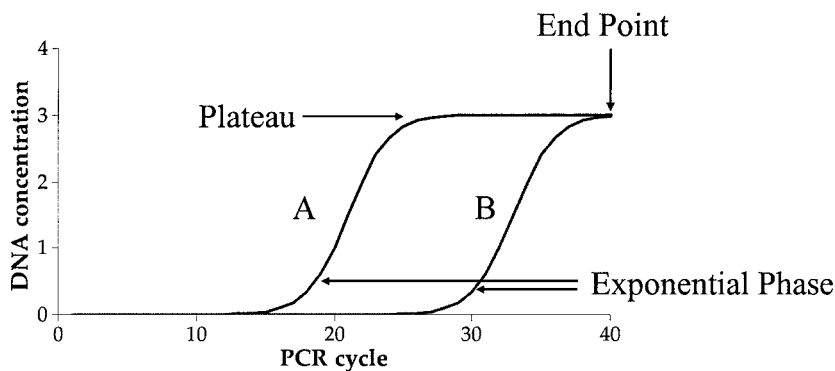


Fig. 1. End point methods of polymerase chain reaction are not suitable for the quantification of gene expression. A high-concentration sample (**A**) is indistinguishable from a lower-concentration sample (**B**) because of the plateau effect. In this example, sample **B** actually contains an initial concentration some 4000 times lower than sample **A**.

Early approaches to quantitative PCR (qPCR) were adopted infrequently, mainly owing to the numerous additional steps or reactions required, necessitating extensive optimization. Competitive PCR, for example, involves addition of known RNA standards to experimental samples before the reverse transcription (RT) step. These standards are typically the same sequence as that to be amplified, with an insertion or deletion enabling discrimination between standard and endogenous template, making this approach laborious, particularly when multiple transcripts are to be investigated (3).

The advent of real-time PCR has brought qPCR to the masses (4–7), and the number of publications featuring the technique has risen with an unerring similarity to the reaction itself. This technique has rapidly become the gold standard for the quantification of nucleic acids, offering exquisite sensitivity, a massive dynamic range (anything up to 8 log units), increased throughput of samples, and improved versatility when compared with traditional approaches such as Northern blotting or RNase protection assays. Consequently qPCR has become widely used for validating expression data produced from microarray experiments (8).

Real-time PCR (also referred to as kinetic PCR) combines improvements in fluorescent chemistry along with platforms typically consisting of a thermal cycler with laser (or other light source), optics and a detector system (typically a charge-coupled device camera, although a photomultiplier may be used), enabling single-sample imaging on a cycle-by-cycle basis (Fig. 2). In its simplest form, a real-time PCR platform may consist of a UV lamp as a light

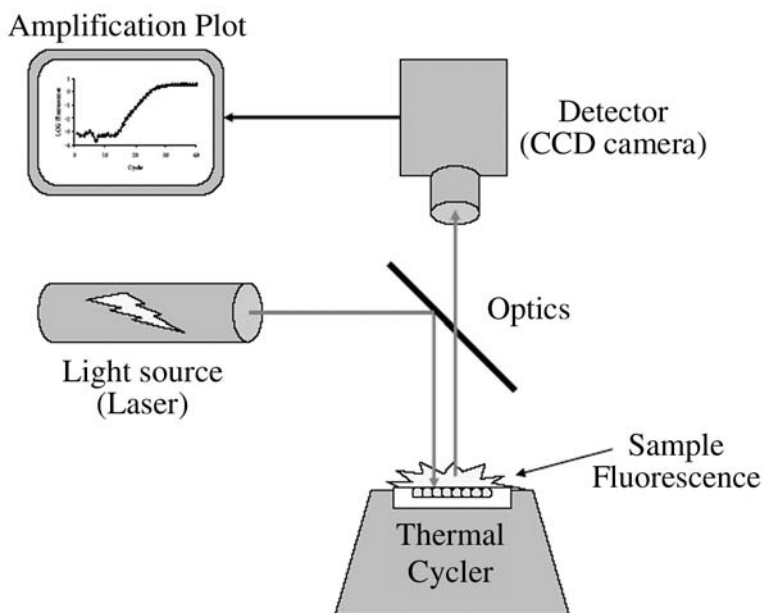


Fig. 2. Real-time polymerase chain reaction setup. All real-time platforms essentially contain four components: a light source, a series of optics, a thermal cycler, and a recording device. The light source may be a white light source with filters or, more typically, a laser. The optics transmit the light from the source to the samples on the thermal cycler, then the fluorescence from the samples to detector. The recording device typically used is a charge-coupled device camera. The output is an amplification plot for each sample, plotting cycle number against fluorescence.

source and charge-coupled device camera as a detector, and by inclusion of ethidium bromide in the actual reactions, the increasing ethidium bromide fluorescence may be monitored as the reaction progresses (9–10). More technologically advanced platforms offer better thermal profiles, improved optics, and more advanced chemistries, enabling improved precision and specificity (for details on real-time chemistries see refs. 4, 7, and 11).

As the same principles underlie qPCR, this chapter will deal with general considerations rather than focusing on any one specific platform or chemistry (although, of course, drawing on personal experience).

1.1. Concepts

The amplification plot is the end result of real-time PCR no matter what platform or chemistry is used. The amplification plot is essentially a growth curve of fluorescence, which is proportional to the DNA concentration within

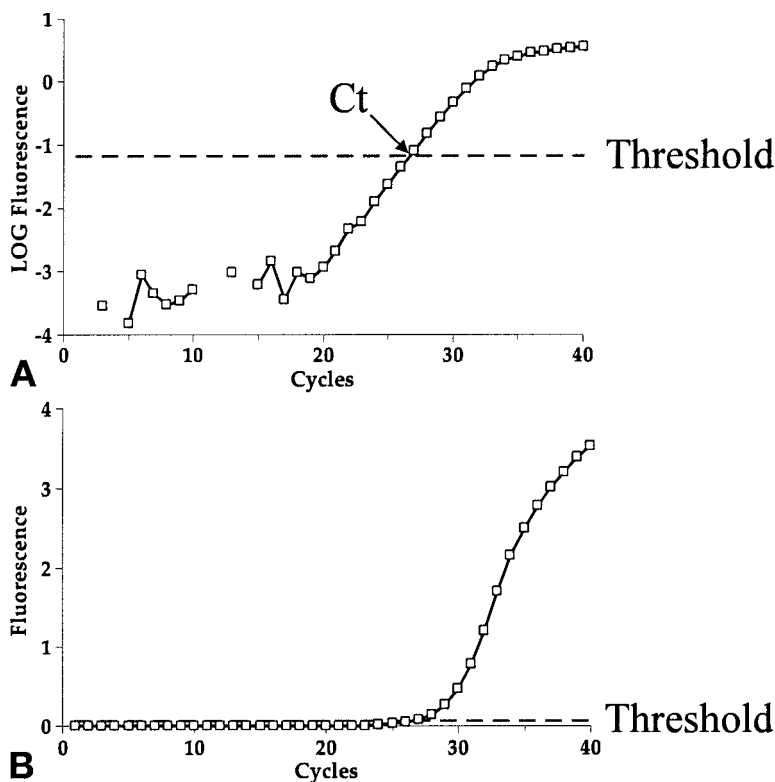


Fig. 3. The end result of real-time polymerase chain reaction (PCR) is the amplification plot. The *x*-axis is the PCR cycle whereas the *y*-axis is the fluorescence that increases throughout the reaction. (A) Amplification plot on a linear scale, demonstrating appropriate threshold. (B) Amplification plot on a logarithmic scale, demonstrating the same threshold. Note that on a logarithmic scale the linear phase appears to be a straight line.

the reaction. As a result, reactions may be analyzed and compared while still in the linear phase of exponential amplification before any plateau occurs. Samples with a high initial target level will demonstrate an earlier rise than those with a low expression level. A typical amplification plot is shown in Fig. 3, on both a linear and a logarithmic scale. The analysis of amplification plots requires two additional concepts, that of fluorescence threshold and the threshold cycle (confusingly for circadian biologists termed *Ct*). The threshold is an arbitrary level of fluorescence within the linear phase at which all amplification plots are analyzed. The *Ct* is the PCR cycle at which each amplification plot reaches this threshold.

Although single-step RT-PCR protocols may be used, including an initial RT step before the PCR is initiated, we will focus here primarily on two-step protocols involving an initial cDNA synthesis followed by a separate PCR reaction. We have adopted this approach to allow the investigation of multiple transcripts, which prevents any differences in RT efficiency from affecting the expression level (3,12). We have found this approach to be particularly useful with circadian studies on clock gene expression, where the number of transcripts of interest increases every year. A final addition advantage is that the labile RNA is converted into more stable cDNA that is more amenable to storage.

1.2. Experimental Design

Because experimental design is an essential aspect of any empirical research, a brief discussion of assay design with respect to qPCR is provided here to address several areas of confusion.

1.2.1. Absolute and Relative Quantification

First, is absolute or relative quantification required? That is, are expression values to be presented as a value relative to an untreated control group (relative) or as a stand-alone number (absolute)? For most biological research, relative quantification is the norm, as the experimental setup includes an untreated, wild-type, or other such control with which all data are subsequently compared (for circadian profiles, ZT or CT 0 may be used). Absolute quantification is far more technically demanding, as it requires a high level of reproducibility. Given problems with the storage, quantification, and handling of exact concentrations of nucleic acids, as well as the exponential amplification of PCR, even slight differences in reaction conditions may produce quite a different result. However, in certain situations, such as viral loading or calculating number of transgene insertions, absolute quantification is essential, and extensive tests of reproducibility and quality control are necessary before quantification can proceed. For absolute quantification a standard curve consisting of a serial dilution of known concentrations of nucleic acid is necessary, usually constructed of plasmid, oligonucleotide, or in vitro transcribed RNA (see **Subheading 3.6.**).

In contrast, relative quantification involves a slightly different approach to data analysis, although the end results in terms of the difference in expression across samples are the same—only the units differ (13). As target gene expression is normally corrected by use of an internal control (typically a housekeeping gene) to account for differences in RNA quality and RT efficiency, the end result of qPCR is an expression ratio of target/internal control, and as such the units of measurement are canceled out (see **Subheading 3.5.**).

1.2.2. Sample Size

Second, what sample size is required for a qPCR experiment? Given the sensitivity and high throughput, the use of single pooled samples is discouraged, as it provides no account of biological variance and enables no statistical analysis of the end result. If one plans for a biological variance of around 50%, a twofold change in expression can be detected in a sample size of four per group—which is not unreasonable. Given only a 1.5-fold change in expression, the sample size increases to around 16 per group, and below this, the sample sizes required rapidly increase, making the detection of such small differences practically unfeasible, unless biological variance is considerably lower (*see Note 1*).

1.2.3. Experimental Setup

The main consideration when setting up a real-time PCR assay is to ensure that all samples between which direct comparisons are to be made are included on the same assay. As slight differences between reaction conditions will occur, splitting an experimental group across multiple assays may cause problems (*see following paragraph*). For relative quantification, the simplest approach is to run each transcript on a separate plate (although several may be run on the same plate, space permitting). Different transcripts (including internal controls) may be run on separate plates, as all calculations are made relative to one group of samples (the control group; *see Note 2*).

The primary cause of the differences that inevitably occur between real-time PCR assays (interassay variability) is the method of deriving the amplification efficiency. Any difference in the efficiency results in an exponential difference to the expression value derived. For example, if a standard curve is constructed on every plate that is run, the efficiency calculated will never be exactly the same, and will vary between assays as a result of measuring errors (*i.e.*, standard curve construction). These differences will conform to a normal distribution, the variance of which is dependent on standard storage, quantification, and pipetting precision. For example, a difference of just 1% in amplification efficiency can result in a 13% difference in expression, whereas a 5% difference in amplification efficiency will produce around a 90% difference (*see Note 3*). As such, where samples must be split across multiple plates, they must be analyzed with the same amplification efficiency to achieve a meaningful result.

When using platforms with a 96-sample capacity, reactions are commonly run in triplicate to provide more accurate resolution of each data point. However, given availability of larger sample groups, the benefits of including additional samples on the same plate far outweigh the disadvantage of not using triplicates (*see Note 4*).

2. Materials

1. Experimental sample RNA.
2. Sample of cDNA for testing and optimization.
3. DEPC or other nuclease-free water.
4. RNase-free DNase for removal of contaminating genomic DNA.
5. RT reagents.
6. Reaction master mix (or individual components).
7. Primers/probes for target gene(s) and internal controls.
8. Reaction consumables (PCR tubes, optical plates, capillaries, etc.).
9. Real-time PCR platform.

3. Methods

3.1. RNA

We have found that RNA quality is not too essential, given careful use of internal controls. However, comparable samples do remove the dependence on internal controls in situations in which housekeeping gene expression may be affected by experimental treatment, so the use of similar concentrations of RNA for each reaction is recommended. If the same amount of RNA is used in every sample, a similar internal control expression is expected. RNA concentration and quality may be assessed in a variety of ways, including spectrophotometry, fluorescent dyes, denaturing gel electrophoresis, or microfluidic analysis (*see Note 5*).

3.2. DNase Treatment

No matter what method is used for RNA extraction, DNase treatment prior to reverse transcription is essential. No method of RNA isolation is entirely free of contaminating genomic DNA, and even if amplification products span introns, genomic amplification is possible (e.g., amplification across small introns, amplification of pseudogenes, or amplification of genomic misprimers).

3.3. Reverse Transcription

The exact reverse transcription kit used should not affect the end result, as long as good quality cDNA is synthesized. If ribosomal RNAs (rRNAs) are to be used for internal controls, the use of random primers is necessary, although the use of mRNA internal controls should not be ruled out (*see Subheading 3.5*). Following RT always test all samples to ensure that amplification-quality cDNA is present. It may be worth investing a little time in designing primers to a housekeeping gene that will amplify across a small intron. This will then enable the testing of all newly synthesized cDNA, and the presence of any gDNA contamination may be detected by the presence of a different-sized product when the samples are run out on an agarose gel.

3.4. Primer Testing

Details for primer design are largely dependent on the chemistry used. Probe-based systems such as TaqMan (Applied Biosystems) often have quite specific primer and probe requirements. For double-stranded DNA (dsDNA) binding dyes, the primers are the sole source of specificity, and as such it is not worth conducting a qPCR assay with suboptimal primers. Use of dsDNA binding dyes enables use of larger products, and this is also beneficial to allow the separation of primer–dimers and specific product by melting curve analysis. Regarding product size, 150 to 200 bp is optimal for dsDNA binding dyes, although we have used products up to 400 bp without a significant decrease in amplification efficiency. As a larger amplicon will bind more dye, larger amplicons should yield a greater increase in fluorescence per cycle, improving assay sensitivity.

All primer and probe sets to be used should be tested on a comparable cDNA to ensure gene-specific amplification, with no spurious products and minimal primer–dimers. This can be done on a normal thermal cycler for dsDNA binding dyes such as SYBR Green I, but for probe-based assays, testing must be conducted on the real-time PCR platform.

When using a dsDNA binding dye, a melting curve should also be conducted on the reaction products (**Fig. 4A**). As nonspecific products such as primer–dimers will also fluoresce with such dyes, they must be prevented from contributing to the measured fluorescence. This may be accomplished by measuring the fluorescence at a higher temperature at which primer–dimers cannot form. As the desired amplicon will be larger than any primer–dimer, nonspecific products may be melted out without denaturing the specific product. To determine the melting temperature of any product, the temperature can be ramped from 60 to 95°C, and data collected. This is then typically plotted as a differentiation plot, as rate of change of fluorescence (**Fig. 4B**). Melting curves also provide a postamplification means of analyzing the products formed in a closed-tube format, enabling confirmation that only a single product has been amplified and greatly reducing the risk of contamination when compared with gel electrophoresis (*see* **ref. 14** for more details on melting curve analysis).

3.5. Internal Controls

The matter of internal controls has been subject to intense debate within the scientific literature surrounding gene expression for a number of years. First, it should be stressed that there is no ideal internal control. All housekeeping genes used for this purpose possess merits and flaws. Although many people recommend the use of rRNAs (especially 18S rRNA), these are not ideal for a number of reasons, including the massive difference in expression between target

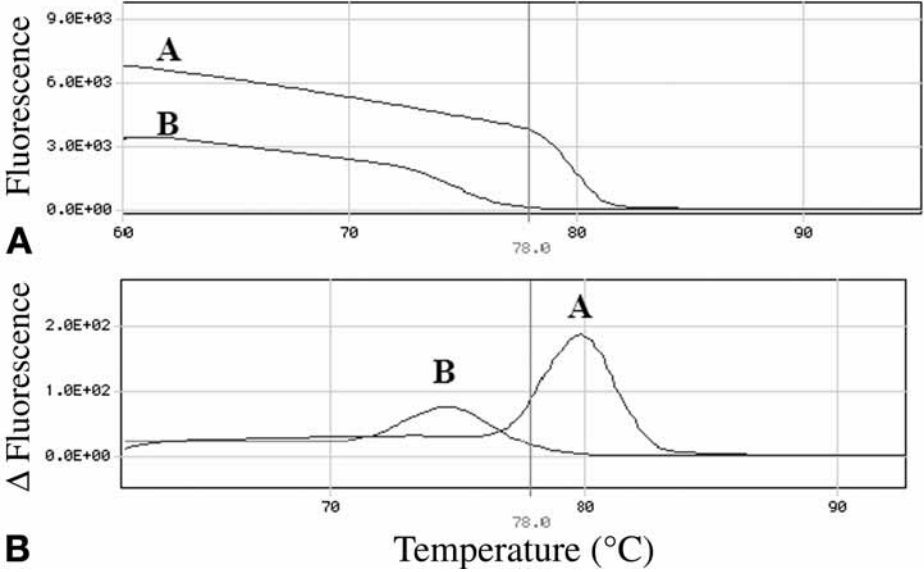


Fig. 4. Melting curve (A) and differentiation plot (B) for the double-stranded DNA binding dye SYBR Green I. Arrow indicates recording temperature to avoid primer-dimers contributing to recorded fluorescence.

genes and controls, as well as the conservation of rRNAs between species providing an ideal opportunity for contamination, and the fact that rRNAs contain no introns, meaning that gDNA contamination is difficult to preclude.

With regard to real-time PCR, the most lucid and common-sense approach to this subject is provided by Vandesompele et al. (15), who recommend the use of multiple internal controls. This enables cross-comparisons to be made to produce a gene-stability index, and a normalization factor based on the geometric mean of suitable internal controls (see **Note 6**). This procedure minimizes outlier effects and, most important, provides the reassurance that the differences in internal control expression between samples are in fact a true measure of RNA loading. Furthermore, by more accurate normalization, the accuracy of the data is improved, reducing error associated with noisy internal control data.

3.6. Data Analysis

Numerous approaches to data analysis have been suggested for quantitative real-time PCR. Unfortunately, data analysis does require use of a little mathematics, although this should not put off the nonmathematically inclined. Once the basis is understood, these calculations may all be simply automated.

The amplification of DNA during a PCR may be described by the following simple equation (**Eq. 1**):

$$X_n = X_0 \times (1 + E)^n \quad (1)$$

When X_n is the DNA concentration at cycle n , X_0 is the initial DNA concentration, E is the reaction efficiency, and n is the number of cycles of PCR (**16**). In real-time PCR, instead of measuring DNA concentration, the fluorescence of each sample is measured, which is proportional to the DNA content. If the amplification efficiency (E), threshold (R_{Ct}), and number of cycles taken to reach this threshold (Ct) are known, one may calculate the theoretical initial fluorescence (R_0) as follows:

$$R_0 = R_{Ct} \times (1 + E)^{-Ct} \quad (2)$$

The simplest way of analyzing qPCR data is to calculate the R_0 value for each sample at a known threshold (R_{Ct} in **Eq. 2**) from its Ct value. This circumvents many problems, as R_0 is a linear unit (as opposed to Ct). Therefore, a sample with an R_0 twice that of the control R_0 contains double the transcript level, and moreover, statistical analysis and measures of variance can be calculated from R_0 values, which are less straightforward when using Ct values.

If threshold (R_{Ct}) and Ct are known, then the only unknown is the amplification efficiency (E). The different approaches to data analysis just represent different approaches to calculating this value (**13**). These can be broken down into three major approaches:

1. Assumed efficiency.
2. Standard curves.
3. Kinetic analysis.

The first method, assumed efficiency, is perhaps more commonly referred to throughout the qPCR literature as the $2^{-\Delta\Delta Ct}$ method (**16,17**). This simply assumed the reaction efficiency to be 1.00 for both target gene and internal control, i.e., a perfect doubling of reaction product every cycle of PCR. The advantage of this approach is its simplicity—there is no need for any additional calculations, and in most cases it provides a good approximation (*see Note 7*). The disadvantage is that in most cases, the amplification efficiency will be lower than 1, and as such, this approach will introduce errors into the exact quantification, as well as exaggerating the magnitude of any differences between groups.

The second approach makes use of standard curves (*see Fig. 5*), typically constructed of either copy numbers (if absolute quantification is required) or a diluted cDNA sample (**18**). The amplification efficiency can be derived from the slope of a standard curve as follows (**Eq. 3**):

$$E = (10^{(1/\text{slope})}) - 1 \quad (3)$$

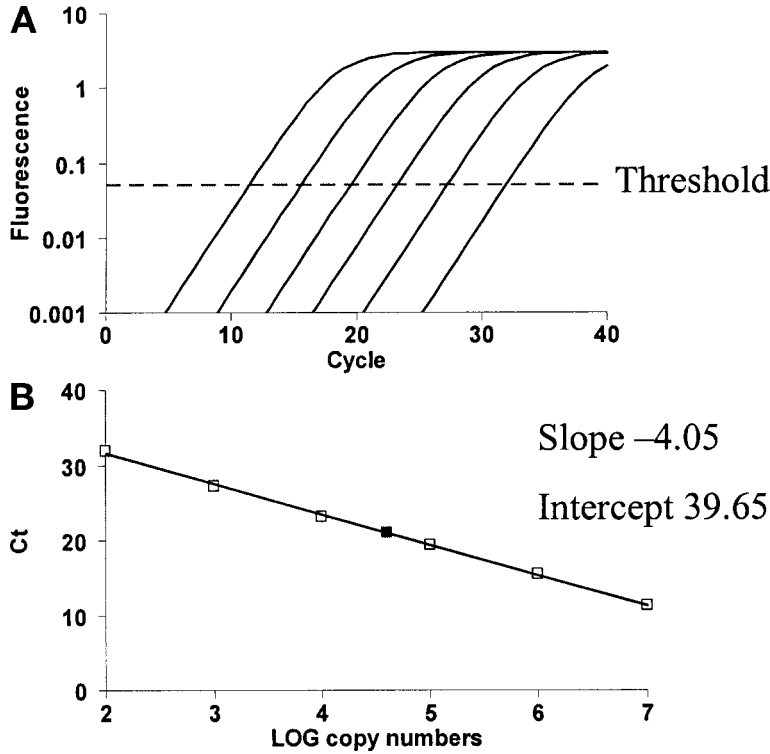


Fig. 5. The use of standard curves involves preparing a serial dilution of template, and plotting C_t vs the initial concentration (on a logarithmic scale). The concentration of unknown samples may then be extrapolated using linear regression. For example, samples containing a range of concentrations between 100 and 100,000,000 copies are amplified (A). The C_t of each known concentration is then plotted against the copy number, and unknowns may then be extrapolated (B). If an unknown sample has a C_t of 21, this would correspond to a concentration of 4.6 (log scale), or 39,811 copies.

When calculating relative expression, if using a standard curve composed of copy numbers, the end result (fold change between control and experimental samples) is mathematically identical whether using **Eq. 2** or deriving the copy number for every sample.

The final approach, kinetic analysis, uses the information that is present in every amplification plot to calculate the amplification efficiency for every sample (19). As there will be an associated measuring error, individual corrections are possible only when this measuring error is very small. Otherwise, slight differences in reaction efficiency result in an exponential addition of this error (see **Subheading 1.2.3.**). In its simplest form, a linear regression may be

conducted to the exponential portion of each amplification plot, the slope of which enables the amplification efficiency to be derived. The advantage of this approach is that no additional standards are required, and furthermore one has multiple measurements of the amplification efficiency for every transcript under study. One may therefore calculate the mean efficiency and test for any deviations in amplification efficiency between groups (13). More advanced models are available, but these are computationally intensive and present additional technical challenges (ref. 20; see Note 8).

4. Notes

1. Sample sizes should be considered before setting up experiments. The University of Vienna Department of Medical Statistics website (www.univie.ac.at/medstat) provides a useful sample size calculator. Whereas the value α is familiar to most as the probability of a false-positive (that a difference exists as would be expected by chance alone, usually set somewhat arbitrarily at 0.05), β is less familiar to biologists. β Represents the probability of a returning a false-negative (the probability of a real difference not being detected). This is most commonly encountered as the power of a test, equal to $1 - \beta$. β Is usually set at 0.20, as false-negatives are usually regarded as less detrimental than false-positives. However, if a sample population is small and has a high variance, the ability to discriminate significant differences is compromised, and false-negatives become more likely (no statistical difference is returned when a real difference may exist, that is the power of the test is low). If conducting a very important or costly trial, one may choose to set β to a higher level, thus requiring larger sample sizes.
2. For example, given two samples, control and treated, if the treated sample demonstrates a twofold increase in target gene expression, and a fourfold increase in internal control expression, then this treated sample obviously contains more RNA than the control. Taking this into account by normalizing the expression level of the target gene demonstrates that the target gene expression actually halves following treatment. Whether the target gene and internal control samples are run on the same plate is irrelevant.
3. These calculations are based on a sample with $C_t = 25$, as the exact difference is dependent on the expression level of the transcript. For example, a 1% difference this may be calculated as follows:

$$(E + 0.99)^{-25} / (E + 1.00)^{-25} = 1.13, \text{ or } 13\% \text{ higher.}$$

Individual corrections may become possible with technical improvements in thermal cycling and detectors, but with most platforms, this is currently inadvisable.

4. This is because the interassay variance (typically around 10%) is far lower than the biological variance typically encountered in gene expression (often as high as 40 to 50%). Therefore, inclusion of additional biological replicates provides far more information than replicating each individual point (which may not be highly representative of the population as a whole).

5. See Chapter 22 for more details on measurement of RNA concentration and quality.
6. Gene stability indices may be calculated from multiple internal controls, and a normalization factor derived from the geometric mean of the most stable internal controls.
7. As such, this method provides a good starting point when becoming accustomed to qPCR data analysis. By simply substituting 2 for the efficiency in **Eq. 2**, this effectively becomes the following (**Eq. 4**):

$$R_0 = \text{Threshold} \times 2^{-C_t} \quad (4)$$

which is, in effect, equivalent to the $2^{-\Delta\Delta C_t}$ method.

8. Sigmoid models may be used, using nonlinear methods to minimize the sum of squares to give the best possible fit.

Acknowledgments

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V

GENE EXPRESSION: PROTEINS

Protein Extraction, Fractionation, and Purification From Cyanobacteria

Natalia B. Ivleva and Susan S. Golden

Summary

This chapter deals with methods of protein extraction from cyanobacterial cells based on work in the circadian model organism *Synechococcus elongatus* PCC 7942. Some of these techniques have already been used successfully for analysis of circadian rhythms in cyanobacteria, whereas others are heretofore unpublished, but may yield exciting results in the near future.

Key Words: cyanobacteria; protein extraction; protein purification; protein interaction.

1. Introduction

Analysis of the protein content of cells is a key complementary approach to genetic studies for understanding circadian regulation in cyanobacteria. In these organisms diurnal changes in protein levels of central clock components likely play a crucial role in generating rhythmicity (1) and, therefore, affect the fitness of the cell (2). The development of a variety of techniques for cyanobacterial protein fractionation and purification has provided us with knowledge of physical interactions among the Kai proteins and between KaiC and SasA (3,4), in vivo phosphorylation properties of KaiC (5), cycling of sigma factors (6), autophosphorylation activity of CikA (7), and intracellular dynamics of the Kai proteins during diurnal cycles (8).

This chapter deals with methods of protein extraction from cyanobacterial cells for a variety of analytical methods. Some of these techniques already have been used successfully for analysis of circadian rhythms in cyanobacteria, whereas others are unpublished, but show potential to yield exciting results in the near future.

A simple way to look at expression levels of protein is to load total disrupted cell contents on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and evaluate the samples by immunoblotting. However, in some cases this approach gives results that are difficult to interpret because of the presence of abundant cell membranes and/or crossreactivity of antisera. In these cases, fractionation of proteins may yield more easily interpreted results. Fractionation can also predict localization of proteins in the cell. Purification of a protein of interest directly from the cyanobacterium is often desirable. A protein can be purified from cyanobacterial cells using affinity purification (e.g., of a functional His-tagged variant) or immunoprecipitation (antibodies raised against the protein or an engineered epitope tag). Depending on the buffer composition used in those purifications, the resulting sample will contain either the pure protein of interest or the protein and its possible interactants (i.e., co-purification).

2. Materials

1. SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer: 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS (electrophoresis grade), 0.1% bromophenol blue, 10% glycerol.
2. Glass beads: diameter 0.2–0.3 mm (Sigma), acid-washed.
3. Phosphate-buffered saline (PBS) buffer: 136 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄; adjust pH to 7.4 with HCl (9).
4. Isopropylthio- β -D-galactoside (IPTG): stock solution, 100 mM in water.
5. Ni-NTA agarose (Qiagen [10]).
6. Lysis buffer: 50 mM NaH₂PO₄, 300 mM NaCl. Adjust pH to 8.0 using NaOH (10).
7. Wash buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole. Adjust pH to 8.0 using NaOH (10).
8. Elution buffer: 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole. Adjust pH to 8.0 using NaOH (10).
9. Interactants (IA) lysis buffer: 50 mM NaH₂PO₄, 5 mM NaCl. Adjust pH to 7.8 using NaOH.
10. IA wash buffer: 50 mM NaH₂PO₄, 5 mM NaCl, 20 mM imidazole. Adjust pH to 7.8 using NaOH.
11. IA elution buffer: 50 mM NaH₂PO₄, 5 mM NaCl, 250 mM imidazole. Adjust pH to 7.8 using NaOH.
12. AffiGel-Hz beads (Bio-Rad).
13. TES–NaOH buffer: 10 mM TES–NaOH, 5 mM NaCl, 5 mM EDTA. Adjust pH to 7.0 using NaOH.
14. IP buffer: 50 mM Tris-HCl, 100 mM KCl, 5 mM MgCl₂, 0.2% glycerol, 0.1 mM EDTA.
15. Bovine serum albumin (BSA).

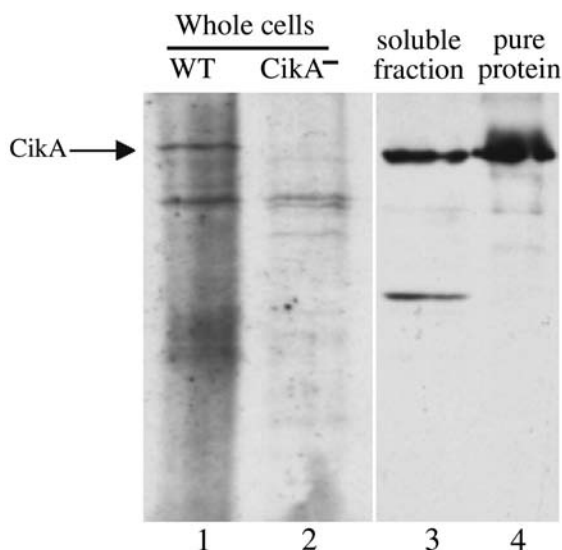


Fig. 1. Immunoanalysis of samples using antisera raised against Cika protein. Lanes 1 and 2: whole cyanobacterial cells (*see Subheading 3.1.1.*). Lane 3: soluble fraction of wild-type cells (*see Subheading 3.2.*). Lane 4: His-tagged Cika protein purified from cyanobacterial cells (*see Subheading 3.3.1.*).

16. SDS-PAGE loading buffer without reducing agent: 50 mM Tris-HCl (pH 6.8), 2% SDS (electrophoresis grade), 10% glycerol.
17. 2-Mercaptoethanol.
18. Bromophenol blue.

3. Methods

3.1. Total Protein Extraction

3.1.1. Analyzing Disrupted Cells on SDS-PAGE

In many cases the quick and easy approach of loading disrupted cells on an SDS-PAGE gel will yield suitable results. If not, the additional fractionation procedures that follow are likely to improve the outcome (**Fig. 1**).

1. To compare protein levels at different time points or in different strains, grow the cell cultures to an optical density of 0.5 at 750 nm (*see Note 1*).
2. Spin down 1 mL of each sample for 1 min at 10,000g and discard the growth medium (pellets may be frozen at this step).
3. Resuspend the pellets in 50 μ L of SDS gel-loading buffer and boil them for 5 min before loading on a gel.

3.1.2. Crude Cell Extract Preparation

This sample preparation results in a more uniform disruption of cells than boiling alone, and removes particulates to effect more accurate separation of proteins on a gel.

1. Grow a 100-mL culture to an optical density of 0.5 at 750 nm.
2. Spin down cells for 10 min at 1500g and wash the pellet by resuspending in 2 mL of PBS buffer and repeating the spin. Resuspend the pellet in 200 μ L of PBS.
3. Freeze the cells at -80°C and quickly thaw them at 37°C to allow partial cell breakage. For the remainder of the procedure, keep the samples on ice and in the presence of protease inhibitors (*see Note 2*).
4. Add sufficient glass beads (*see Note 3*) to leave 2 to 3 mm of cell suspension above the level of settled beads. Break the cells by vigorous vortex mixing of the tube for 10 min in cycles of mixing for 30 s and cooling on ice for 30 s.
5. Add 100 μ L of PBS buffer, briefly spin the suspension at 1000g and collect the supernatant fraction (*see Note 4*). Spin the resulting supernatant fraction for 10 s at 1000g to pellet residual glass beads. The resulting green supernatant fraction is a crude cell extract.
6. Determine protein concentration using a Lowry or similar assay (*9,11*). The cell extract may be used for further protein fractionation or directly loaded on a gel for immunoblotting.

3.2. Fractionation of Crude Cell Extract

The soluble fractions may be used for further protein purification. Fractionation may also be useful for localization of proteins involved in regulation of circadian rhythmicity (*8*).

1. Subject the crude cell extract (*see Subheading 3.1.2.*) to centrifugation at 100,000g for 30 min at 4°C . Collect the bright-blue supernatant (soluble fraction).
2. Wash the deep-green pellet in 2 mL ice-cold PBS buffer twice and use it as a total membrane fraction.
3. Verify the purity of each fraction by examining the absorption spectrum (*8*) from 250 to 750 nm. The membrane fraction should contain chlorophyll exclusively, which has an absorbance peak around 681 nm; the soluble fraction should contain phycobiliproteins exclusively, which absorb maximally around 620 nm.

Several methods for further localization of proteins in cyanobacteria are available (*see Note 5*).

3.3. Purification of a Soluble Protein of Interest

Two protein purification approaches have been used for studying circadian rhythms in cyanobacteria. The protein can be purified from cyanobacterial cells

using an affinity purification tag (e.g., engineered 6-His) or immunoprecipitation (antibodies raised against the protein or epitope tag). In the case of epitope-tag-based purification, it is important to demonstrate genetically that the tag does not affect protein function. This is easily accomplished in *Synechococcus elongatus* by complementation of a null mutant with the allele that encodes the tagged protein (7). In addition, the wild-type strain, which does not carry the modified allele, should be used as a negative control to identify nonspecific proteins that may bind to the column. In our experience, two polypeptides of molecular weights of 55 and 13 kDa, appropriate in size to be subunits of the abundant Rubisco enzyme, might be eluted from nickel columns along with His-tagged proteins and their interactants. When performing immunoprecipitation, a strain that carries a deletion of the gene of interest should be used as a negative control. For further details on immunoprecipitation procedure refer to **Subheading 3.4.2.**

3.3.1. Affinity Purification

1. Induce expression with IPTG, if the promoter of the affinity-tagged allele is under *lac* repressor control (see **Note 6**).
2. Proceed with cell disruption and fractionation as described in **Subheadings 3.1.2.** and **3.2.**, with the exception of substituting lysis buffer for PBS buffer.
3. Pass the soluble protein fraction through a column prepacked with 2 mL Ni-NTA-agarose.
4. Wash the column five times with 10 mL of wash buffer.
5. Elute with 4 mL of elution buffer, collecting each 1-mL fraction in a separate tube. Analyze the eluted fractions by immunoblotting or by staining with Coomassie brilliant blue (9) to see which one has the highest concentration of the target protein.

3.4. Co-Purification of Interacting Proteins

These approaches are useful for identifying novel proteins that interact with a protein of interest, and for detecting time-specific interactions for proteins that have been shown by other methods to interact. Co-purification procedures are very similar to the purification protocols described in **Subheading 3.3.** However, co-purification should be performed using buffers with lower salt concentration than used in buffers in simple purification of the target protein. Relatively low salt concentration does not disrupt protein interactions and allows co-purification of the target protein and its interactants.

3.4.1. Affinity Co-Purification

The protocol is presented for His-tagged proteins, but other affinity tags and matrices can be substituted.

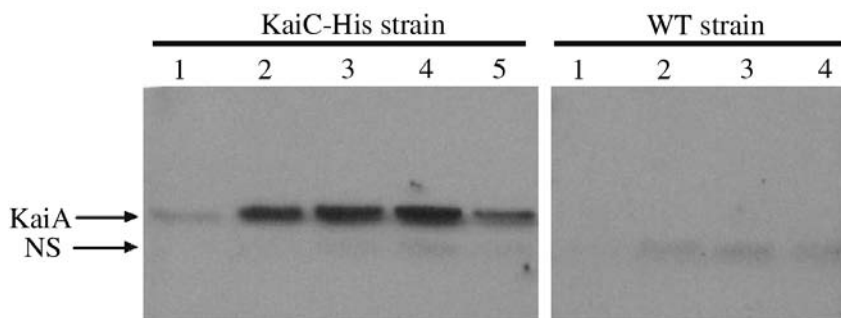


Fig. 2. KaiA protein copurifies with His-tagged KaiC protein (*see Subheading 3.4.1.*). The proteins were eluted with 5 mL of IA elution buffer. Each 1-mL fraction was collected in a separate tube. Immunoblot of samples using antisera raised against KaiA protein is shown. Numbers correspond to the eluate fraction. NS, nonspecific band.

1. Induce expression of the His-tagged protein if it is under control of a regulated promoter (*see Note 6*).
2. Prepare the cyanobacterial soluble protein extract as described in **Subheadings 3.1.2.** and **3.2.**, with the exception of substituting IA lysis buffer for PBS buffer.
3. Load the soluble protein fraction on a column prepacked with 2 mL Ni-NTA-agarose.
4. Wash the column five times with 10 mL of IA wash buffer.
5. Elute with 5 mL of IA elution buffer, collecting each 1-mL fraction in a separate tube (**Fig. 2**). Proceed with analysis of the eluted fractions to see which one has the highest concentration of target protein (*see Note 7*).

3.4.2. Coimmunoprecipitation

The following procedure was compiled from the conditions used by Kitayama et al. (**8**).

1. In advance of coimmunoprecipitation reactions, couple 25 μ L bed volume of AffiGel-Hz beads to purified antibodies raised to the protein of interest. To block nonspecific interactions wash the coupled resin with 0.5% BSA in IP buffer.
2. Harvest 0.1 g of cyanobacterial cells (about 100 mL of culture at optical density of 0.5 at 750 nm). Proceed with cell disruption and fractionation as described in **Subheadings 3.1.2.** and **3.2.**, with the exception of substituting TES-NaOH buffer for PBS.
3. Adjust the protein content of soluble extract for each sample to the same concentration (0.2 mg/mL is suggested). Proceed with 700 μ L of the soluble fraction.
4. Add 700 μ L of IP buffer. Incubate the resulting 1400 μ L with the coupled resin at 4°C for 2 h.

5. Wash the beads twice with 1 mL of IP buffer that contains 0.5% w/v BSA to minimize nonspecific interaction, and four times with 1 mL of IP buffer without BSA.
6. Resuspend the beads in 80 μ L of SDS-PAGE loading buffer without reducing agent. Elute proteins by vortexing gently for 10 min at room temperature. Spin briefly to collect the supernatant fraction.
7. Supplement the collected fraction with reducing agent (0.1% 2-mercaptoethanol) and 0.1% bromophenol blue to load on SDS-PAGE for further analysis (*see* **Note 7**).

4. Notes

1. The amount of cells used for this assay depends greatly on the sensitivity of the antibodies used. A series of 1:10 dilutions of concentrated culture may be used in the first experiment. Optical density measurements must be made at longer wavelengths for cyanobacteria than for *Escherichia coli* so that light scattering, rather than absorbance by the photosynthetic machinery, will be detected.
2. Addition of 1 mM phenylmethylsulfonyl fluoride (prepare stock solution of 100 mM in isopropanol) or 1/1000 vol of protease inhibitor cocktail for general use (Sigma) or other protease inhibitors (9) significantly improves stability of proteins in the extract. Phenylmethylsulfonyl fluoride permanently inhibits proteases, but it is quickly inactivated in aqueous solutions; therefore, it should be added directly to the sample containing proteases, not to the buffer before adding it to the sample. For long-term storage, freezing of the sample is recommended. Also, to decrease degradation rates of proteins during the protein extraction and purification protocols described in this chapter, it is recommended to use ice-cold buffers and keep the samples at 4°C.
3. A few methods for disrupting cyanobacterial cells have been described. Approaches other than breakage with glass beads as outlined in this chapter include sonication (3) or passing cells through a French pressure cell press (8).
4. If it is important to extract most of the total protein from the sample, and low protein concentration in the extract is not an issue (for example, when you are planning to proceed with protein purification; *see* **Subheading 3.3.**), add an additional 500 μ L of PBS after collecting the first supernatant fraction, spin briefly, and collect the supernatant fraction again. Repeat a few times until the supernatant fraction is clear. Proceed with centrifugation of the collected green supernatant fraction for 10 s at 1000g.
5. Methods for localization of proteins in the periplasm and thylakoid or plasma membranes of cyanobacteria are also available (12,13). Proteins from membrane fractions may also be separated by their hydrophilic or hydrophobic nature, and the strength of protein attachment to the membrane can be evaluated (14). Control proteins of known localization should be assayed to assure purity of the fractions. The protein fractionation methods mentioned here are based on solubility of proteins and their attachment to membranes. In addition, the cell extract can be subdivided based on size of proteins and protein complexes using gel filtration chromatography (3).

6. Affinity-tagged alleles are commonly driven in *S. elongatus* by a promoter under *lac* repressor control. Addition of 1 mM IPTG for 1 h is normally sufficient for protein expression controlled by pTrc promoter. However, concentration of IPTG and time of induction greatly depend on the individual protein, experimental variables, and the specific promoter, and should be adjusted accordingly. If the His-tagged protein is being expressed for purification of its possible interactants, it is important to keep expression levels relatively low, because gross overexpression may increase nonspecific protein interaction in the cell. Titration of IPTG with phenotypic monitoring should be used to keep the protein within physiologically relevant concentrations.
7. The eluted fractions can be analyzed by immunoblotting using the antibody raised against proteins known to be involved in circadian rhythmicity. The eluted fractions can be concentrated by precipitation of proteins with trichloroacetic acid. Add trichloroacetic acid to the sample to final concentration of 15%, freeze the sample at -80°C , then thaw and spin at 16,000g to precipitate the protein. Carefully remove all liquid and air-dry the sample. Resuspend the pellets in SDS gel-loading buffer and boil before loading on a SDS-PAGE gel for further staining with Coomassie brilliant blue (9). Co-purifying proteins may be identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

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Protein Extraction From *Drosophila* Heads

Patrick Emery

Summary

In *Drosophila*, the concentration and phosphorylation levels of several important circadian proteins (e.g., PERIOD, TIMELESS) oscillate on a 24-h basis. A simple and rapid method for extracting proteins from fly heads is presented here. The extracts can immediately be loaded onto an sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel to assay the effects of mutations, genetic manipulations, or environmental conditions on the oscillations of circadian proteins by Western blotting. They can also be used for immunoprecipitation experiments.

Key Words: Protein extraction; protein cycling; phosphorylation; immunoprecipitation.

1. Introduction

Fly heads contain a high concentration of circadian proteins (*1*). They are easy to separate from the rest of the body and their proteins can be rapidly extracted. In brief, flies are usually synchronized to a light–dark cycle for a few days, collected, and frozen on dry ice. Heads are separated from the bodies by vigorous shaking. The lysate obtained by grinding the heads in an extraction buffer can be used for Western blotting or immunoprecipitation experiments (*see* Chapter 31). As mentioned in Chapter 20 for RNA extraction from *Drosophila*, it is important to remember that most of the circadian proteins in head extracts come from the eyes, a peripheral oscillator, and not from the neurons controlling circadian behavior (*see* **ref. 1** and **Note 1**).

2. Materials

1. Incubator with light and temperature control.
2. More than 20 flies per time point, 3 to 7 d old (*see* **Note 2**).
3. Dry ice.
4. One medium-sized and one small funnel, chilled at -80°C .
5. Plastic mesh or brass sieves (nos. 25 and 40), chilled at -80°C .

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6. Pellet pestle motor and pellet pestles (Kontes; *see Note 3*).
7. Extraction buffer (store at 4°C; *see Notes 4–6*): 20 mM HEPES, pH 7.5, 100 mM KCl, 5% glycerol, 10 mM EDTA, 0.1% Triton, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mg/mL aprotinin, 5 mg/mL leupeptin, 5 mg/mL pepstatin A.
8. 5X Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (store at –20°C): 300 mM Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 5% β -mercaptoethanol, 0.01% bromophenol blue.

3. Methods

3.1. Light and Temperature Treatments and Fly Collection

The most commonly used conditions for synchronizing flies is a 12-h light:12-h dark regime at a constant temperature of 25°C. Two full light–dark cycles are sufficient to achieve synchronization. Flies can thus be collected on the third day, under the desired conditions (e.g., light–dark, constant darkness). Temperature can also be used as a synchronization cue, with, for example, 25°C for the day and 20°C for the night. At the time of collection, flies are transferred to 15-mL tubes chilled on dry ice with the help of a funnel. Flies should be collected in the dark or under safe red light. They can be stored at –80°C indefinitely.

3.2. Protein Extraction From Whole Heads

1. Samples must be kept as much as possible on dry ice until extraction actually begins.
2. Flies are decapitated by vigorously vortexing the 15-mL tubes twice for 15 s.
3. If the number of heads per time-point is small, heads are counted on a plastic mesh placed over dry ice and transferred into microcentrifuge tubes with a small brush. If the number of heads is large, brass sieves can be used to separate the heads from the bodies and other small body parts like legs and antennae. The top sieve (no. 25) will let the heads go to the bottom sieve (no. 40), which will separate the small fly fragments from the heads. The heads can then be transferred to microcentrifuge tubes with a small funnel. The sieves and the funnel need to be cooled at –80°C prior to use.
4. Extraction buffer is added to the heads. Usually, extraction is done with a volume of 1 μ L per head (but no less than 30 μ L).
5. The heads are ground three times during 30 s with the homogenizer. The liquid is spun down with a nanocentrifuge after each cycle of homogenization.
6. If the extract is going to be used for immunoprecipitation, at least one 10-min microcentrifugation step at 4°C is required to clear the supernatant from the debris. For Western blots, the appropriate amount of 5X SDS loading buffer can directly be added to the homogenate (*see Note 7*). Extracts are boiled for 5 min at 100°C, microcentrifuged for 5 min at 12,000g, and loaded on an SDS-PAGE gel. Alternatively they can be stored at –80°C before analysis.

4. Notes

1. About 66 to 75% of the circadian proteins extracted from whole heads are coming from the eyes, a peripheral oscillator (1). The neurons controlling circadian behavior contribute to only a small fraction of the extracted proteins. One possibility is to use mutant flies without eyes (*eyes-absent*, for example) as a source of protein extracts to increase the pacemaker neurons' contribution. Another is to isolate the brains by dissection. However, the contribution from these neurons in eyeless flies or in brains has not been determined and might still be small, as other groups of neurons as well as glial cells express circadian proteins (2–4). To observe protein oscillations in the ventral lateral neurons directly, *in situ* immunohistochemistry is the method of choice (see Chapter 12).
2. The number of heads should not be smaller than 20 for Western blots, to ensure reproducible extractions. Usually, loading the equivalent of about 8 heads is sufficient. For immunoprecipitations, the amount of heads required will depend on the strength of the interaction and protein concentrations. A good starting point is 400 heads.
3. The pellet pestle can be rinsed with water between sample extractions and reused.
4. For immunoprecipitations, the extraction buffer might require adjustment (type and concentration of salt and detergent; see Chapter 31).
5. Dithiothreitol and the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin A must be added just before use. Commercially available cocktails of protease inhibitors (e.g., Complete protease inhibitors, Roche) can be used instead (add just before use).
6. To inhibit serine/threonine phosphatases, add 20 mM β -glycerophosphate to the extraction buffer. To inhibit tyrosine phosphatases, add 100 mM Na_3VO_4 (sodium orthovanadate).
7. For extract to be used on Western blots, a 10-min microcentrifugation to remove cuticle and cellular debris, although not necessary, is recommended. This reduces the number and intensity of crossreacting bands.

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Plant Protein Extraction

Helen E. Conlon and Michael G. Salter

Summary

A method is presented for the extraction of total protein from *Arabidopsis thaliana* tissue. The protocol was designed for the solubilization of a range of proteins and their efficient and quantitative recovery. It is especially compatible with the small quantities of available tissue often associated with this species and was originally intended for Western blot preparations. Samples extracted using this method can be quantitated directly using a commercially available kit.

Key Words: *Arabidopsis thaliana*; total protein extraction; efficient; limited tissue; Western blot.

1. Introduction

Arabidopsis protein extraction methods typically have drawbacks such as recovery of only soluble proteins (1) or protein precipitation steps to enhance extraction efficiency of low-abundance proteins (2). Furthermore, methods that attempt to conquer such extraction inefficiency can result in their own problems. Large quantities (e.g., 1–2 g) of fresh tissue are often used to increase the protein input and minimize the losses; however, this may require many adult plant leaves or seedlings and can be impractical (2). Alternatively, high concentrations of sodium dodecyl sulfate (SDS) can be used to increase protein solubility, but this approach results in the inability to proceed directly to protein quantitation (3).

The following is a rapid, efficient, and quantitative total protein extraction method (4) suitable for the limited quantities of plant tissue often available from *Arabidopsis*. The extraction buffer contains a low concentration of SDS and the reducing agent sodium metabisulfite, making it compatible with direct quantitation methods and negating the requirement for SDS-polyacrylamide gel electrophoresis comparison or trichloroacetic acid precipitation and

resuspension prior to quantitation. The protocol is equally suitable for use with either seedlings or adult plant tissue.

In brief, an extraction buffer is added to fresh tissue prior to homogenization. Subsequent to centrifugation, a proportion of the supernatant is used for protein quantitation so that input into subsequent applications can be normalized. A loading buffer can be added to the remaining supernatant for Western blot analysis.

2. Materials

1. Forceps (FST, Vancouver, British Columbia, Canada).
2. Disposable micropestles, 1.5 mL (Kimble-Kontes, Vineland, NJ, cat. no. 749521-1590)
3. Complete protease inhibitor cocktail tablets (Roche Applied Science, cat. no. 1836153; *see Note 1*).
4. Buffer E: 125 mM Tris-HCl, pH 8.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50 mM $\text{Na}_2\text{S}_2\text{O}_5$. Prepare in a fume hood, as both SDS and $\text{Na}_2\text{S}_2\text{O}_5$ (sodium metabisulfite) are irritants. Store tightly capped and at room temperature to prevent $\text{Na}_2\text{S}_2\text{O}_5$ oxidation and SDS precipitation, respectively.
5. Buffer Z: 125 mM Tris-HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v) β -mercaptoethanol, 0.001% (w/v) bromophenol blue.
6. Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA).

3. Methods

Products of circadian regulated genes will obviously vary in abundance over a time course. Moreover, some products will be present in larger quantities than others. To ensure optimal representation of the protein of interest, the amount of tissue required for the extraction and subsequent supernatant volume for Western blot or other applications should be worked out empirically (for guidelines, *see Note 2*). For other applications *see Note 3*.

3.1. Extraction Procedure

1. Collect whole seedlings or adult plant leaves using forceps.
2. Add the material to 200 μL of buffer E in a 1.5-mL microfuge tube.
3. Homogenize at room temperature using a micropestle. A homogeneous suspension is achieved in approx 30 s to 1 min.
4. If processing more than one sample, immediately transfer the suspension to ice while the remaining extracts are prepared.
5. Warm the samples to room temperature once more, to allow resolubilization of any precipitated SDS (*see Note 4*).
6. Centrifuge the extracts at maximum speed for 10 min in a microfuge and transfer the supernatant to a fresh tube.
7. If required, this method can be scaled up (*see Note 5*).

3.2. Total Protein Quantitation

1. Take 10 μL of the extraction supernatant to use in the Bio-Rad *DC* Protein Assay. This is a Lowry-based method of protein quantitation (5) adapted for compatibility with a range of detergents. The total protein concentration typically ranges from approx 0.5 to 10 $\mu\text{g}/\mu\text{L}$, depending on the amount and nature of the starting material (see Note 2).
2. Samples can be stored at -20°C if required.

3.3. Extract Preparation for Western Blot Analysis

1. Add one-tenth volume of buffer Z to the remaining extract supernatant. Samples at this stage can be stored at -20°C if required.
2. Adjust the loading volume of each sample to take into account the total protein concentration and the abundance of the protein of interest.

4. Notes

1. The concentration of SDS in the buffer is usually sufficient for recovery of undegraded proteins. However, this method is compatible with the addition of protease inhibitors should you experience proteolysis of your protein of interest. Complete mini-protease inhibitor cocktail tablets (Roche) are a convenient source of a wide range of inhibitors. The tablets include inhibitors of serine and cysteine proteases, which are the most prevalent protease classes in plant extracts.
2. This method results in approx 0.5 to 1.5 $\mu\text{g}/\mu\text{L}$ of total protein being extracted from 60 to 70 seedlings at the 4-d stage. Four to five leaves from adult plants (3 wk) generate an extract of 5 to 10 $\mu\text{g}/\mu\text{L}$. For proteins present at an extremely low abundance, 200 μg of total protein may be required for visualization on a Western blot. For highly abundant proteins, however, a total protein loading of approx 15 to 20 μg will be sufficient. This protocol compares favorably with many other reports in terms of amounts of starting material required for protein visualization (2,6). It must be stated that the protein levels required will in part be subject to the affinity of the primary antibody to be used and the film exposure time can also be adjusted accordingly.
3. The method is suitable for immunochemical techniques where a linear epitope is detected by the primary antibody. This is because the SDS, which aids the extraction efficiency, will also denature the protein and abolish any conformational epitope. If an ELISA is to be used, the alkalinity of the extraction buffer E would be optimal for plate binding; however, the extract must be added to the plate before the antibodies (a sandwich ELISA format, where a capture antibody is adhered to the plate prior to extract, would not be possible). The washing steps prior to blocking would remove the SDS and therefore permit antigen-antibody binding; however, the proteins would not return to their native conformation, hence the requirement for a linear epitope-specific antibody.

The SDS in the extraction buffer also abolishes the protein charge, and therefore its isoelectric point, as it confers a negative charge throughout the length of

the polypeptide. This is incompatible with the isoelectric focusing dimension of two-dimensional gel electrophoresis, which separates proteins on the basis of their isoelectric point. Any contaminating phenolic compounds in the extract will also affect this stage. Whatever the extraction procedure used, it is possible to clean up the samples for two-dimensional gel electrophoresis if required (e.g., 2-D Clean Up Kit, Amersham Biosciences, cat. no. 80-6484-51).

For other applications, consideration should be taken as to whether the pH, SDS, or sodium metabisulfite will have a detrimental effect. The technical support department of your company of choice should be contacted for further information.

4. It is important to ensure complete solubilization of the SDS in the extraction buffer, particularly prior to the extraction procedure. SDS precipitates at low temperatures, so gentle warming for a limited period (e.g., 37°C, 1–2 min) will aid its solubilization if necessary. Care should be taken not to overheat the buffer, however, as prolonged exposure to temperatures higher than 25°C or fluctuation of temperature can affect the reducing capability of the sodium metabisulfite within the buffer.

Failure to fully solubilize the SDS after the incubation on ice will result in the compound pelleting with the other insoluble matter. This will also occur if a refrigerated centrifuge is used that has not been warmed to, and set at, room temperature.

5. If multiple extractions on the normal scale are impractical for the amount of total protein required, a larger mortar and pestle may be used and the volume of extraction buffer E scaled up. If possible, the homogenization should be carried out in a fume hood or goggles and a mask should be worn. Once ground up, the sample can then be transferred to an appropriately sized tube on ice and the subsequent steps of the procedure followed.

Alternatively, the tissue can be ground up in liquid nitrogen and mixed with an appropriate volume of buffer E at room temperature promptly to prevent proteolysis. A spatula dipped regularly in liquid nitrogen will aid the transfer of the ground tissue from both the mortar and the pestle and minimise sample losses. Goggles should be worn and general care taken when using liquid nitrogen.

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Protein Extraction From Mammalian Tissues

Choogon Lee

Summary

For Western blotting and coimmunoprecipitation (coIP), protein samples must be extracted from tissues. The protocol described in this chapter has been used to extract clock proteins from mammalian tissues as diverse as liver, kidney, and brain. The extraction protocol is mild enough to be used for coIP as well as Western blotting. Simply, clock proteins are extracted from tissues by freezing and thawing, and homogenizing with a handheld homogenizer. This procedure extracts most (>90%) of the clock proteins from mammalian tissues.

Key Words: Clock proteins; Western blotting; coimmunoprecipitation; extraction; mammalian tissue.

1. Introduction

Protein extraction is the first step for many biochemical procedures, such as immunoassays, protein kinase assays, and protein purification. For the best results, extraction conditions must be adjusted according to the nature of the proteins to be studied (e.g., membrane vs cytoplasmic proteins) and the assay to be used (e.g., Western blotting vs coimmunoprecipitation [coIP]). If protein–protein interactions are examined by coIP, harsh conditions employing ionic detergents and high concentrations of salt should be avoided, because they can disrupt protein–protein interactions. However, harsh conditions may be more efficient for extracting certain proteins. For example, extraction of integral membrane proteins requires harsher conditions than does extraction of cytoplasmic proteins.

All the known mammalian clock proteins can be extracted in mild conditions (*1*), which can preserve integrity of clock protein complexes for coIP. When extraction is performed, protease inhibitors must be added to block the possible degradation of proteins caused by various cellular proteases. This can

be achieved by adding a cocktail of protease inhibitors and EDTA to the extraction buffer. To extract proteins from tissues, soluble intracellular contents must be released from cells. Cell disruption can be easily accomplished by freezing–thawing and mechanical shearing with a homogenizer. Cell debris and chromosomal DNA are removed by centrifugation.

Relative or absolute amounts of total protein in tissue extracts must be determined by a total protein quantitation method such as the Bradford method (2). This is important for measuring quantitative changes in clock proteins in a specific tissue over a circadian time course. Once the concentration of total protein is determined, the extracts must be processed immediately, or frozen and kept at -80°C to prevent possible degradation of the protein or deterioration of posttranslational modifications (e.g., phosphate groups).

2. Materials

1. Mini-homogenizer (e.g., Kontes).
2. Plastic pestles (e.g., Kontes).
3. Extraction buffer (EB; *see* **Notes 1** and **2**): 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.05% Triton X-100, 1 mM dithiothreitol (DTT), 5 mM sodium β -glycerophosphate, 0.5 mM sodium orthovanadate, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{mL}$ aprotinin, 5 $\mu\text{g}/\text{mL}$ leupeptin, 2 $\mu\text{g}/\text{mL}$ pepstatin.
4. Total protein quantitation reagent (e.g., Coomassie Plus solution, Pierce).
5. Bovine serum albumin (BSA).

3. Methods

When tissues are collected, they should be frozen in dry ice and kept at -80°C until they are used. All procedures must be done with prechilled reagents on ice or in a cold room.

3.1. Homogenization

1. Break a big piece of frozen tissue into small pieces and transfer them into a 1.5-mL microcentrifuge tube (*see* **Note 3**).
2. Add 5 vol of EB to the tube.
3. Homogenize the tissue with 10 to 15 strokes (3–4 s/stroke) using a mini-homogenizer and plastic pestle on ice.
4. Spin at 12,000g for 15 min at 4°C .
5. Transfer the supernatant to a fresh tube. Try not to take any lipid from the surface layer or any precipitated particle from the bottom. They may interfere later with Western blotting and coIP. Save the pellet, if the efficiency of extraction needs to be determined (*see* **Note 4**).
6. Spin again at 12,000g for 10 min at 4°C .
7. Transfer supernatant to a fresh tube.

3.2. Quantitation of Total Protein in Extracts

The following is a method to quantify total protein in extracts using Coomassie Plus Protein Assay Reagent (Pierce).

1. Pipet 998 μL of H_2O into appropriately labeled tubes that can hold more than 2 mL liquid.
2. Add 2 μL of protein extract to each tube. Make a blank control by adding 2 μL of EB instead. To build a standard curve, prepare 3 to 5 samples by adding 2 μL of a BSA solution at known concentration (e.g., 0.05, 0.2, 0.5, and 2 mg/mL) to the tubes.
3. Add 1 mL Coomassie Plus solution to each tube and mix well.
4. Set a spectrophotometer at 595 nm and calibrate the “zero” using the blank.
5. Measure the absorbance of the samples. If BSA controls were used, create a standard curve to determine the protein concentration of the samples (*see Note 5*).

4. Notes

1. EB is made using the following stock solutions:
 - a. 500 mM HEPES: dissolve 23.8 g of HEPES (free acid form) in 200 mL of H_2O , adjust pH to 7.5 with HCl or NaOH, filter, and store at 4°C.
 - b. 4 M NaCl: add 58.4 g of NaCl to 250 mL of H_2O and filter.
 - c. 10% Triton X-100: dissolve 10 mL of Triton X-100 in 90 mL H_2O .
 - d. 1 M DTT: dissolve 1.54 g of DTT in 10 mL of 20 mM sodium acetate, pH 5.2, filter, make 1 mL aliquots, and store at -20°C .
 - e. 1 M sodium β -glycerophosphate in H_2O .
 - f. 0.5 M sodium orthovanadate: make 500 mM solution in H_2O , adjust pH to 10.0 with HCl or NaOH, boil the solution until it turns colorless, and let it cool down to room temperature. Measure pH. If pH has changed significantly, readjust pH to 10.0 and boil again until it becomes colorless. Repeat this step until pH stabilizes near 10.0. Make 1-mL aliquots and store them at -20°C .
 - g. 500 mM EDTA: make 500 mM solution in H_2O and filter.
 - h. 100 mM PMSF: make 100 mM solution in isopropanol and store at -20°C .
 - i. 10 mg/mL aprotinin: dissolve 10 mg in 1 mL H_2O and store at -20°C .
 - j. 5 mg/mL leupeptin: dissolve 5 mg in 1 mL H_2O and store at -20°C .
 - k. 2 mg/mL pepstatin: dissolve 2 mg in 1 mL ethanol and store at -20°C .
2. DTT (reducing agent), sodium β -glycerophosphate (general phosphatase inhibitor), sodium orthovanadate (tyrosine phosphatase inhibitor), EDTA (chelant) and PMSF, aprotinin, leupeptin, and pepstatin (protease inhibitors) need to be added to the EB immediately prior to use.
3. When transferring the pulverized material into a microcentrifuge tube, make sure not to add more than the equivalent of 100 μL of tissue, as the sample may splash out during homogenization. Select a round-bottom tube so that the pestle (used in **step 3**) can touch the bottom of the tube. This facilitates complete homogenization of the tissue.

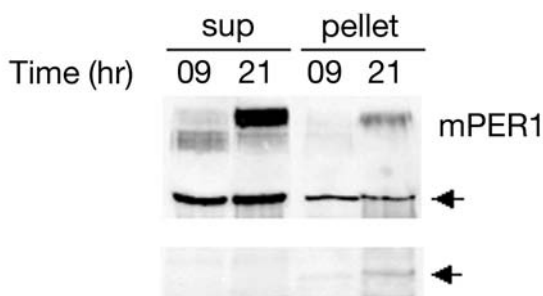


Fig. 1. Determination of the efficiency of protein extraction. Proteins were extracted from mouse liver collected at two time points (9 and 21 h after lights-on in a 12-h light:12-h dark environmental cycle). Tissue debris (pellet) after the first centrifugation were resuspended in 1X sample buffer in the same volume as the supernatant (sup), boiled for 3 min at 95°C, and sonicated briefly. Both the supernatant and the resuspended pellet were run on the same gel, blotted, and immunoassayed with anti-mPER1 antibodies to assess the efficiency of the extraction condition. The arrows indicate nonspecific bands. mPER1 and the top nonspecific protein were extracted efficiently, whereas only a minor portion of the bottom nonspecific protein was extracted under these conditions.

4. When extracting a protein for the first time, it is important to assess the efficiency of extraction, because potential poor solubility in the EB used can seriously bias any downstream application. To determine the efficiency of extraction a comparison is made (by Western blot) between the soluble (in the supernatant) and the insoluble (in the pellet) fractions (**Fig. 1**). First the pellet is washed with EB to remove any remaining soluble protein, then the insoluble fraction is extracted from the tissue debris with 1X sodium dodecyl sulfate (SDS) sample buffer (diluted from a 2X SDS sample buffer stock: 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 2 mM EDTA, 0.1 mg/mL bromophenol blue). To remove the soluble proteins, resuspend the pellet in 5 to 10 vol of EB, spin at 12,000g at 4°C for 5 min, and remove the supernatant. To extract the insoluble fraction add 3 to 5 vol of 1X SDS sample buffer (the high concentration of SDS and 2-mercaptoethanol does solubilize most proteins except cytoskeletal proteins) to the tissue debris and homogenize as described in the protein extraction methods. Sonicate the sample if it is too viscous. Before loading on the gel, heat the sample at 95°C for 3 min, and centrifuge at 12,000g for 5 min. If a minor fraction of the protein was extracted, increase the concentration of detergent and salt in the EB and/or change the homogenization method. 1X SDS sample buffer can be directly used to extract proteins for Western analysis. In this case, however, it is difficult to measure protein concentration.
5. If the same protein quantitation method is used repeatedly, it is not necessary to include the BSA controls for every experiment. The BSA controls need to be

done for only the first two or three experiments. Absorbance data of samples can be used to estimate amounts of total protein present in the samples based on previous standard curves.

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Western Blotting

Choogon Lee

Summary

Western blotting is one of the most commonly used biochemical techniques to detect a specific protein from a mixture of proteins such as tissue extracts. Antibodies to the specific antigen are used to detect the protein. The mixture of proteins is resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a membrane. A specific antigen immobilized on the membrane is detected and visualized by a primary antibody, a secondary antibody–peroxidase conjugate, and a chemiluminescent reagent.

Key Words: Western blotting; SDS-PAGE; primary antibody; secondary antibody; chemiluminescence.

1. Introduction

Western blotting is a very sensitive and efficient assay to detect and characterize *in vivo* proteins present in small amounts, such as clock proteins. In combination with other biochemical techniques, Western blotting can also be used to determine molar amounts, posttranslational modifications, half-life, and other properties of clock proteins (1–3). Western blotting consists of four parts: extraction of protein samples, gel electrophoresis, electroblotting, and detection of a specific antigen. Detailed protocols for the extraction of proteins from different model organisms can be found in Chapters 26–28 and 32.

Extracted proteins from tissues are resolved by a form of gel electrophoresis known as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; ref. 4), in which proteins are mixed with a buffer containing SDS prior to loading onto a polyacrylamide gel. SDS binds proteins and confers negative charge to the proteins. Because SDS uniformly binds proteins, most proteins will be negatively charged in proportion to their molecular mass. When an electrical field is applied to a polyacrylamide gel matrix, the negatively charged proteins migrate through the polyacrylamide gel matrix toward the anode.

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Because the negative charge per unit mass of protein is roughly uniform, the migration rate of the proteins will depend primarily on the size of the proteins. Thus, proteins are separated according to their molecular mass.

The separated proteins are then transferred from the gel to a membrane by electroblotting. Polyvinylidene fluoride and nitrocellulose membranes are most commonly used. Although each membrane has advantages and disadvantages, nitrocellulose membranes are easier to use and are more commonly used in laboratories studying circadian clocks. Electroblotting can be performed using either a wet transfer or a semidry transfer system. The semidry system requires less time and reagents, and produces results comparable with the wet transfer system.

The final step of Western blotting is to detect a specific antigen immobilized on the membrane using primary and secondary antibodies and a chemiluminescent reagent. The antigen is specifically recognized and bound by a primary antibody, which is also specifically associated with a secondary antibody. The secondary antibody is conjugated with the enzyme horseradish peroxidase, which catalyzes a reaction with a chemiluminescent reagent to produce light. The light output can be imaged on films or by a charge-coupled device imaging system.

2. Materials

1. 30% Acrylamide: 29.2% acrylamide/0.8% *bis*-acrylamide in H₂O (*see Note 1*).
2. H₂O-saturated isobutyl alcohol (*see Note 2*).
3. 1 M Tris-HCl, pH 8.8 (*see Note 3*).
4. 1 M Tris-HCl, pH 6.8 (*see Notes 3 and 4*).
5. 10% SDS (*see Note 3*).
6. 25% ammonium persulfate (APS; *see Note 5*).
7. TEMED.
8. 2X SDS sample buffer: 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol (2-ME), 2 mM EDTA, 0.1 mg/mL bromophenol blue (*see Note 6*).
9. 5X Electrophoresis buffer: 15.1 g Tris base, 72 g glycine, and 5 g SDS in 1 L of H₂O.
10. Prestained molecular-weight (MW)-marker mixture (e.g., Kaleidoscope standards from Bio-Rad).
11. Electrophoresis system: Bio-Rad Mini-PROTEAN 3 or equivalent; 0.75-mm spacers are recommended.
12. Power supply capable of providing constant voltage of 150 V or higher.
13. 5X transfer buffer: 15.1 g Tris and 72 g glycine in 1 L H₂O.
14. 1X transfer buffer: Mix 300 mL of H₂O, 100 mL of 5X transfer buffer, 100 mL of methanol, and 1.9 mL of 10% SDS.
15. Semidry transfer apparatus (e.g., Trans-Blot SD, Bio-Rad).
16. Nitrocellulose membrane (e.g., Protran nitrocellulose membrane BA-85, S&S).

17. Extra-thick blot paper (e.g., Bio-Rad); 2–3 mm thickness.
18. Ponceau S staining solution: 0.25% (w/v) Ponceau S in 1% acetic acid.
19. Primary antibody: antigen-specific.
20. Secondary antibody–horseradish peroxidase conjugate (e.g., Jackson Immuno-laboratories): species-specific for the animal in which the primary antibody has been raised.
21. Nonfat dry milk (e.g., Bio-Rad or Carnation).
22. Tris-buffered saline–Tween-20(TBS-T): 0.9% NaCl, 20 mM Tris-HCl, pH 7.5, and 0.05% Tween-20.
23. Blocking and antibody dilution solution: dissolve 5 g of nonfat dry milk in 100 mL of TBS-T.
24. 10% Thimerosal (a preservative).
25. Chemiluminescence reagent (e.g., ECL, Amersham).
26. X-ray film (e.g., X-O-MAT AR, Kodak).
27. Stripping solution: 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM 2-ME.

3. Methods

3.1. SDS-PAGE

1. Prepare samples as described in the protein extraction method of your choice. Mix the extracts with 1 vol of 2X sample buffer and boil at 95°C for 3 min (*see Note 7*). Centrifuge samples at 12,000g for 30 s after boiling to pellet undissolved particles and bring down moisture from the wall of the tube into the solution. Allow the samples to cool down to room temperature before loading onto a gel.
2. Assemble a glass plate sandwich on a casting stand according to instructions provided by the manufacturer of the electrophoresis system. The two glass plates will be separated by spacer strips at two opposite edges, and the gel will be poured into the space between the plates.
3. Prepare resolving and stacking gel solutions according to [Table 1](#). Volumes given are for two gels with the Bio-Rad Mini-Protean system. Volumes should be adjusted, if different systems are used.
4. Add 15 μL of 25% APS and 10 μL TEMED to the resolving solution and use immediately. Acrylamide should begin to polymerize within 5 min.
5. Pour the gel solution into the sandwich with a pipet until the height of the solution reaches three-fourths of that of the small glass plate (*see Note 8*).
6. Overlay the solution with 500 μL of H_2O -saturated isobutanol. Be careful not to disturb the surface of the solution (*see Note 9*).
7. Let the solution polymerize for 30 min. A line will be visible between the polymerized gel and the isobutanol layer because of differential light transmission of the two layers (*see Note 10*).
8. Pour off any liquid from the top of the gel and rinse the surface with H_2O . Remove H_2O as much as possible. Remaining H_2O may cause the stacking gel to shrink.

Table 1
Recipes for Resolving and Stacking Gel Solutions

% Polyacrylamide (resolving gel)	6	8	10	15	Stacking gel
30% Acrylamide/ bis-acrylamide (mL)	2	2.7	3.3	5	0.75
1 M Tris-HCl, pH 8.8 (mL)	4	4	4	4	—
1 M Tris-HCl, pH 6.8 (mL)	—	—	—	—	0.5
10% SDS (mL)	0.1	0.1	0.1	0.1	0.05
H ₂ O (mL)	3.9	3.2	2.6	0.9	3.7
Total volume (mL)	10	10	10	10	5

This recipe produces enough gel solutions to make two mini-gels for Bio-Rad Mini-PROTEAN 3 system. If a different system is used, adjust the volumes of the solutions according to instruction manuals.

All solutions must be prepared with Milli-Q-purified or double-distilled H₂O, and filtered through a 0.45- μ m filter.

After all components are added, mix the solution gently. Be careful not to make foam.

9. Add 7.5 μ L of 25% APS and 7.5 μ L TEMED to the stacking gel solution and pour immediately into the glass plate sandwich, until the solution reaches the top of the small glass plate. Pay attention not to introduce bubbles. The solution should start to polymerize within 5 min.
10. Insert a 0.75-mm comb into the sandwich. If bubbles are trapped below the comb, remove the comb, add more stacking gel solution to the top of the small glass plate, remove bubbles, and insert the comb again. Because acrylamide polymerizes quickly, the whole procedure should be done as quickly as possible.
11. Allow the stacking gel to polymerize for 30 min. Make sure that the gel has polymerized by checking the leftover solution in the original container (*see Note 10*).
12. Attach the glass sandwich to the electrophoresis system according to the instruction manual.
13. Pour 1X electrophoresis buffer into the upper buffer chamber and the lower chamber. The top and bottom of the gel should be submerged in buffer for electric current to flow through the gel matrix. If excessive bubbles are trapped in the bottom of the gel, they should be removed by a syringe with a curved needle (*see Note 11*).
14. Remove the comb carefully, making sure not to disrupt wells. Rinse the wells with 1X electrophoresis buffer using a syringe with a thin needle to get rid of gel pieces that may be present. These gel pieces can result in uneven migration of proteins.
15. Load samples and a mixture of prestained MW markers into separate wells (*see Notes 12 and 13*). The mixture of MW markers should be also boiled until the

precipitate dissolves completely. If possible, use an equal volume of samples across the lanes on a gel (*see Note 14*). Load an equal volume of 1X sample buffer into unused wells.

16. Check buffer levels before connecting the electrophoresis cell to a power supply. The bottom and top of the gel, and both cathode and anode electrodes, should be submerged under the electrophoresis buffer.
17. Make sure that the power supply is off before it is connected to the electrophoresis tank. Connect the electrode of the upper buffer chamber to the cathode (–) outlet of the power supply and that of the lower buffer chamber to the anode (+) outlet.
18. Set the power supply at 150 V of constant voltage and start running (*see Note 15*).
19. Stop the power supply when bromophenol blue dye reaches the bottom of the gel or a desired resolution is achieved (*see Note 16*).
20. Remove the glass sandwich from the electrophoresis tank and take off one of the two glass plates. Do not take off the gel from the other glass plate because it is easier to handle the gel while it is attached. Be careful not to tear the gel.
21. Remove the stacking gel along with the top 1 to 2 mm of the separating gel. It is difficult to align a whole gel on a blot membrane because the stacking gel is sticky and will adhere to the membrane.

3.2. Electroblotting

1. Place the gel along with the glass plate in a tray containing 1X transfer buffer.
2. Remove the gel gently from the glass plate. The gel usually comes off the glass plate when the glass plate is gently shaken in the transfer buffer.
3. Prepare a nitrocellulose membrane and two layers of blot paper. Each blot paper layer should be 2- to 3-mm thick (*see Note 17*). Cut the membrane and the blot paper so that their length and width are each 1 cm larger than the gel. If two gels are transferred together on the same membrane, double the area of the membrane and the blot paper. Wet the membrane and blot papers with 1X transfer buffer.
4. Sandwich the gel and the membrane between the two blot paper layers and arrange this sandwich on the anode plate of the semidry blotting apparatus as shown in **Fig. 1** (*see Note 18*).
5. Remove bubbles from the gel-membrane blot paper sandwich by rolling a plastic pipet on the top blot paper from one end to the other. Repeat this in the other direction. Do not push too hard while rolling the pipet; it may squeeze the gel out of the sandwich.
6. Wipe off transfer buffer from the surrounding area of the sandwich.
7. Place the cathode plate on the sandwich. Avoid sideways movement of the cathode plate, as it may misalign the sandwich.
8. Connect the blotting apparatus to a power supply. Do not switch the polarity. Unlike SDS-PAGE, electroblotting requires low voltage and high current. A power supply for electroblotting should be able to produce 2 A or higher.
9. Set the power supply at 20 to 23 V of constant voltage and start running (*see Note 19*).

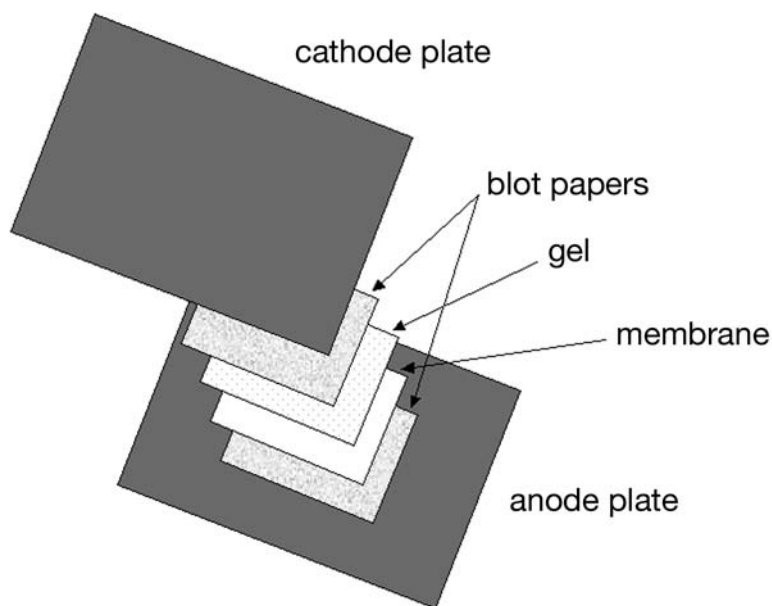


Fig. 1. Assembly of the transfer sandwich for protein gel electroblotting.

10. Stop the power supply and take off the upper electrode carefully.
11. Remove the top blot paper and the gel. Check remaining prestained markers on the gel and transferred markers on the membrane. If most of the markers were transferred, it indicates that most of your proteins of interest were also transferred.
12. Wash the membrane briefly with TBS-T.
13. Remove TBS-T and add Ponceau S solution just enough to cover the membrane.
14. Shake the tray by hand for a couple of minutes. The protein bands should be readily visible. Visually assess the efficiency of the transfer (*see Note 20*).
15. Remove the Ponceau S solution and wash the membrane with TBS-T until the staining is completely washed off.
16. Add blocking solution and incubate at room temperature for 30 min.

3.3. Immunodetection

1. Remove the blocking solution and add the primary antibody diluted in blocking solution (*see Notes 21 and 22*).
2. Incubate the primary antibody at room temp for 2 to 3 h or at 4°C overnight with gentle shaking.
3. Remove the primary antibody (*see Note 23*).
4. Wash the membrane with TBS-T at room temp for 10 min. Repeat this procedure three times.

5. Add secondary antibody diluted in blocking solution. Incubate at room temperature for 1 h with gentle shaking.
6. Remove the secondary antibody.
7. Wash the membrane with TBS-T at room temperature for 10 min. Repeat this procedure six to eight times.
8. After the final wash, drain TBS-T as much as possible and add a chemiluminescent reagent (e.g., ECL, Amersham).
9. After 1 to 2 min incubation, pick up the membrane with a pair of forceps and drain the chemiluminescent reagent as much as possible by allowing the membrane to touch an absorbent such as a paper towel.
10. Put the membrane between two sheets of plastic wrap to prevent films from getting wet.
11. Record the signal by exposing the blot to an X-ray film in a dark room or by using a charge-coupled device imaging system. Signals should be visible within 30 min (*see Note 24*).
12. If necessary, the blot can be stripped and reprobed with a primary antibody against a different antigen, saving time and samples (*see Note 25*).

4. Notes

1. Dissolve 29.2 g acrylamide and 0.8 g *bis*-acrylamide in 70 mL H₂O, add H₂O to 100 mL, and filter the solution with a 0.45- μ m filter. Acrylamide is light-sensitive. The container should be covered with foil or otherwise shielded from light. Acrylamide is also a neurotoxin. When weighing acrylamide powder, a mask and gloves should be worn. When handling acrylamide solution, gloves should be worn.
2. Mix 1 vol of isobutyl alcohol and 1 vol of H₂O by a vigorous shaking and allow to stand overnight. The top layer is water-saturated isobutyl alcohol and the bottom layer is water. Use only the top layer.
3. Filter the solution with a 0.45- μ m filter.
4. pH may change during storage. If pH changes more than 0.5, discard and make a fresh batch.
5. Dissolve 2.5 g APS in 8 mL H₂O, add H₂O to 10 mL, filter (0.45 μ m) and make 1-mL aliquots. Store a working aliquot at 4°C and the rest of the aliquots at -80°C.
6. Filter and store in 1.0-mL aliquots at -20°C.
7. SDS and 2-ME will denature proteins and reduce intra- or intermolecular disulfide bonds, which also inactivates most, if not all, proteases present in the extracts. After boiling, the samples can be stored at -80°C indefinitely and repeatedly thawed and frozen. If protein samples were already mixed with 2X sample buffer and are taken from -80°C, they just need to be boiled for 1 min or until precipitate dissolves completely.
8. Care should be taken in avoiding to introduce bubbles, as the solution contains SDS, which is a detergent and prone to foaming.
9. The H₂O-saturated isobutanol layer ensures that the top of the gel is flat after polymerization.

10. If the gel does not polymerize within 30 min, 25% APS and/or TEMED should be replaced with fresh aliquots or fresh reagents should be purchased.
11. If there is a leak from the upper buffer chamber, it should be fixed or the system will have to be reassembled. Insufficient buffer in the upper chamber can cause partial overheating of the glass plates, which can lead to breakage of the glass plates during the run. It is always safer to monitor the level of the upper buffer during run and replenish the buffer if necessary.
12. When 10 well combs are used in the Bio-Rad Mini-PROTEAN system, 20 to 50 μ g total protein is recommended. If too much protein is loaded, resolution will be poor.
13. Samples can be loaded using either a Hamilton syringe or a pipettor with a disposable gel-loading tip. The tip of the needle or the disposable tip should be thin enough to be inserted between the two glass plates. When samples are applied, the tip of a needle or a pipet tip should be as close as possible to the bottom of the well. This minimizes mixing of the sample with electrophoresis buffer during loading.
14. If too much or too little sample volume is used compared with adjacent wells, protein samples will spread into adjacent wells or will be compressed by protein samples in adjacent wells, respectively. If the volume of a sample is less than half of that in adjacent lanes, add 1X SDS sample buffer to normalize the volume.
15. It is more convenient to use constant voltage than constant current because the voltage does not need to be changed according to the number of gels run using the same power supply. Current will be proportionally increased as the number of gels connected to the power supply increases. If current reads too high or too low, it is most likely that there is a bad connection or that the electrophoresis buffer was not correctly made.
16. An adequate percentage of polyacrylamide should be used to obtain well-resolved Western blot results. This is particularly important to detect different isoforms of clock proteins as a result of phosphorylation. The following is recommended:
 - 6%: proteins of MW 100 kDa or more.
 - 8%: MW 50–100 kDa.
 - 10%: MW 50 kDa or lower.
17. If the blot paper is too thin, the transfer buffer will dry out during the procedure. If paper that is thinner than 2 to 3 mm is used, stack multiple sheets together to achieve the appropriate thickness.
18. If prestained markers were run on the gel, there is no need for marking the membrane for lane orientation. If two gels are transferred on the same membrane, each gel can be identified by using different amounts of prestained markers or using different lanes for the markers on two gels.
19. It is more convenient to use constant voltage than constant current because the current will need to be adjusted according to the number of gels being electroblotted. When the appropriate percentage of polyacrylamide and the Bio-

Rad apparatus are used, all the known mammalian clock proteins can be successfully transferred at 23 V within 30 min.

20. If uneven transfer is observed, check the anode and cathode plates. They can distort or sag over a long period of use. This can cause uneven transfer of proteins. If this occurs, replace the defective plate(s) with new one(s).
21. To save primary antibody, the membrane can be trimmed or cut into pieces. Moreover, heat-sealable plastic bags can be used instead of trays.
22. If the size of two clock proteins is substantially different, they can be assayed at the same time using a single gel. Run SDS-PAGE long enough to separate the two proteins by a reasonable distance. Transfer proteins to a membrane, stain the membrane with Ponceau S and cut the membrane between the expected positions of the two clock proteins using the prestained MW markers as a reference.
23. If primary antibody is to be used more than once, add thimerosal to the solution. Make a 10% thimerosal stock solution and add it to the primary antibody solution to make 0.1% thimerosal. Freeze primary antibodies in dry ice and store them at -80°C .
24. If no signal (including background signal) is visible, it is most likely that either the primary or the secondary antibody have not been added, or that the secondary antibody is not compatible with the primary antibody. This could happen, for example, if the primary antibody was generated in rabbits, and the secondary antibody was generated against rat IgG.
25. Wash the blot with TBS-T twice before incubating it in stripping solution. Incubate the blot in stripping solution at 50°C for 30 min with gentle shaking. Remove the stripping solution. Add TBS-T and incubate at room temperature for 10 min. Repeat this step three times to remove remaining SDS and 2-ME from the blot. Incubate the blot in blocking solution at room temperature for 30 min. The blot is ready for incubation with a different primary antibody. After the second immunodetection, the blot can be used again for a third time. However, signal intensity will be significantly reduced after each stripping compared with a fresh blot.

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Coimmunoprecipitation Assay

Choogon Lee

Summary

As with most other proteins, clock proteins physically interact with one another. Coimmunoprecipitation (coIP) is the most straightforward technique to study protein–protein interactions *in vivo*, if antibodies against the proteins of interest are available. To perform coIP, first an antibody against a target protein is coupled to Sepharose beads through protein A or G, then the complexes containing the target protein are immunoprecipitated with the antibody-coupled beads by centrifugation. Protein components in the complexes are visualized by Western blotting using antibodies specific to the different components.

Key Words: Coimmunoprecipitation; protein–protein interactions; protein A/G; protein complexes.

1. Introduction

Coimmunoprecipitation (coIP) has been crucial in understanding protein function in many areas, including circadian biology. Although coIP is a simple, yet powerful, technique to study the function of proteins *in vivo*, sometimes it is not an option because an antibody against the protein of interest is not available. If interaction between two proteins is suspected but antibodies are not available, coIP can still be performed using tagged proteins expressed in cultured cells or *in vitro*. DNA sequences coding for short (10–20 amino acids) peptide tags, such as the hemagglutinin or myc epitopes, can be inserted onto the C- or N-terminus of proteins by recombinant DNA technology. Antibodies to these tags are commercially available.

CoIP consists of four steps: preparation of protein extract, coupling of antibody to beads, isolation of protein complexes, and analysis of the protein complexes. Mammalian clock protein complexes are readily extracted from tissues by the method described in Chapter 29. Sometimes the conditions of extraction

used for Western blotting do not work for coIP, because they are too harsh to preserve the integrity of protein complexes. The best conditions for coIP are those that extract most of the proteins of interest, and yet are mild enough to leave the complexes intact during extraction. The conditions described in Chapter 29 have been successfully used for coIP with *Drosophila* and mammalian tissues. If conditions for protein extraction described in other sections are suspected to be too harsh, they can be modified by decreasing detergent and/or salt concentrations. The modified conditions should be tested to determine whether they improve the results of a coIP assay.

Antibodies can be covalently or noncovalently coupled to beads. Beads are normally made of Sepharose (an agarose derivative) or crosslinked Sepharose for rigidity and are readily pelleted by centrifugation. If antibodies are covalently linked to beads, they are not released during antigen elution and thus can be reused several times. However, their activities drop significantly during the crosslinking procedure, which results in poor recovery of target antigens. Antibodies are readily noncovalently linked to Sepharose beads through bacterial proteins called protein A or G. Beads crosslinked with these proteins are commercially available through a number of vendors (e.g., Amersham). These proteins bind to the Fc region of IgG (1,2).

In the next step, the protein extract and the beads coupled to the antibody are mixed and incubated. During the course of incubation, the protein complexes containing the target antigen become attached to the beads via the bound antibody–protein A/G (Fig. 1). The immune complexes attached to the beads are precipitated by centrifugation and the unbound proteins are washed off. After washing, the immune complexes are released by 2X sodium dodecyl sulfate (SDS) sample buffer and analyzed by Western blotting. More extensive background information for coIP can be found in ref. 3.

2. Materials

1. Protein A- or G-coupled beads (e.g., protein A/G-coupled Sepharose 4 Fast Flow, Amersham; see Table 1 for species specificity of protein A or G).
2. Mini-homogenizer (e.g., Kontes).
3. Plastic pestles (e.g., Kontes).
4. Extraction buffer (EB; see Note 1 and Chapter 29): 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.05% Triton X-100, 1 mM dithiothreitol (DTT), 5 mM sodium β -glycerophosphate, 0.5 mM sodium orthovanadate, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/mL aprotinin, 5 μ g/mL leupeptin, 2 μ g/mL pepstatin.
5. Rotating wheel.
6. 2X SDS sample buffer: 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 2 mM EDTA, 0.1 mg/mL bromophenol blue.

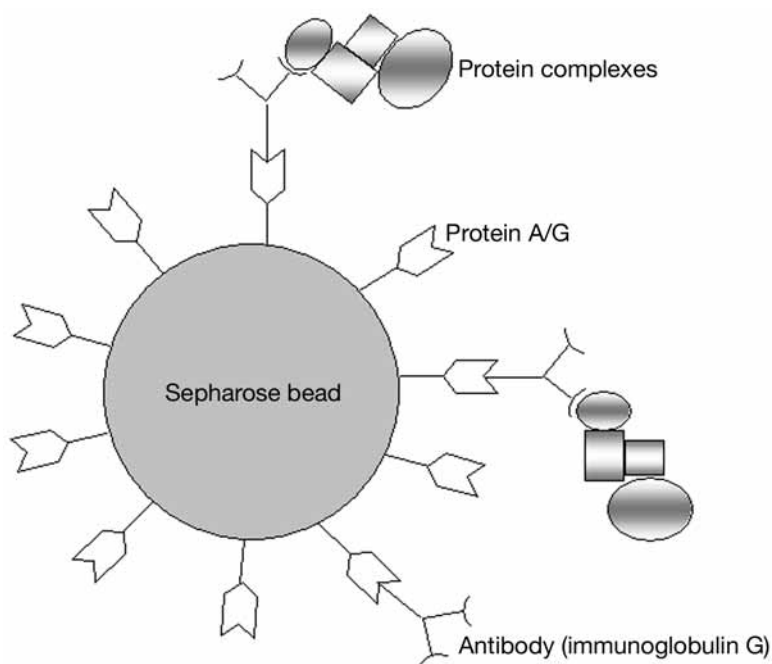


Fig. 1. The principle of the co-immunoprecipitation assay. Protein A/G-coupled beads are commercially available (*see* **Heading 2**). First, an antibody is attached to protein A/G-bearing beads. The antibody-coupled beads are then incubated with tissue extract. During the incubation, protein complexes containing the target antigen for the antibody are bound to the antibody–protein A/G-beads.

3. Methods

All procedures must be performed with prechilled reagents either in a cold room or on ice. However the coupling of the antibody to the beads is performed at room temperature.

3.1. Preparation of Extracts and Preclearing

1. Prepare the protein extract from the tissue of choice as described in Chapter 29. Usually 50 to 100 mg of tissue will yield enough samples to repeat the final Western blot three or four times.
2. Save 10% of the extract, mix with 1 vol of 2X SDS sample buffer, and boil at 95°C for 3 min. This will serve as the “starting sample.”
3. Prepare the beads. Take 20 μ L of beads per reaction (equivalent to 40 μ L of a 1:1 slurry; the beads are normally sold as a 1:1 slurry in 20% EtOH) and equilibrate with 500 μ L of EB for 15 min on a rotating wheel. Centrifuge at 3000g for 10 s

Table 1
Relative Binding Affinity of Polyclonal Antibodies
From Various Species to Protein A or G

Species	Protein A	Protein G
Chicken	–	–
Cow	+	++
Donkey	–	++
Goat	+	++
Guinea pig	++	+
Horse	–	++
Human	++	++
Mouse	+	+
Rabbit	++	++
Rat	+/-	+/-
Sheep	–	++

–, no binding; +/-, weak binding; +, medium binding; ++, strong binding.

and remove the supernatant (*see Note 2*). Repeat this wash step two more times. After the final wash, remove as much liquid as possible and add 2 vol of EB (*see Note 3*).

4. Preclear the extract. Add 10 μL of the equilibrated beads to the extract (equivalent to 30 μL of the EB equilibrated slurry), incubate for 20 min on the rotating wheel, centrifuge at 12,000g for 3 min, and transfer the precleared extract to a new tube.

3.2. Coupling of Antibody to Beads

1. Add the equilibrated beads (10 μL /reaction) to a microcentrifuge tube containing 300 μL of EB. If a same antibody is used for multiple reactions, the coupling for these reactions can be performed in one tube (*see Note 4*).
2. Add the antibody (3–5 μL whole antiserum or 0.5–1 μg affinity-purified antibody/reaction) to the tube and incubate at room temperature for 1 h on a rotating wheel.
3. Centrifuge at 3000g for 10 s (*see Note 2*).
4. Remove supernatant.

3.3. Isolation of Protein Complexes

1. Add the protein extract into the tube containing the antibody–protein A/G beads. If the tube contains antibody-coupled beads for more than one reaction, dispense the beads into an appropriate number of tubes before adding the protein extract.
2. Incubate the reaction at 4°C for 3 to 6 h with rotation (*see Note 5*).

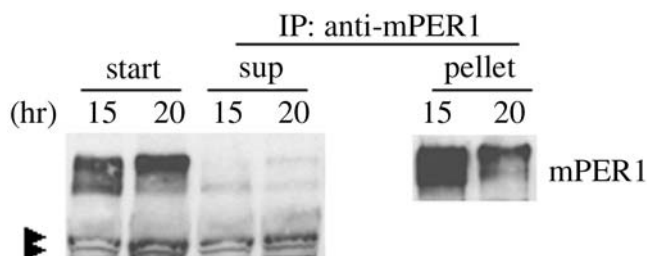


Fig. 2. Determination of the efficiency of immunoprecipitation (IP). Liver tissue was collected at two different time points (ZT 15 and 18) and subjected to IP with an anti-mPER1 antibody. The original extracts (start), the supernatants (sup), and the immune complexes (pellet) were run on the same gel, and immunoblotted to reveal mPER1. The arrows indicate nonspecific bands, which remained in the supernatant fractions. mPER1, on the other hand, was almost immunodepleted from the starting extracts.

3. Centrifuge the tubes at 3000g for 10 s.
4. Remove the supernatant (*see Note 6*).
5. Add 1 mL of EB and incubate for 20 min with rotation.
6. Centrifuge at 3000g for 10 s, remove supernatant, add 1 mL EB, and incubate for 20 min with rotation.
7. Repeat **step 6** four more times.
8. Remove the supernatant as much as possible after the final wash. Be careful not to take any beads while pipetting.
9. Add 20 μ L 2X sample buffer and boil at 95°C for 3 min.
10. Shake the tubes for 5 min. This will ensure release of most immunoprecipitated proteins.
11. Centrifuge at 12,000g for 1 min. The samples are now ready for Western blot analysis.

3.4. Western Blotting

1. Follow the procedure for Western blotting in Chapter 30.
2. If possible, the primary antibody used for detection should have been raised in a species different from the one used to raise the antibody for coIP (*see Note 7*).
3. To determine the efficiency of immunoprecipitation, run supernatant samples along with starting samples (**Fig. 2**).

4. Notes

1. DTT (reducing agent), sodium β -glycerophosphate (general phosphatase inhibitor), sodium orthovanadate (tyrosine phosphatase inhibitor), EDTA (chelant), and PMSF, aprotinin, leupeptin, and pepstatin (protease inhibitors) need to be added to the EB immediately prior to use.

2. High centrifugal force may compromise the integrity of the beads. Consult the instruction manual of your microfuge to infer the conditions of centrifugation (the force will depend on the radius of your centrifuge and the rotational speed).
3. The beads must be well mixed before dispensing; it is helpful to cut off the end of the tip.
4. For example, if five reactions are performed with anti-mCLOCK antibody, 50 μ L beads can be coupled with the antibody in one tube. However, if more than 10 reactions with the same antibody are performed, use a separate tube.
5. Antigen–antibody association is normally completed within 3 to 4 h at 4°C. The incubation can be also done overnight at 4°C. However, overnight incubation may cause deterioration of posttranslational modifications and degradation of the proteins themselves. Epitope(s) of the target antigen should be exposed to antibody; otherwise, the target antigen can not be immunoprecipitated. This is one of reasons that some antibodies work for Western blotting but do not work for immunoprecipitation. In Western blotting, epitopes are less likely to be hidden or inaccessible because the proteins are denatured and immobilized on a membrane. In general, antibodies generated against long peptides (100 amino acids or more) are more efficient for coIP than antibodies against short peptides (10–30 amino acids), because the former can recognize more epitopes of a given protein.
6. If efficiency of immunoprecipitation needs to be determined, save the supernatant. Take an aliquot and mix with the same volume of 2X SDS sample buffer. Boil the sample at 95°C for 3 min.
7. This is especially important to detect proteins whose sizes are similar to IgG heavy chain (~55 kDa) such as CK1 ϵ/δ . The antibody for coIP will be dissolved together with immunoprecipitated protein complexes in 2X SDS sample buffer and run on SDS-polyacrylamide gel electrophoresis. The heavy chain of the antibody for coIP will strongly react with the Western blot secondary antibody, if the same animal species were used for both coIP and detection. This strong signal may obscure nearby bands of lower intensity.

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In Vitro Phosphorylation and Kinase Assays in *Neurospora crassa*

Lisa Franchi and Giuseppe Macino

Summary

Phosphorylation assay is a widespread technique usually necessary for the identification of a specific kinase substrate and/or for the measurement of kinase activity. As an example of the technique, here we describe an assay aimed to test the phosphorylation of the myelin basic protein (MBP) by protein kinase C (PKC), which is overexpressed and purified from *Neurospora*. The kinase is immunopurified from *Neurospora* using the expression vector pMYX2 and the FLAG epitope. The purified PKC and the MBP are then incubated in the presence of radioactive ATP, and the phosphorylated product is separated using the polyacrylamide gel electrophoresis technique.

Key Words: *Neurospora*; PKC; protein expression; phosphorylation; kinase assay.

1. Introduction

Two major aspects of *Neurospora* physiology that are extensively studied are the circadian clock and the mechanism of light perception and regulation. Both these phenomena are tightly regulated at various levels, and posttranslational modifications such as phosphorylation have been shown to be crucial ([1](#)). For these reasons the role of phosphorylation in the regulation of *Neurospora* physiology has become an important field of study, and, consequently, the identification of protocols aimed to study and characterize this activity became necessary. One possible approach to study the catalytic activity of a kinase of interest on a specific substrate is to perform in vitro phosphorylation assays. Here we describe a detailed protocol designed to test the catalytic activity of protein kinase C (PKC), which is overexpressed and purified from *Neurospora*, on the myelin basic protein (MBP, commonly known to be a PKC

substrate). The kinase is cloned in-frame with a sequence encoding the FLAG epitope under an inducible promoter (*qa2*), which is part of the *Neurospora* expression vector pMYX2 (see **Note 1**). This plasmid is transformed into a *Neurospora* wild-type (WT) strain, and its expression is induced using quinic acid. The kinase is purified by immunoprecipitation using a commercially available agarose-conjugated anti-FLAG resin. The phosphorylation reaction takes place when the immunopurified PKC is incubated with radioactive ATP and the substrate MBP. The result is visualized radiographically.

2. Materials

1. *Neurospora* WT strain 74a transformed with pMYX2fK: pMYX2 containing the cDNA of *pkc* fused to the FLAG coding sequence.
2. 30% Quinic acid, filter-sterilized, pH 5.5, adjusted with NaOH.
3. Vacuum pump.
4. Filter paper, 38–43- μ m diameter, porous.
5. Vogel's 50X salts (2; see **Note 2**).
6. Vogel's medium N (minimal): 1X Vogel's 50X salts, 2% (w/v) sucrose.
7. Selective minimal medium: 1X Vogel's 50X salts, 2% (w/v) sucrose, 100 μ g/mL benomyl.
8. Pestle and mortar.
9. Liquid nitrogen.
10. Protein lysis buffer: 100 mM Tris-HCl, pH 7.5, 0.05% Triton-X100, 0.5 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 10 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin (see **Note 3**).
11. Homogenizer.
12. Flag M2 antibody conjugated to agarose beads (Sigma).
13. FLAG competing peptide (Sigma).
14. 5X PKC reaction buffer: 100 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM CaCl_2 , 20 mM MgCl_2 , 20 μ M ATP.
15. [γ - ^{32}P]ATP: a typical commercial source of [γ - ^{32}P]ATP has a specific activity of 3,000 Ci/mmol and a concentration of 10 μ Ci/ μ L.
16. 1 mg/mL MBP (Sigma).
17. 2X Laemmli buffer: 60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% sodium dodecyl sulfate (SDS), 14.4 mM β -mercaptoethanol, 1% bromophenol blue.
18. Acrylamide and SDS-polyacrylamide gel electrophoresis (PAGE) equipment.
19. Phosphoimager.

3. Methods

The methods described below are subdivided into the following sections:

1. *Neurospora* growth and induction of kinase expression.
2. Protein extraction and immunoprecipitation.
3. Phosphorylation reaction and SDS-PAGE.

3.1. *Neurospora* Growth and Induction of Kinase Expression

1. Inoculate 10^7 *Neurospora* conidia (originating from WT strain 74a transformed with pMYX2fK grown in solid flasks or from a frozen stock in water) in 200-mL flasks containing 100 mL of selective minimal media (see **Notes 4** and **5**).
2. Incubate the flasks at 28°C with constant shaking (150 rpm) for about 44 h. In these conditions *Neurospora* grows producing hyphae and at least 1.5 to 2 g of mycelia are usually obtained.
3. Filter the growing mycelia using a vacuum pump and filter paper and resuspend in selective minimal media without sucrose but containing 0.03% quinic acid (see **Note 6**).
4. Incubate the flasks at 28°C with constant shaking (150 rpm) for about 4 h. This is usually sufficient to induce about a 50-fold increase in the expression of *pkc* RNA compared with the endogenous levels (see **Note 7**).
5. Filter the mycelia using a vacuum pump and filter paper and freeze in liquid nitrogen in a polypropylene tube.
6. Grind the frozen mycelia to a powder using a ceramic mortar and pestle, in the constant presence of liquid nitrogen. Mycelia are usually kept at -80°C, where they can be stored for up to 1 mo (see **Note 8**).

3.2. Protein Extraction and Immunoprecipitation

The procedure illustrated above is a general initial step, described in the majority of *Neurospora* protocols, necessary for the production of the starting material from which proteins, RNA, DNA, and so on can be extracted. This section describes the protein extraction and immunoprecipitation steps. Here the general techniques of cell lysis and protein extraction and purification are carried out using buffers that specifically preserve the PKC catalytic activity.

1. Weigh 200 mg of mycelial powder in a vial and resuspend in 1 mL of ice-cold protein lysis buffer.
2. Gently blend the mycelia in lysis buffer with a homogenizer. This step is necessary to destroy the cell walls and release the proteins.
3. Pellet the debris by centrifugation at 12,000g for 15 min at 4°C, move the supernatant (containing the proteins) to a clean vial (see **Note 9**).
4. Immunoprecipitate PKC incubating the protein lysate with 50 μ L of FLAG M2 antibody resin resuspended 1:1 in lysis buffer (50% slurry). The incubation is carried out for 3 h at 4°C on a rotatory wheel at the lowest speed.
5. Precipitate the beads by centrifugation at 10,000g for 10 s. Replace the supernatant with 1 mL of lysis buffer. Repeat this wash two more times.
6. Precipitate the beads by centrifugation at 10,000g for 10 s. Resuspend the beads in 200 μ L of lysis buffer containing 100 μ g/mL of FLAG peptide to elute PKC (see **Note 10**).
7. Precipitate the beads by centrifugation at 10,000g for 20 s and move the supernatant containing the eluted kinase to a clean vial. The eluted PKC can now be used to perform the phosphorylation reaction.

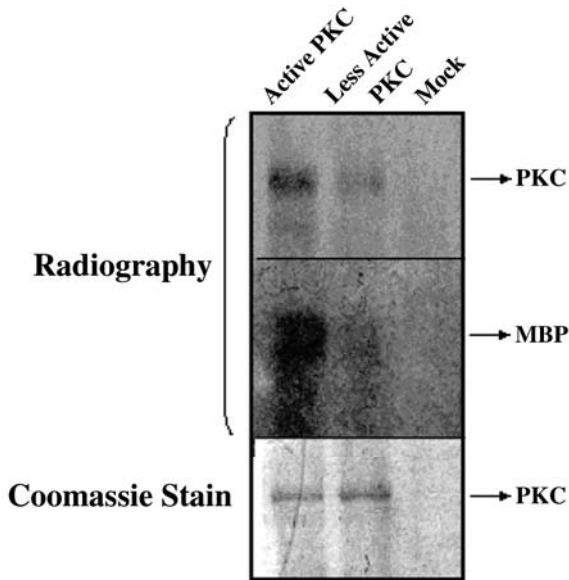


Fig. 1. Immunopurified protein kinase C (PKC) phosphorylates myelin basic protein (MBP). Top panel: phosphorylated PKC. Middle panel: phosphorylated MBP. Lower panel: total PKC protein levels obtained by immunoprecipitation.

3.3. Phosphorylation Reaction and SDS-PAGE

1. To measure the activity of the eluted kinase mix: 40 μ L of eluted kinase, 12 μ L of 5X PKC reaction buffer, 1 μ L of 1 mg/mL MBP, 5 μ Ci [γ - ^{32} P]ATP, H₂O to 60 μ L. Incubate at 30°C for 30 min.
2. Stop the reaction by adding 60 μ L of 2X Laemmli buffer.
3. Denature the proteins by incubating at 95°C for 5 min and separate by running on a 7.5% SDS-PAGE. Usually the whole reaction is loaded on the gel. For optimal separation of the protein bands, the use of a large protein gel apparatus (i.e., Hoefer) is recommended.
4. Wrap the gel in ceramic wrap and analyze in a phosphorimager. The bands detected correspond to proteins (present in the phosphorylation mix) that have incorporated radioactive ATP. This reaction was mediated by the activity of the immunopurified kinase; thus, keeping constant every other condition, the intensity of the bands is proportional to the activity of the kinase. This allows assessment of the activity of the kinase produced under different physiological conditions. In the case where the kinase is active, two bands should appear—the MBP and the autophosphorylated PKC—whereas in the case where the kinase is not active, no bands or lower intensity bands of both MBP and PKC are expected (Fig. 1).

4. Notes

1. See the website of the Fungal Genetics Stock Center, www.fgsc.net/fgn41/campbell.html, for a description of the pMYX2 plasmid.
2. To prepare 1 L of Vogel's 50X salts, dissolve in 750 mL of distilled water (at room temperature) the following (in this order): 125 g $\text{Na}_3\text{citrate}\cdot 2\text{H}_2\text{O}$, 250 g KH_2PO_4 anhydrous, 100 g NH_4NO_3 anhydrous, 10 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 5 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 5 mL of trace element solution (see below), 2.5 mL of biotin solution. Add 2 mL of chloroform as a preservative and store at room temperature. To prepare 100 mL of trace element solution, dissolve in 95 mL of distilled water (at room temperature) the following (in this order): 5 g citric acid $\cdot 1\text{H}_2\text{O}$, 5 g $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 1 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$, 0.25 g $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 0.05 g $\text{MnSO}_4\cdot 1\text{H}_2\text{O}$, 0.05 g H_3BO_3 anhydrous, 0.05 g $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$. Add 1 mL of chloroform as a preservative and store at room temperature. The biotin solution is prepared by dissolving at room temperature, 5 mg of biotin in 50 mL of distilled water. Aliquot and store at -20°C .
3. Add the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and pepstatin immediately before use.
4. *Neurospora* growth conditions may vary slightly from one laboratory to another, depending on the requirements of each experiment. Among the variables are the storage conditions of the conidia. Most experiments require that exactly the same growth conditions are used for all tests done, and it is well known that cycles of freezing and unfreezing of the conidia stored in water reduce the viability of the conidia dramatically. This results in variability of the number of viable conidia inoculated for each experiment. It is, therefore, recommended to prepare aliquots of the stock and test the viability of the conidia for each experiment. It is alternatively possible to use freshly harvested conidia for each experiment, isolated from *Neurospora* growing in solid media.
5. The selection is given by the presence of 100 $\mu\text{g}/\text{mL}$ benomyl, a fungicide to which the strains transformed with the pMYX2fK plasmid are resistant.
6. To induce the *qa2* promoter on the pMYX2fK plasmid and the expression of PKC, the growing mycelia are incubated in the presence of 0.03% quinic acid. The activating effect of the quinic acid on the *qa2* promoter is inhibited by the presence of the carbohydrate contained in the minimal medium. To prevent this inhibitory effect, the mycelia are filtered and then resuspended in minimal medium without sucrose, containing 0.03% quinic acid.
7. Expression levels induced by the *qa2* promoter are very variable and can be very different in transformants generated from the same plasmid. This is why transformants are usually screened for the preferred type of expression: highest, stringent, low, and so on. When a vector is transformed into *Neurospora*, it is randomly integrated into the genome by nonhomologous recombination. Thus, insertions occur by chance in heterochromatic or euchromatic regions, which explains the consequent variability of expression. It is therefore recommended to test the expression level of recombinants every time, preferably by Western blot-

ting, as it is necessary to have high amounts of kinase to perform the kinase assay.

8. It is very important to grind the mycelia very finely, as more proteins will be extracted. However, once the mycelia are grinded they can more easily thaw and it is important to always keep the vials in liquid nitrogen. All subsequent steps are on ice or at 4°C; this is to preserve the activity of the kinase as close as possible to its original state. This is particularly important when the kinase activity is tested in specific conditions (such as light, dark, different circadian times, etc.). Also, mycelia are sometimes stored for longer than 1 mo at -80°C, especially when not needed for delicate experiments; however, it is recommended not to store the samples for too long.
9. This protocol is based mostly on mechanical disruption of the cell, so that it becomes very important to accurately grind and homogenize the mycelia. However this does not favor the isolation of membrane associated proteins. It is known that PKCs are usually found associated to the plasma membrane; therefore, here we add the detergent Triton-100X to the lysis buffer to a final concentration of 0.05%. Higher concentrations would favor the isolation of a higher amount of membrane protein, but it would decrease the efficiency of the following immunoprecipitation. It is therefore important to empirically find the Triton concentration that allows for purification of sufficient amount of PKC and that does not interfere with the immunoprecipitation.
10. Higher amounts of eluted kinase would be obtained by resuspending the pellet in larger volumes, and repeating the step two or more times. This would result in very large final volumes of low-concentration purified kinase. As it is not recommended to perform the phosphorylation assay in very large volumes, it is important to obtain the purified kinase in a highly concentrated small volume. We find that the elution step described here results in an amount of purified kinase sufficient to perform at least five phosphorylation assays. It is important to verify the success of the immunoprecipitation by Western blot.

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VI ---

IN VITRO SYSTEMS

Basic Protocols for *Drosophila* S2 Cell Line

Maintenance and Transfection

M. Fernanda Ceriani

Summary

Cells in culture have been increasingly employed in the dissection of intracellular processes. They are generally easier to handle than the organism of study and certainly less complex, which facilitates testing for specific functions and protein–protein interactions. This chapter will describe the extremely simple steps required to keep a healthy S2 cell culture going.

Key Words: Schneider's cells; S2 cells; transient transfections; stable lines.

1. Introduction

In recent years the explosion of interest in and understanding of the molecular underpinnings of the biological clock made it an absolute requirement to possess an alternative system, ideally less complex than the organism under study, to test specific functions or interactions. Usually it is tempting to resort to lower organisms for that task, although the strategy has not always proven successful. In this regard, the clock community has found an ideal venue on the so-called S2 cells, or Schneider's *Drosophila* line 2 cell line. This cell line was established from late (20–24 h) Oregon-R embryos more than 30 yr ago ([1](#)). Originally three independent embryonic lines were established, of which line 2 is the most widespread used. Notwithstanding their somewhat heterogeneous origin the S2 cells are relatively similar in morphology, predominantly epithelial-like in appearance, and range from 5 to 11 μm in diameter and 11 to 35 μm in length. They grow in a loose monolayer with some tendency to remain in suspension. They are mostly diploid, although they have a tendency to become

tetraploid if seeded too thinly on transfer (**1**). According to the American Type Culture Collection, currently they are 60 to 80% tetraploid and they carry exclusively XX chromosomes (*see* **Note 1**).

This cell line has been used to perform transient expression assays to assess subcellular localization (**2**), transcriptional assays (**3**), and immunoprecipitations (**4**), some of which are described in Chapters 34 and 37.

2. Materials

1. Schneider's S2 cells. The cells can be obtained from the American Type Culture Collection (www.atcc.org/) or purchased from Invitrogen (www.invitrogen.com, cat. no. R690-07).
2. Schneider's cells medium. This medium can be purchased from a number of vendors; we found the most reasonably priced to be Sigma-Aldrich's (cat. no. S 0146). The composition of the original medium is included in **Table 1**.
3. Fetal calf serum (FCS) or fetal bovine serum (FBS) heat-inactivated at 56°C for 30 min.
4. Antibiotics: penicillin G 50 U/mL, streptomycin sulfate 50 µg/mL.
5. T25, T75, and T150 flasks (Corning).
6. Sterile pipets and technique.
7. Sterile polypropylene tubes (Falcon).
8. Laminar flow hood.
9. Drawer at room temperature (22–25°C) or incubator (28°C).
10. Dimethyl sulfoxide.
11. Freezing medium: Schneider's cells medium supplemented with 20% heat-inactivated FCS and 10% dimethyl sulfoxide.
12. 2-mL Sterile vials.
13. Freezer boxes with foam inside
14. 0.25 M CaCl₂ filter-sterilized and aliquoted into 15-mL polypropylene tubes at –20°C.
15. 2X HEBES: 16g/L NaCl, 0.7g/L KCl, 0.4g/L Na₂HPO₄, 2g/L dextrose, 10g/L HEPES (as free acid), pH 7.1. After adjusting the pH with NaOH, filter-sterilize and aliquot in polypropylene tubes at –20°C (*see* **Note 2**).
16. 60-mm Petri dishes (Falcon).
17. 17 × 10 mm polycarbonate tubes (Falcon).
18. Selection vectors: pCoHygro or pCoBlast (Invitrogen)
19. Selective drugs: hygromycin or blasticidin (*see* **Note 3**).
20. Lipofectin (Invitrogen) or other lipid-based reagents.
21. Tissue culture-treated Corning (Costar) 6- and 12-well culture clusters.
22. Neubauer chamber.

3. Methods

This section outlines how to (1) keep and (2) transiently or stably transfect S2 cells.

Table 1
Composition of Schneider's *Drosophila* Medium

	Molecular weight	Concentration (mg/L)	Molarity (mM)
Inorganic salts			
Calcium chloride (CaCl ₂)	111	600	5.4
Magnesium sulfate (MgSO ₄ ·7H ₂ O)	246	3700	15
Potassium chloride (KCl)	75	1600	21
Potassium phosphate (KH ₂ PO ₄)	136	450	2.59
Sodium bicarbonate (NaHCO ₃)	84	400	4.76
Sodium chloride (NaCl)	58	2100	35.90
Sodium phosphate, dibasic (Na ₂ HPO ₄ ·7H ₂ O)	268	1321	9.57
Other compounds			
α-ketoglutaric acid	146	200	1.37
D-Glucose	180	2000	11.10
Fumaric acid	116	100	0.862
Malic acid	134	100	0.746
Succinic acid	118	100	0.847
Trehalose	342	2000	5.85
Yeastolate	Nd	2000	Nd
Amino acids			
β-Alanine	89	500	5.6
L-Alanine	89	400	2.3
L-Aspartic acid	133	400	3.01
L-Cysteine	121	60	0.496
L-Cystine	240	100	0.417
L-Glutamic acid	147	800	5.44
Glycine	75	250	3.33
L-Histidine	155	400	2.58
L-Isoleucine	131	150	1.15
L-Leucine	131	150	1.15
L-Lysine hydrochloride	183	1650	9.02
L-Methionine	149	800	5.37
L-Phenylalanine	165	150	0.909
L-Proline	115	1700	14.80
L-Serine	105	250	2.38
L-Threonine	119	350	2.94
L-Tryptophan	204	100	0.49
L-Tyrosine	181	500	2.76
L-Valine	117	300	2.65

3.1. Maintenance

1. Seed cells in Schneider's cells medium supplemented with 10% heat-inactivated FCS (or FBS) and antibiotics at 22 to 25°C without gas exchange (*see* **Notes 4 and 5**).
2. Cells are maintained in 25- or 75-cm² T-flasks with lids tightly closed. Up to 5 and 10 mL of cells in culture medium can be kept in a T-25 and T-75 flask, respectively.
3. Grow the cells to a density of 1 to 5×10^6 cells/mL.
4. Split the culture into fresh medium at a 1:4 or 1:5 dilution every 3 d. Splitting can be pushed to the limit by doing a 1:10 dilution once a week (this for cells kept at 22–23°C; *see* **Note 6**).

S2 cells do not attach well to the plastic surface (or any other solid substrate) and so they are easily resuspended by gently pipetting up and down; alternatively, a rubber policeman can be employed. No trypsinization is required. Doubling time is about 40 h.

For protein expression purposes these cells can be adapted to grow mostly in suspension-employing spinners or shake flasks.

Because S2 cells do not completely adhere to surfaces it is difficult to rinse the cells if needed. To exchange cells into new medium or to wash cells prior to lysis:

1. Resuspend cells in the conditioned medium and centrifuge at 100g for 2 to 3 min. Decant the medium.
2. Resuspend the cells in fresh medium (or PBS) and centrifuge as above.
3. Repeat.
4. Add fresh medium (or buffer) and replate the cells (or lyse them).

3.1.1. Freezing and Thawing

As with any other cell line, it is highly recommended to keep track of the number of passages that have taken place since the S2 cells in use were first subcultured (*see* **Note 7**).

To freeze cells down:

1. Grow cells to a density of 3 to 5×10^6 cells/mL (log phase) in 30 to 50 mL of medium in a 150-cm² T-flask. Alternatively, two T-75 flasks containing approx 15 mL of medium each could be combined into one.
2. Resuspend cells by pipetting with a sterile technique and transfer the medium into a sterile polypropylene tube. Spin in a tabletop centrifuge at 200g (about 1000 rpm in an Eppendorf 5810R) for 1 to 2 min.
3. Remove the medium by aspiration and resuspend in 1.5 mL of freezing medium.
4. Aliquot 0.5 mL of cells into 2-mL sterile vials. Label and transfer to a freezer box with foam inside, to allow for slow cooling.

5. Transfer to a -70°C freezer overnight (may be longer).
6. For permanent storage transfer the vials to a liquid nitrogen tank.

To thaw:

1. Remove the vial from liquid nitrogen and warm in a water bath at 25°C (or room temperature).
2. Immediately after the medium is thawed, transfer to a 25-cm² (or the equivalent of two vials to a 75-cm²) T-flask with 5 to 10 mL of Schneider's cells medium with 10% FCS.
3. Allow the cells to loosely attach (about 3 h, but may take longer) and replace the medium with a fresh aliquot.
4. Incubate at 25°C for 3 to 5 d.

After thawing cells may have a long lag period (3 to 7 d) before they start to grow.

3.2. Schneider's Cells Transfection

Drosophila Schneider's cells can be transfected with the expression vector alone for transient expression studies or in combination with a selection vector to create stable cell lines. It is advisable to confirm that there is enough expression of the protein of interest by transient transfection before undertaking selection of stable cell lines. Stable lines are useful for long-term storage, increased expression of the desired protein, and large-scale production. Usually stable cell lines contain several copies of the desired construct, which can be manipulated by varying the ratio of expression and selection plasmids (according to Invitrogen's recommendations; *see Note 8*).

Nowadays there are a number of transfection reagents and kits available to transfect this cell line either transiently or stably, the most common ones being from Invitrogen (Lipofectin and Cellfectin), and Qiagen (Effectene). A protocol for transfection with Lipofectin will be described below.

3.2.1. Transfection Assays With CaCl_2 Method

1. Seed 5 mL of Schneider's cells medium supplemented with 10% FCS (or FBS) and antibiotics in a 60-mm dish with 0.2 to 0.3 mL of cell culture (5 to 8×10^6 cells/mL).
2. Incubate at 25°C for at least 6 h or overnight before transfection.
3. Mix 10 μg of plasmid DNA (expression vector) with 0.4 mL 0.25 M CaCl_2 and add to 0.4 mL 2X HEBES dropwise, swirling the mix in 17×10 -mm polycarbonate tubes. Incubate at room temperature for 20 to 30 min; the solution should become slightly cloudy.
4. Add 0.8 mL of this solution per 60-mm dish, swirl, and incubate at 25°C (*see Note 9*).

To select for stably transformed lines:

1. Repeat the procedure above. However, the plasmid DNA used for transfection is now a combination of expression vector and selection vector (*see Note 8*).
2. After 24 h, split cells 1:4 into fresh Schneider's cells medium supplemented with 10% FCS (or FBS) and antibiotics.
3. Wait 24 h longer to add the selective drug.
4. Split cells every 7 to 10 d into fresh Schneider's cells medium supplemented with 10% FCS (or FBS), antibiotics and selective agent (selective medium).
5. Grow cell lines as mixed cultures in selective medium. Eventually the transformed cells should take over the culture.

To clone:

Dilute cells into microtiter plate wells, growing them in a 1:1 ratio of new:conditioned media (sterile-filtered).

3.2.2. Transfection With Lipofectin

3.2.2.1. DAY 1: PLATING

1. Under sterile conditions resuspend the S2 cells and proceed to count a 10 μ L aliquot in a Neubauer chamber (hemacytometer).
2. Dilute cells to a final concentration of 1×10^6 per mL of fresh Schneider's cells medium supplemented with 10% FCS and antibiotics, seed 0.8 mL per well in a 12-well culture cluster. Cells should derive from a recent subculture (*see Note 10*).

3.2.2.2. DAY 2: TRANSFECTION

1. Prepare a 1:5 dilution of lipofectin by adding 8 μ L of lipofectin per well to 32 μ L of Schneider's cells medium per well. Let it sit for 30 to 45 min (*see Note 9*).
2. Dilute the recombinant DNA (include the selection vector if stable transfections are sought) in Schneider's cells medium at the proper concentration (*see Note 11*). The diluted DNA mix should make up 40 μ L per well.
3. Add the diluted lipofectin to the DNA mix. Let it sit for about 10 min.
4. In the meantime, remove the culture media from the wells with a sterile cotton-plugged Pasteur pipet connected to a vacuum device in a laminar flow. Make sure not to remove the loosely attached cells (*see Note 12*).
5. Dilute the lipid-DNA complexes up to 400 μ L/well in Schneider's cells medium and quickly add dropwise to the side of the wells.
6. Cover with Parafilm. Place in an incubator (or quiet drawer) at room temperature. There is no need to worry about gas exchange.

3.2.2.3. DAY 3: POST-TRANSFECTION

1. Add 400 μ L of Schneider's cells medium supplemented with 20% FCS.
2. For transient transfections a time course is recommended to determine the optimal harvesting time (*see Note 13*).

Special considerations when generating stable cell lines:

1. Wait at least 72 to 96 h after transfection before starting selection.
2. Resuspend the cells, pipetting up and down three or four times. Transfer the cells to a sterile Eppendorf tube and centrifuge at 100 to 200g (1000–2000 rpm in an Eppendorf 5415D) for 2 min. Keep the well in the original plate wet by adding 0.5 mL fresh medium.
3. Remove old media and replace with fresh Schneider's cells medium supplemented with 10% FCS and the appropriate selection agent. Add the cells back to the same well.
4. Wrap in Parafilm.
5. Replace selective medium every 4 to 5 d until resistant cells start growing out (generally it takes between 2 and 4 wk depending on the selection agent).

3.2.2.4. WEEKS 2–3: EXPANSION (STABLE TRANSFECTION)

1. Wait until the culture reaches a density of 6 to 20×10^7 cells/mL.
2. Centrifuge the cells and resuspend in Schneider's cells medium supplemented with 10% FCS and containing the appropriate selection agent. Passage the cells at a 1:2 dilution plating into smaller plates or wells to promote cell growth.
3. Passage the cells several times before expanding them for large-scale expression or preparing frozen stocks as to remove dead cells.
4. Expand resistant cells into 6-well plates to test for expression or into T-flasks to prepare frozen stocks. Always use the appropriate selection agent when maintaining stable S2 cell lines.

4. Notes

1. Another observation that supports the notion that the Schneider's cells have experienced chromosomal rearrangements along the years in culture is the fact that *CLOCK* overexpression leads to the induction of the endogenous *timeless* gene (at the mRNA and protein level); meanwhile no expression from the *period* locus (another target of that transcription factor) can be detected (Lino Saez, unpublished observations).
2. When thawing aliquots for use readjust the pH and resterilize right before use.
3. Two common selection vectors are pCoHygro and pCoBlast, both available from Invitrogen. They express the hygromycin or blasticidin resistance genes, respectively, from the *copia* promoter (5). According to Invitrogen's recommendations, hygromycin is used to a final concentration of 300 $\mu\text{g/mL}$ and blasticidin is used to a final concentration of 25 $\mu\text{g/mL}$. If using different selection vectors it is advisable to test varying concentrations of the selection agent on the S2 cell line to determine the concentration that kills the cells (kill curve).
4. The S2 cells can be kept in an incubator or even a quiet drawer.
5. Growth can be sped up by culturing at 28°C (not higher).
6. S2 cells grow better when some conditioned medium is brought along with the passage.

7. Cells that have been passaged for an extended time tend to change their growth behavior, morphology, and transfectability. When cells with high passage numbers are used for replicate experiments, decreased transfection efficiencies may be observed in later experiments. We recommend using cells with low passage number (<50 splitting cycles). I have experienced some lack of reproducibility when not taking this matter into account.
8. We used a 19:1 (w/w) ratio of expression vector to selection vector and lipid-based transfection reagents, although the calcium phosphate method is the method of choice in a number of situations.
9. Make sure to include a negative control (empty vector where the recombinant construct was cloned) as well as a positive control (reporter genes such as *luciferase*, green fluorescent protein or *lacZ* are widely used).
10. Ideally the subculture should be 2 to 3 d old but certainly not older than 1 wk.
11. For each new plasmid a titration experiment should be performed. We never used concentrations above 1 μg of plasmid for 10^6 cells (therefore 0.8 μg /well in the 12-well/plate format).
12. It is recommended to transfect no more than four wells at a time to avoid drying out the cells, which will be detrimental to cell viability.
13. When employing lipid-based reagents, transfection efficiency of S2 cells is around 10%. This efficiency can be corroborated employing reporters such as green fluorescent protein or *lacZ* under a constitutive promoter. A number of parameters such as amount of reagent and DNA, length of exposure of cells to the DNA–reagent complex can be optimized for each particular construct. Certain reagents (such as Effectene, Qiagen) provide slightly higher transfection efficiency, although they seem to cause higher degree of cell death (which might not affect certain applications).

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Coimmunoprecipitation on *Drosophila* Cells in Culture

M. Fernanda Ceriani

Summary

Coimmunoprecipitation (coIP) provides evidence that two or more proteins can be found in the same complex. It can be performed in vitro (employing in vitro transcribed and translated proteins, or proteins expressed in *Escherichia coli*) or from transfected cells, which assess whether the interaction takes place in a more functional context. This chapter includes a general description and guidelines to carry out coIP in transfected Schneider's cells.

Key Words: Coimmunoprecipitation; coIP; protein G-Sepharose; S2 cells.

1. Introduction

It may prove difficult to assess the nature of certain protein–protein interactions in intact organisms. The complexity brought about by a restricted spatial distribution, the abundance of the proteins of interest, and particularly the quality and availability of specific antibodies may hinder detection. On the other hand, heterologous systems such as yeast two-hybrid assays, powerful and simple to perform as they are, may not always reflect the in vivo scenario. In that regard, immunoprecipitation from transiently or stably transfected cell lines offers distinct advantages: proteins of interest may be tagged (no protein-specific antibodies are required) and expressed at different levels from native, constitutive, or inducible promoters. Native promoters will ensure a condition closest to in vivo. However, this approach involves much more construct-building, and expression will ultimately depend on the presence of specific transcription factors in the selected cell line. Coimmunoprecipitation (coIP) from cells in culture offer another advantage: the identification of the interaction domains by deletion mapping or critical point mutations; a number of constructs can be tested at once without the requirement of generating transgenic organisms for each particular one.

Immunoprecipitations from transfected Schneider 2 (S2) cells have been employed to confirm interactions between CRYPTOCHROME (CRY) and TIMELESS (TIM) (1,2) and CRY and PERIOD (PER) (3). These groups reported coIP to work employing a stable S2 line expressing either *tim* (1) or *per* (3) under the *Drosophila actin5C* promoter, which was then transfected transiently together with a tagged version of *cry* (either CRY-GFP or HA-CRY). Noteworthy, certain interactions depend on the proper environmental condition, such as absence or presence of light; it is important to take this matter into account when trying to reproduce the in vivo scenario.

2. Materials

1. Schneider's S2 cells.
2. Serum-containing medium (SCM): Schneider's cells medium, 10% fetal calf serum heat-inactivated at 56°C for 30 min, 50 U penicillin G, 50 µg/mL streptomycin sulfate, filter-sterilized.
3. Sterile pipets and technique.
4. Laminar flow hood.
5. Quiet drawer or incubator: 22 to 25°C.
6. Purified plasmid DNA resuspended in TE buffer.
7. Lipid-based reagents: Effectene (Qiagen).
8. Tissue-culture treated Corning (Costar) 6- and 12-well culture clusters.
9. 10X Phosphate-buffered saline (PBS) buffer: 11.5 g/L Na₂HPO₄, 2 g/L KH₂PO₄, 80 g/L NaCl, 2 g/L KCl, pH 7.4. Dilute to 1X PBS before use.
10. ES2 protein extraction buffer: 20 mM HEPES, pH 7.5, 100 mM KCl, 0.05% Triton X-100, 2.5 mM EDTA, 5 mM dithiothreitol, 5% glycerol, 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 2 mg/mL pepstatin (4).
11. Protein G Sepharose (Gammabind Plus Sepharose, Pharmacia Biotech) (see Note 1).
12. Rotisserie shaker.
13. Bicinchoninic acid (BCA) protein assay reagent kit (Pierce cat. no. 23227).
14. ELISA plate reader (or spectrophotometer).
15. TBS: 35 mM Tris-HCl, pH 7.4, 140 mM NaCl.
16. 0.05 M Tris-HCl, pH 6.8.
17. 10X Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel running buffer: 30.3 g/L Tris base, 144 g/L glycine, 100 mL/L SDS 10%. Dilute to 1X before use.
18. Tris-glycine transfer buffer: 3.03 g/L Tris base, 14.4 g/L glycine, 150 mL/L methanol; pH 8.4 (see Note 2).
19. 2X Laemmli sample buffer: 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 710 mM β-mercaptoethanol.

3. Methods

A number of transfection protocols are provided in Chapter 33. An alternative for transient transfections of Schneider S2 cells will be included here.

3.1. Transient Transfection of Suspension Cells With Effectene (see Note 3)

1. Split the cells 1:2 the day before transfection.
2. On the day of transfection, remove the cells by pipetting up and down, harvest by centrifugation (100g), remove the medium, and wash once with 1X PBS (see Note 4).
3. Seed the cells at a density of 1.6×10^6 cells per well in a 6-well microtiter plate in 0.8 mL SCM (see Note 5).
4. Dilute 1.6 μ g of DNA dissolved in TE (see Notes 6 and 7), with the DNA-condensation buffer EC (provided in the Effectene kit), to a total volume of 100 μ L. Add 12.8 μ L of enhancer (provided in the Effectene kit) and mix by vortexing for 1 s (see Note 8).
5. Let it sit for 5 min at room temperature (15–25°C).
6. Add 30 μ L of Effectene to each DNA-enhancer mix and pipet up and down five times, allowing 5 to 10 min at room temperature to allow transfection complex to form.
7. Dilute the complexes up to 0.8 mL in 10% SCM and add to each well dropwise (see Note 9). Gently swirl the dish to ensure uniform distribution of the complexes.
8. Cover with Parafilm. Place in an incubator at room temperature (22–25°C).
9. Determine the optimal harvesting time (see Note 10). Transfection usually proceeds for 24 to 48 h (see Note 11).

3.2. Protein Extraction and colP

1. Resuspend the transfected cells by gently pipetting up and down, and transfer to a 1.5-mL microcentrifuge tube. If too many cells were left in the well, add 1X PBS and repeat the operation.
2. Spin down at 100g for 2 min.
3. Remove the SCM and add an equal volume of 1X PBS to wash out residual SCM. Resuspend the cells carefully, so as not to break them open.
4. Spin down at 100g for 2 min and remove most of the PBS.
5. Resuspend the cells in approx 100 μ L/well of ice-cold ES2 (4) lysis buffer (see Note 12). Detergent-insoluble material is removed by centrifugation at maximum speed in a refrigerated microcentrifuge, and the soluble fraction is transferred to a new 1.5-mL microcentrifuge tube.
6. Set aside a 5- to 10- μ L aliquot to determine protein concentration using the BCA assay (BCA protein assay kit), and 10 to 20 μ L as a positive control for Western blots.
7. In the meantime, follow the manufacturer's recommendation to resuspend the protein-G Sepharose beads and generate a 1:1 (v/v) slurry.
8. Wash the beads with 1X PBS; centrifuge at 100 to 200g for 2 min and remove the excess PBS to return to the original 1:1 slurry.
9. Incubate the lysate with 10 μ L of beads for 30 min in a rotisserie shaker in a slow head-to-tail motion (see Note 13).

10. Spin down at 100g for 2 min and transfer the supernatant to a new microcentrifuge tube.
11. Add 0.5 to 5 μ L of specific antibody to each lysate. Incubate on ice (or in a rotisserie shaker at 4°C) for 1 to 3 h. Include proper controls (lysates lacking the proteins of interest immunoprecipitated in parallel; a primary antibody against another protein known to be present or complexed in the lysate).
12. Add up to 50 μ L of the slurry per mL lysate. Mix with gentle shaking for 1 h at 4°C.
13. Wash the antigen–antibody–protein G complexes by centrifuging for 1 min at 200g. Carefully aspirate the supernatant with a fine tipped Pasteur pipet. Gently resuspend in 1 mL ES2. Repeat the wash. Wash in TBS and in 0.05 M Tris-HCl, pH 6.8 (*see Note 14*).

3.3. Preparation for SDS-PAGE

1. Microcentrifuge at 200g for 1 to 2 min. Gently, without splatter, add 20 to –50 μ L 1X Laemmli sample buffer. **Do not vortex.** Heat for 5 min at 100°C. Microcentrifuge as above and apply supernatant directly to an SDS-PAGE. Make sure to include positive controls (transfected cells that have not been immunoprecipitated) as well as negative controls.
2. Run a 6% SDS-PAGE to detect large proteins such as PER, TIM, or CLOCK, or a gradient gel (4–12%) when analyzing proteins of very different molecular weights.
3. Transfer to supported nitrocellulose in a Tris–glycine buffer. Perform Western blots as usual.

4. Notes

1. According to Pharmacia, the binding capacity of Gammabind Plus is relatively low to pull down hamster, rat, and mouse immunoglobulins. When employing primary antibodies from any of those species it is highly recommended to switch to an anti-species-IgG coupled to agarose beads.
2. When preparing transfer buffer measure the pH but do not adjust it. If it is not the one indicated, prepare a new solution.
3. Preliminary experiments showed a somewhat higher transfection efficiency employing Effectene, although cell integrity appeared more affected than in the presence of other lipid-based reagents. Transfection efficiency might affect to a higher extent on coIP than on transcriptional assays and may hamper the detection of the desired interaction. Keep in mind that the overall efficiency revolves around 10% of the transfected cells. In such cases it is recommended to generate a stable cell line expressing a tagged protein (to facilitate further detection) and transiently transfect the other constructs. Cells transfected in different wells can be pooled after transfection to improve detection of the desired interaction.
4. The wash may be skipped without a substantial loss in transfection efficiency.
5. Cells can be plated at 1 to 3.5×10^6 cells in 1.6 mL SCM per well in a 6-well microtiter plate.

6. DNA should be resuspended in TE, pH 7.0 to 8.0, at a minimum concentration of 0.1 $\mu\text{g}/\mu\text{L}$. When transfecting two constructs altogether use half of the total DNA amount of each.
7. Plasmid DNA quality strongly influences several transfection parameters such as efficiency, reproducibility, and toxicity, as well as interpretation of results. Therefore only plasmid DNA of the highest purity should be used.
8. It is important to always keep the ratio of DNA to enhancer constant.
9. It is not necessary to stress the cells by placing them in serum-free medium, as serum does not inhibit Effectene.
10. In many cases, removal of transfection complexes is not necessary. However, if cytotoxicity is observed, centrifuge cells after 6 to 18 h, remove medium containing the complexes, wash the cells with 1X PBS, then resuspend in 1.6 mL fresh growth medium.
11. When transfecting a reporter such as GFP the optimal harvesting time can be easily determined by inspecting the cells under an inverted fluorescent microscope. It will require at least 6 to 8 h posttransfection to identify GFP-expressing cells.
12. The detection of the desired protein–protein interaction will depend greatly on the salt concentration of the medium. Higher salt concentration will select for more specific interactions, but weak interactors may be lost. When no information is available, it is recommended to perform coIP in buffers of different ionic strength ranging from 25 to 150 mM KCl. Add the protease inhibitors right before use.
13. This clearing step in the absence of the specific primary antibody allows the removal of the complexes formed between the protein G coupled to the beads and any protein present in the lysate.
14. Make sure to perform at least four washes.

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Basic Protocols for Zebrafish Cell Lines

Maintenance and Transfection

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Summary

Cell lines derived from zebrafish embryos show great potential as cell culture tools to study the regulation and function of the vertebrate circadian clock. They exhibit directly light-entrainable rhythms of clock gene expression that can be established by simply exposing cultures to light–dark cycles. Mammalian cell lines require treatments with serum or activators of signaling pathways to initiate transient, rapidly dampening clock rhythms. Furthermore, zebrafish cells grow at room temperature, are viable for long periods at confluence, and do not require a CO₂-enriched atmosphere, greatly simplifying culture conditions. Here we describe detailed methods for establishing zebrafish cell cultures as well as optimizing transient and stable transfections. These protocols have been successfully used to introduce luciferase reporter constructs into the cells and thereby monitor clock gene expression *in vivo*. The bioluminescence assay described here lends itself particularly well to high-throughput analysis.

Key Words: Zebrafish cells; electroporation; luciferase; circadian; clock; light.

1. Introduction

In vertebrates, the circadian clock was originally thought to reside in a small number of specialized organs termed pacemakers, such as the suprachiasmatic nucleus, the retina, and in lower vertebrates, the pineal gland ([1,2](#)). Subsequently, rhythmic clock gene expression was encountered in most tissues and shown to persist *in vitro* ([3–5](#)). Cell lines derived from embryonic zebrafish harbor a directly light-entrainable clock and thus represent a powerful *in vitro* tool to study vertebrate circadian rhythms ([6](#)). Sustained circadian rhythms of gene expression can be established by simply exposing cultures to light–dark cycles ([7](#)). This chapter describes protocols to generate, maintain, and transfect zebrafish embryonic cell lines. Using these protocols, we have established

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PAC-2 luciferase reporter cell lines to visualize, in real time, the promoter activity of clock genes such as the novel zebrafish period gene *zfper4* (7). These luciferase reporter cells have proven ideal for this study for the following reasons:

1. The bioluminescence rhythms observed in the reporter cells have impressive regularity and smoothness, most likely the result of light emission from a static monolayer of uniformly expressing cells (Fig. 1).
2. The cells are viable and do not detach from the substrate even following long periods at confluence without medium changes.
3. Luciferin is stable in the culture medium for up to 3 wk and can diffuse easily into the cells. Thus, a single addition of luciferin is sufficient for long experiments.
4. The cells grow in atmospheric levels of CO₂ at room temperature, greatly simplifying the in vivo luciferase assay conditions.
5. Growing the cells in a 96-well plate and using an automatic scintillation counter to measure bioluminescence allows high-throughput analysis over long time periods under various lighting regimes. For an example of the data obtained, see Fig. 1.

2. Materials

2.1. Establishing Zebrafish Cell Cultures

1. E3 medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 10⁻⁵% methylene blue.
2. Bleach (Roth): 12% sodium hypochlorite solution.
3. Pronase (Roche): 30 mg/mL dissolved in water.
4. Breeding stock of zebrafish maintained according to standard procedures (8).
5. Dissection microscope (Zeiss).
6. Materials for routine passaging of zebrafish cell cultures (Subheading 2.2.).

2.2. Routine Passaging of Zebrafish Cell Cultures

1. Sterile, disposable tissue culture flasks and plates (no specialized coating), conical tubes, and pipets.
2. Laminar flow hood for sterile tissue culture work.
3. L15 complete culture medium: L15 (Leibovitz) culture medium (Gibco), 15% fetal bovine serum (Biochrom KG), 100 U/mL penicillin/100 mg/mL streptomycin (Gibco), 50 mg/mL gentamicin (Gibco).
4. 1X phosphate-buffered saline (PBS) without calcium and magnesium (Gibco).
5. 1X Trypsin-EDTA: 10X stock (Gibco) diluted in 1X PBS.

2.3. Optimizing Electroporation Conditions for Transfection of Zebrafish Cell Lines

1. β -Galactosidase-encoding plasmid (e.g., pcDNA3.1myc-his lacZ, Invitrogen).
2. Carrier DNA plasmid pBluescript II SK(-) (Stratagene).

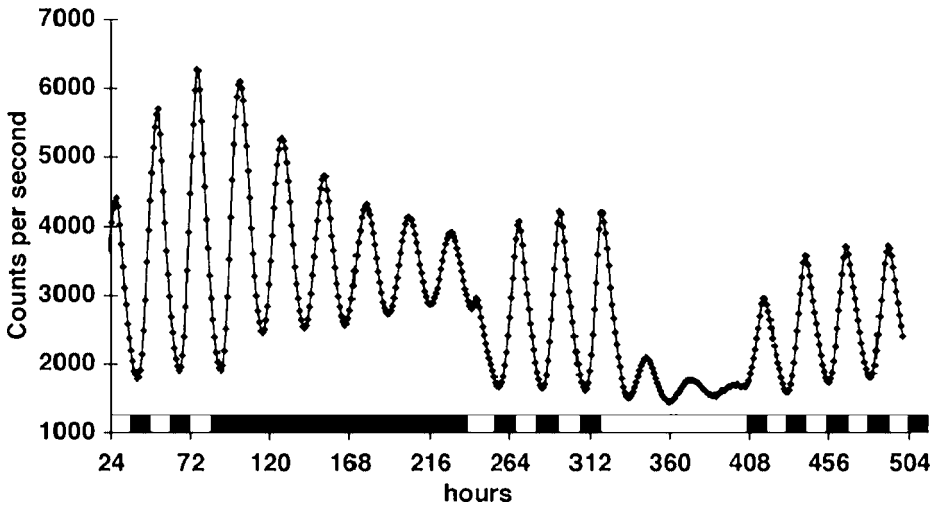


Fig. 1. In vivo bioluminescence assay of a zebrafish luciferase reporter cell line. Regulation of gene expression by the circadian clock in response to light has been visualized by stably transfecting a luciferase reporter construct, driven by the *zfp*period4 clock gene promoter (7). The cells are seeded into a 96-well plate and then a single addition of 0.5 mM beetle luciferin is made at the start of the assay. The assay continues uninterrupted for 20 d. The plate is counted automatically using a Packard TopCount scintillation counter. Between counts, the plates are exposed to various lighting conditions. Bioluminescence is plotted on the y-axis (counts per second) and time on the x-axis (hours from start of the experiment), where a white/black bar shows the duration of light–dark periods respectively. Apart from the extended periods of constant dark and light, each light and dark period lasts 12 h. The graph represents the mean of data obtained from 16 independent wells. The data is entirely consistent with the mRNA expression profile of the endogenous *zfp*period4 gene (7).

3. TNE: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0.
4. 0.25 M Tris-HCl, pH 7.8.
5. Protein assay dye reagent (Bio-Rad).
6. PM2, pH 7.0: 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂.
7. ONPG solution, pH 7.0: 8 mg/mL *O*-nitrophenyl-β-D-galactopyranoside (ONPG), 60 mM Na₂HPO₄, 40 mM NaH₂PO₄. Store at –20°C in the dark as small aliquots.
8. β-Mercaptoethanol.
9. 1 M Na₂CO₃.
10. Gene pulser apparatus (Bio-Rad).
11. Hemocytometer.
12. 4-mm Sterile electroporation cuvettes (Pqclab).

2.4. Transfection of Luciferase Reporter Plasmid Into PAC-2 Zebrafish Cells

1. Luciferase reporter plasmid (based on the vector pGL3Basic; Promega).
2. G-418 resistance plasmid (based on pcDNA3,1myc-His[A]; Invitrogen).
3. Geneticin G-418 sulfate (Gibco).

2.5. Long-Term Storage of Cells

1. Freezing medium: L15 culture medium, 30% fetal bovine serum, 10% dimethyl sulfoxide (DMSO; Sigma), 100 U/mL penicillin/100 mg/mL streptomycin (Gibco), 50 mg/mL gentamicin (Gibco).

2.6. In Vivo Luciferase Assay

1. 96-Multiwell fluoplate (Nunc).
2. TopSeal A sealing plastic (Packard, PerkinElmer).
3. Beetle luciferin, potassium salt (Promega).
4. Packard TopCount NXT counter (PerkinElmer).
5. For data analysis: Microsoft Excel for Windows using the Import and Analysis macro (Steve Kay, www.scripps.edu/cb/kay/shareware/, Scripps Research Institute). Chrono for Mac, version 4.4 (Till Roenneberg, till.roenneberg.imp.med.uni-muenchen.de, Munich).

3. Methods

3.1. Establishing Zebrafish Cell Cultures

The zebrafish is an excellent model for studying early vertebrate development because large numbers of embryos are immediately accessible and easily maintainable. Several protocols exist for establishing cell cultures from zebrafish embryos (8,9). We have routinely used the following protocol to establish cell cultures that can be efficiently transfected by electroporation.

1. Collect eggs 3 h after fertilization and wash three times with filtered E3 medium in a plastic tea strainer.
2. Transfer the eggs with 10 mL E3 buffer into a 10-cm Petri dish and allow the embryos to develop at 28°C for 16 to 24 h.
3. Again transfer the eggs to a tea strainer and immerse them in E3 buffer supplemented with 0.5% (v/v) bleach for 2 min (*see Note 1*).
4. Rinse the eggs three times with sterile 1X PBS to remove traces of bleach. From this step on, work in a laminar flow tissue culture hood using sterile plastic pipets, plates, and flasks.
5. Transfer the embryos into a 10-cm tissue-culture Petri dish in 10 mL of filtered E3 medium.
6. Remove the chorion by adding 20 μ L of 30 mg/mL pronase. Incubate at 27°C for at least 1 h (*see Note 2*).

7. Remove the pronase solution and incubate the embryos twice for 5 min in 10 mL sterile 1X PBS with gentle swirling (*see Note 3*).
8. Transfer 50 embryos to a 6-cm tissue-culture Petri dish and remove as much of the residual 1X PBS as possible. Add 1 mL of 1X trypsin–EDTA and incubate at room temperature. Check for dissociation by examining with a dissection microscope (should take 10 to 15 min). Disrupt the embryos by pipetting up and down several times through the tip of a 1-mL automatic pipet.
9. Continue the dissociation reaction until a suspension of predominantly single cells is obtained.
10. Add 1 mL of L15 complete culture medium and transfer the cell suspension into a 15-mL conical tube. Dilute the cells with an additional 13 mL of L15 complete culture medium to ensure inactivation of the trypsin.
11. Harvest the cells by centrifugation at 200g for 3 min at room temperature.
12. Remove the supernatant and resuspend the cells in L15 complete culture medium (1 mL of medium for each 15 embryos processed).
13. Transfer the cell suspension to a 25-cm² tissue culture flask. Seal this and then incubate the cells at 25°C (*see Note 4*). The medium should be changed after 20 d.

3.2. Routine Passaging of Zebrafish Cell Cultures

All cell culture work should be performed in a laminar flow tissue culture hood using sterile solutions and plasticware.

1. Aspirate the medium from a confluent cell monolayer and then gently wash the cells twice with 1X PBS (*see Note 5*).
2. Detach the cells from the culture flask substrate by a 5-min treatment with 1X trypsin–EDTA at room temperature. Use a volume of trypsin just sufficient to cover the cell layer. Dilute the suspension of detached cells with L15 complete culture medium (*see Note 6*) and pipet the cell suspension vigorously up and down through a 5-mL or 10-mL pipet to break up cell clumps.
3. Transfer 20% of the suspension to a new flask and dilute with more L15 complete culture medium to ensure that the medium completely covers the new growth surface.
4. Seal the flasks and incubate at room temperature or in a 25°C incubator.
5. Passage the cells again when they reach confluence. Alternatively, change the culture medium each 7 d until ready to repassage the cells. The confluent cell monolayers remain viable for up to 1 mo.

3.3. Optimizing Transient Transfection of DNA Into Zebrafish Cells

Here, we describe a simple and reliable procedure for the introduction of DNA into zebrafish primary cultures by electroporation. Electroporation involves the exposure of cells to a pulsed electric field to create transient pores in the plasma membrane and thereby facilitate access of DNA ([10,11](#)). It is advisable to optimize the conditions for newly derived fish cell lines before embarking on

stable transfections (*see* **Notes 7 and 8**). In order to assay electroporation efficiency we transiently transfect an expression plasmid encoding *E. coli* β -galactosidase under a range of different electroporation conditions and assay transfected cells for β -galactosidase activity (*see* **Note 9**) (*12*).

1. Plate cells at 40 to 50% confluence in L15 complete culture medium 2 d before electroporation. This ensures that the cells are in exponential growth phase the day of the transfection (*see* **Subheading 3.2.**).
2. Aspirate the medium from the cell monolayer and then detach the cells from the flask by trypsin treatment to produce a suspension of single cells (*see* **Subheading 3.2.**).
3. Determine the cell density by counting cells in a hemocytometer. Enough cells for several electroporation reactions (10^7 cells are required for one transfection reaction) are centrifuged at 200g for 5 min at 4°C and washed twice with 1X PBS. Each cell pellet is resuspended by pipetting in serum-free medium (0.5 mL of medium for each transfection reaction) and stored on ice for a maximum of 30 min before electroporation.
4. Prepare a range of aliquots of β -galactosidase-encoding plasmid (from 5 to 30 μ g) mixed with sufficient DNA carrier (pBluescript II SK [–], Stratagene) to provide a total of 35 μ g of plasmid DNA per aliquot. Dilute each aliquot in 50 μ L of water and mix with 500 μ L of zebrafish cell suspension (**step 3**; *see* **Note 10**).
5. Transfer each aliquot of cells mixed with DNA to a cuvet for electroporation. Aliquots are electroporated at a range of voltages between 0.2 and 0.3 KV, with capacitance set to 960 μ F and resistance set to 0 Ω , all at room temperature using a gene pulser apparatus. Following electroporation dilute each cell aliquot to 10 mL with L15 complete culture medium and then transfer to a 10-cm tissue-culture Petri dish to allow attachment of viable cells.
6. The day after transfection, remove the culture medium, wash the cell monolayer twice with 1X PBS in order to eliminate cell debris, and then add fresh complete medium.
7. Maximum levels of β -galactosidase activity are detected between 48 and 72 h following transfection. Therefore, cells should be harvested at this stage. The medium is aspirated from the cell monolayer and then the cells are gently washed twice with ice-cold 1X PBS.
8. Detach the cells from the culture flask substrate by a 5 min treatment with 1 mL of ice-cold TNE solution and then harvesting with a cell scraper.
9. Transfer the suspension of detached cells to a 1.5-mL Eppendorf tube and centrifuge at 200g for 3 min.
10. Resuspend the cell pellet by pipetting in 0.1 mL of 0.25 M Tris-HCl, pH 7.8, and then disrupt the cells by three cycles of freezing in dry ice (3 min) and thawing at 37°C (1 min).
11. Centrifuge the lysate at 12,000g for 5 min at 4°C and transfer the supernatant to a new 1.5-mL tube.

12. Measure the protein concentration by Bradford assay (**13**) using the Bio-Rad protein assay dye reagent precisely according to the manufacturer's instructions.
13. Assay the β -galactosidase activity by mixing 50 μ g of protein with 1 mL of PM2 buffer, 50 μ L of the substrate ONPG (8 mg/mL), and a 50 mM final concentration of β -mercaptoethanol.
14. Incubate the mixtures together at 37°C from 5 min to 1 h until the appearance of a clear yellow color in most of the tubes (indicator of β -galactosidase activity).
15. Stop the reactions by adding 500 μ L of 1 M Na₂CO₃.
16. Read the optical density of the reactions at a wavelength of 420 nm. Readings between 0.2 and 0.4 optical density units fall within the linear range of the assay.
17. Select the electroporation conditions that result in maximal β -galactosidase activity and use these for all subsequent electroporation experiments.

3.4. Transfection of Luciferase Reporter Plasmid Into PAC-2 Zebrafish Cells

We have studied the PAC-2 cell line extensively (**6,7,14,15**) and so have determined a set of electroporation conditions that provide optimal transfection efficiency. All subsequent protocols employ these conditions.

3.4.1. Transient Transfection

1. Harvest the cells and prepare them for electroporation exactly as described in **Subheading 3.3**.
2. Dissolve 35 μ g of plasmid DNA containing 25 μ g of the luciferase reporter plasmid and 10 μ g of the DNA carrier in 50 μ L of water and mix with a 500- μ L aliquot of resuspended cells (*see Note 10*).
3. Transfer each aliquot of cells mixed with DNA to a cuvet for electroporation and perform the electroporation at 0.29 KV, with capacitance set to 960 μ F and resistance set to 0 Ω , at room temperature using a Bio-Rad gene pulser apparatus.
4. Following electroporation dilute each reaction to 2.5 mL with L15 complete culture medium and then aliquot 250 μ L per well of a 96-well fluoplate.
5. The day after transfection, remove the culture medium, wash the cell monolayer twice with 1X PBS in order to eliminate cell debris, and then add fresh medium supplemented with 0.5 mM beetle luciferin.
6. Forty-eight hours after transfection, follow the protocol described in **Subheading 3.6**, for monitoring luciferase activity in vivo using the TopCount NXT counter.

3.4.2. Stable Transfection

1. Harvest the cells and prepare them for electroporation exactly as described in **Subheading 3.3**.
2. To each 500 μ L of cells, add 35 μ g of plasmid DNA, containing 10 μ g of carrier DNA and 25 μ g of luciferase reporter (when the neo cassette is integrated into the same plasmid) or 22.5 μ g of reporter and 2.5 μ g of plasmid containing a neo

resistance cassette (*see Note 10*). Also, include a negative control cell aliquot lacking plasmid DNA.

3. Perform the electroporation at 0.29 kV, 960 μ F, 0 Ω . Following electroporation dilute each cell aliquot to 10 mL with L15 complete culture medium and then transfer to a 10-cm tissue-culture Petri dish to allow attachment of viable cells.
4. The day after transfection, remove the culture medium, wash the cell monolayer twice with 1X PBS in order to eliminate cell debris, and then add fresh medium.
5. Seventy-two hours following electroporation, start the selection for antibiotic resistance by supplementing the medium with the highest antibiotic concentration, 800 μ g/mL of G-418 (*see Note 11*).
6. Each 5 d wash the cells with 1X PBS and change the selection medium.
7. After a period of 10 to 15 d, all nonresistant cells on the negative-control plate (nontransfected cells) should have detached (*see Note 11*). At this stage, reduce the concentration of G-418 to 400 μ g/mL.
8. Following 1 mo of selection reduce the G-418 concentration to 250 μ g/mL (the maintenance concentration). At this stage, 100 to 200 colonies of resistant cells should be clearly visible for each electroporation reaction. Colonies typically consist of a monolayer of densely packed cells, unlike other mammalian transformed cell lines, where colonies are typically composed of multiple layers of cells.
9. It is possible to visualize G-418 resistant colonies that successfully express luciferase reporter plasmids using a simple film autoradiographic assay. Following addition of 0.5 mM beetle luciferin to the culture medium, transfer the plates of cells to a dark room, place them directly onto a sheet of X-ray film, and then expose for 8 to 12 h. Dark spots on the developed film lie below bioluminescent, luciferase-positive colonies (*see Fig. 2*).
10. When positive clones contain several hundred cells, they are large enough to be individually trypsinized and transferred to single wells of a 96-multiwell plate. Upon reaching confluence, transfer the cells to larger multiwell plates and then ultimately maintain them in 25-cm² flasks. Alternatively, the clones can be trypsinized, mixed together, and subsequently propagated as a pool (*see Note 12*).
11. The clones should be propagated always at the maintenance concentration of G-418 and the medium changed every 5 d.

3.5. Long-Term Storage of Cells (*see Note 13*)

3.5.1. Freezing Cells

1. Wash a subconfluent cell monolayer twice with 1X PBS, trypsinize, and then resuspend the cells in 10 mL of L15 complete culture medium (*see Subheading 3.2.*).
2. Determine the density of the cell suspension by counting an aliquot with a hemocytometer and then harvest the cells by centrifugation at 200g for 5 min at 4°C and wash twice with serum-free medium.

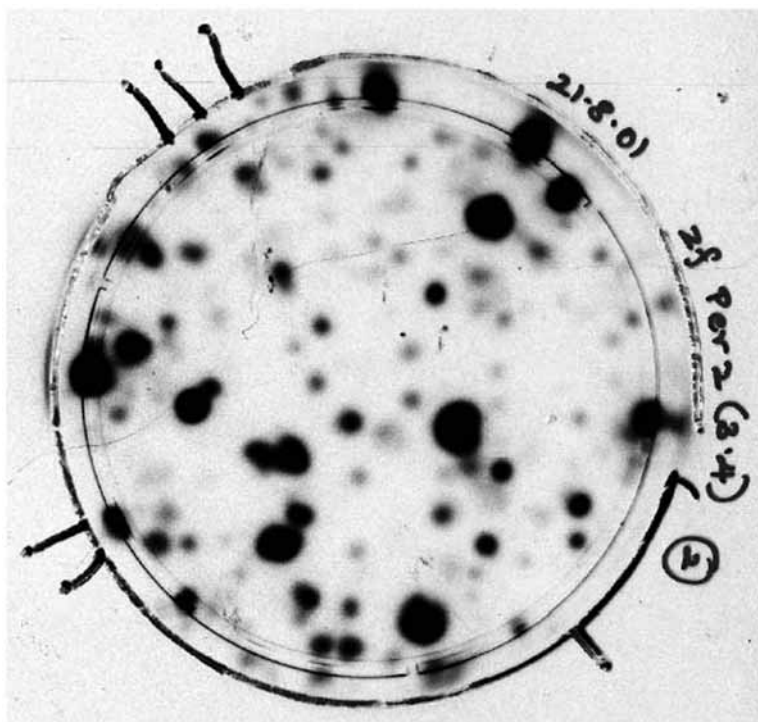


Fig. 2. Visualizing luciferase-positive, G-418-resistant colonies. The culture medium of a Petri dish containing hundreds of G-418-resistant, stably transfected PAC-2 cell clones was supplemented with 0.5 mM luciferin and then exposed on top of an X-ray film overnight in a dark room. After developing the film, dark spots represent individual colonies positive for luciferase activity. The variation in intensity from clone to clone is probably due to differences in the number of copies of the gene inserted into the genome or their sites of integration. The markings on the film are for alignment of the plate with respect to the film.

3. Finally, resuspend the cells at a density of 2×10^6 cells/mL in freezing medium (lacking G-418). Transfer 1-mL aliquots to cryopreservation vials and place them in a -80°C freezer for 2 to 3 d.
4. Transfer frozen aliquots to liquid nitrogen for storage.

3.5.2. Thawing Cryopreserved Cells

1. Remove frozen aliquots from liquid nitrogen and place them immediately on ice for 10 to 15 min.
2. Remove the thawed cell suspension from the vial and immediately dilute it in 10 mL of complete culture medium at room temperature.

3. Centrifuge the cells at 200g for 5 min at 4°C and discard the supernatant (*see Note 14*).
4. Resuspend the cells in L15 complete medium (lacking G-418) and transfer them to a tissue culture flask. Typically, more than 50% of the cells should attach.
5. After 24 h change the medium for fresh L15 complete culture medium (include 250 µg/mL G-418 for resistant clones) to remove dead, floating cells.

3.6. In Vivo Luciferase Assay

1. Seed 3×10^4 cells of the luciferase reporter clones into individual wells of a 96-well Fluoplate in 250 µL complete culture medium containing 250 µg/mL G-418. For transiently transfected cells, omit G-418.
2. After 12 h, replace the medium with 250 µL complete culture medium supplemented with 0.5 mM beetle luciferin.
3. Seal the plates using TopSeal A sealing plastic and attach a bar code identifier label to the plate.
4. Load the plates into the TopCount stacker units, with each plate sandwiched between two transparent 96-well plates to allow light to access the opaque sample plates (*see Note 15*).
5. Program the counter to count each plate, a minimum of once each hour, and each well for a duration of 5 s in an uninterrupted cycle. Add extra empty plates if necessary to adjust the plate-counting interval.
6. Save the data from each plate as a single ASCII file for each counting cycle. Multiple files from a complete experiment can then either be imported into Microsoft Excel using the I and A import macro or alternatively, imported directly into the Chrono program for more detailed analysis (*see Note 16*).

4. Notes

1. It is important not to leave the eggs for too long (longer than 5 min) in the working bleach solution because excess treatment can damage the embryos.
2. The time required for the pronase step to successfully dechorionate the embryos can vary from clutch to clutch of eggs. Ideally, watch the embryos under the microscope during this step and check when the chorion starts to become fragile.
3. Embryos usually separate from their chorion with gentle swirling during this rinsing step.
4. Cells from dissociated embryos look round while they are still in suspension and the majority will not adhere to the culture vessel for several days.
5. This step removes all traces of serum from the cell monolayer. Serum contains potent inhibitors of trypsin. The relatively high concentration of serum used to culture these cells makes this step crucial.
6. Once the cells have detached from the substrate, trypsin is neutralized by addition of at least a 10X excess volume of L15 complete culture medium. The duration of trypsin treatment should be reduced to the minimum required to detach the cells; otherwise, the viability of the cells and transfection efficiency can be reduced.

7. The efficiency of electroporation depends on a combination of factors (16): the total amount and concentration of the DNA transfected, the cell density during electroporation, as well as electrical properties such as voltage, capacitance, and the ionic composition of the medium (17–19). The growth properties of the cells can also influence the transfection efficiency. Prior to electroporation, the cells should be in exponential growth phase. Following successful transfection there is often significant mortality (16). Finally, some authors report maximal levels of transient expression in cells maintained at room temperature during electroporation (20), whereas others have obtained better results with cells maintained at 0°C (21).
8. For all zebrafish cell lines tested, we have found that maintaining the cells at room temperature during and after electroporation consistently results in optimal electroporation efficiencies.
9. Like most vertebrates, zebrafish cells contain relatively low levels of endogenous β -galactosidase activity, and an increase in enzyme activity of up to 100-fold can be detected in a transient transfection. The ease of the β -galactosidase assay makes it feasible to test large numbers of samples rapidly, and so to explore a wide range of electroporation conditions.
10. Although supercoiled plasmid DNA can be efficiently used for transient assays it is necessary to linearize the DNA for stable transfections to ensure efficient integration into the genome. Furthermore, in the latter case, the transfected DNA must carry a dominant-selectable marker (e.g., genes conferring resistance to geneticin [G-418] or hygromycin). The plasmid of interest can directly carry an antibiotic resistance cassette or can be cotransfected in excess with a second plasmid including the selectable marker at a ratio of between 1:10 and 1:7. This increases the probability that all antibiotic-resistant clones will also contain the plasmid of interest.
11. The optimal antibiotic concentration for selection and maintenance must be determined empirically for each cell line and antibiotic. This is best done by treating transfected (using 25 μ g antibiotic resistant plasmid plus 10 μ g carrier plasmid) and not transfected cells with a range of antibiotic concentrations from 0.5 to 1 mg/mL. The ideal antibiotic concentration leaves only stably transfected, antibiotic-resistant cells after a maximum of 15 d of selection. During selection, the medium should be changed every 5 d to ensure that the concentration of active antibiotic does not decrease significantly.
12. Analysis of a pool of clones averages out the clone-to-clone variability that is normally observed as the result of differences in the copy number and sites of integration of the plasmids. It is advisable to limit the number of passages of cell pools to avoid significant changes in the pool composition that can result from differences in the growth rates of the component clones.
13. Following the establishment of a cell line, it is particularly important to freeze down aliquots of the cells in liquid nitrogen as soon as possible. In the event of contamination, the line can be repropagated from cryopreserved cells that have a reduced number of passages. It is also generally advisable not to propagate the

same batch of cells over extended periods of time (e.g., more than 1 yr) in order to avoid major changes in the properties of the cells. For this reason it is important to prepare frozen cell aliquots from batches that have experienced a low number of passages and then to use these to periodically reconstitute the line.

14. After thawing, it is important to dilute out DMSO when growing the cells. DMSO can be toxic under normal culture conditions even at low concentrations.
15. Between counting, the plates are held outside of the counter chamber in stacking units. Thus, cells can be illuminated with a tungsten light source ($20 \mu\text{W}/\text{cm}^2$) connected to a programmable timer. Ideally, the counter should be located in a thermostatically controlled dark room.
16. Low background fluorescence is detected even in empty wells during the light period owing to a low level of autofluorescence from the white plastic of the culture plate. This should be considered in situations where levels of cell bioluminescence are low.

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Manipulation of Mammalian Cell Lines for Circadian Studies

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Summary

In mammals, the central circadian pacemaker resides in the hypothalamic suprachiasmatic nucleus (SCN), but circadian oscillators also exist in peripheral tissues. We have used wild-type and *cryptochrome* (*mCry*)-deficient mouse embryonic fibroblasts (MEFs) to demonstrate that the peripheral oscillator is mechanistically very similar to the oscillator in the SCN. Following serum shock activation, fibroblasts are able to sustain an SCN-like temporal expression profile of all known genes (i.e., antiphase oscillation of *Bmal1* and *Dbp* genes), but are not able to produce oscillations in the absence of functional *mCry* genes. Remarkably, the analysis of *mCry1*^{-/-} and *mCry2*^{-/-} MEFs revealed the capacity to control period length in immortalized cell lines. Thus, the use of mammalian cells has become one of the most convenient methods for monitoring the molecular clock machinery and analyzing clock proteins at the functional/structural level. Here, we present the necessary protocols to (1) derive and culture a fibroblast cell line from wild-type and knockout mouse skin and (2) transfect cells at high efficiency to use in functional clock-protein studies.

Key Words: Cell culture; mammalian; transfection; primary cell lines; circadian.

1. Introduction

In the mouse, the master core oscillator in the suprachiasmatic nucleus (SCN) is composed of interacting positive and negative transcription–translation feedback loops, which have a circadian (almost 24-h) rhythm and involve a set of clock genes (*Clock*, *Bmal1*, *mCry1/2*, *mPer1/2/3*, *Rev-Erb α*, *CK e/d*) (1). A key step in this feedback loop is the shutdown of CLOCK/BMAL1-driven transcription by mCRY proteins (2,3). Molecular oscillators also exist in peripheral tissues (liver, kidney, heart), where they also show a transcriptional cycle of 24 h, but with a 6- to 8-h delay with respect to the central pacemaker in the SCN.

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In vitro, brief treatment of cultured immortalized mammalian cells with various compounds (horse serum, forskolin, 12-*O*-tetradecanoylphorbol 13-acetate, dexamethasone, temperature shifts) induce rhythmic expression of the clock genes for two to three cycles (4,5). Given the power to control the clock machinery in cultured cells, many investigators are considering this system to answer fundamental questions in a systematic and less time-consuming manner. In **Subheading 3.1.** we will describe in detail the methods to growth expand and maintain a mammalian adherent cell line.

By using spontaneously immortalized mouse embryonic fibroblasts (MEFs) from wild-type and *mCry1^{-/-}mCry2^{-/-}* mice we have provided evidence that the gene oscillation induced in culture is a true circadian mechanism (6,7). Indeed, no rhythm of *Bmal1*, *Per1*, and *Dbp* genes expression was detected in *mCry1^{-/-}mCry2^{-/-}* cells following serum shock. Moreover, the effect of the homozygous inactivation of the *mCry1* or the *mCry2* gene, known to accelerate or retard the biological clock at the animal level, was astonishingly conserved in *mCry1^{-/-}* and *mCry2^{-/-}* MEFs grown in culture dishes. It is therefore important to learn how to develop a cell line from the available clock-gene knockout animals. In **Subheading 3.2.** we will report a simple and efficient method to obtain a new primary fibroblast cell line from the skin of adult mice. Based on the above findings, we are now actively testing the oscillation period of the clock machinery in primary fibroblasts derived from the skin of human patients with advanced and delayed sleep phase syndromes.

Because most clock genes have been discovered by genetic screenings in *Drosophila* and gene knockout in mice, not much knowledge is available on the structure and function of the proteins coded by those genes. In recent years we have seen a major effort in the circadian field for developing new methods to tackle this issue. One of these techniques is the transfection of DNA plasmids (represented by a vector that carries a certain clock gene) in mammalian cells (see **Subheading 3.3.**) (3,8). When cells take up DNA, they express it transiently over a period of several days, and eventually the DNA is lost from the population. The ability to express this DNA over a short period is called “transient expression;” it is a convenient and rapid method for studying the expression of foreign genes in mammalian cells. In **Subheading 3.4.** we will see that selection from stable integration of plasmid DNA into the host chromosome permits the generation of stably transfected cell lines that indefinitely express a desired gene product. Stable cell lines express better physiological levels of a foreign protein, as high-level expression obtained in transient transfection is often toxic to the cell in the long term.

2. Materials

1. Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose with Ultra-Glutamine I (Cambrex, cat. no. 12-604F/U1); store at 4°C.
2. Ham's F-10 medium with Ultra-Glutamine I, without thymidine (Cambrex, cat. no. 02-014F); store at 4°C.
3. RPMI-1640 with Ultra-Glutamine I and 25 mM HEPES buffer (Cambrex, cat. no. 12-115F/U1).
4. Medium for transfection: OPTI-MEM I reduced serum medium (Invitrogen, cat. no. 31985); store at 4°C.
5. Fetal calf serum (FCS; Invitrogen); store at -20°C.
6. Penicillin-streptomycin mixture (Cambrex): 10,000 U/mL penicillin, 10,000 µg/mL streptomycin; store at -20°C. Working dilution 1:100.
7. Dulbecco's phosphate-buffered saline (PBS; 1X): 0.0095 M phosphate without calcium and magnesium (Cambrex, cat. no. 17-512F).
8. Trypsin-verseine (EDTA) mixture: 0.5 g/L trypsin, 0.2 g/L EDTA (Cambrex, cat. no. 17-161F).
9. Freezing medium: 6 mL FCS (30% final concentration [f.c.]), 2 mL dimethyl sulfoxide (DMSO; 10% f.c.), 12 mL DMEM without antibiotics.
10. Collagenase type V (Sigma, cat. no. 02014): dissolve in PBS at 25 mg/mL, store in small aliquots at -20°C.
11. Hyaluronidase (Sigma, cat. no. H-3506): 10 mg/mL, store in small aliquots at -20°C.
12. TCH mix: 0.25 mL trypsin (0.25% f.c.), 40 µL collagenase type V (1 mg/mL f.c.), 30 µL hyaluronidase (0.3 mg/mL f.c.), 0.68 mL PBS.
13. Fibro-medium: 100 mL Ham's F10, 100 mL DMEM, 20% FCS.
14. Fugene6 (Roche).
15. Polyethylenimine (PEI; average molecular weight ~25) stock solution: 100 mg/mL in H₂O, extensively dialyzed (cut-off = 14 kD) to remove toxic low-molecular-weight contaminants.
16. Hanks' balanced salt (HBS) solution: 20 mM HEPES, 150 mM NaCl.
17. HBS solution (10X; Invitrogen, cat. no. 14065049).
18. G-418: 400 µg/mL.
19. Hygromycin B: 200 µg/mL.

3. Methods

3.1. Culture and Maintenance of Mammalian Cells

Cell cultures are derived from either primary tissue explants (skin, kidney) or cell suspension (lymphocytes). Primary cells typically will have a finite life span in culture, whereas continuous cell lines (COS, HEK293, NIH3T3, MRC5, CHO) are often transformed cell lines and carry an abnormal chromosome karyotype. Cultures should be examined daily, observing the morphol-

ogy, the color of the medium, and the density of the cells. A tissue culture log should be maintained that is separate from your regular laboratory notebook. The log should contain the name of the cell line, the medium components and any alterations to the standard medium, the date on which the cells were split and/or fed, and any observation relative to morphology. The cells are typically grown at 37°C in an atmosphere of 5 to 10% CO₂ and 20% O₂ because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained. The humidity must also be maintained for adherent cells growing in tissue culture dishes, so a pan of water is kept filled in the incubator at all times.

3.1.1. Growth and Subculture of Adherent Cells

The following protocol can be applied to most cells, both primary fibroblast and transformed cell lines. The medium composition is generally DMEM (or DMEM/F10) supplements with 10% heat-inactivated FCS and antibiotics. Cells are harvested when they have reached a population density that suppresses growth. Ideally, cells are harvested when they are in semiconfluent density. Some cells that are allowed to grow and stay to a confluent state for long time may never recover. On the other hand, primary fibroblasts seeded at high dilutions will not grow (*see Note 1*).

1. Place medium supplements with serum and antibiotics, trypsin/EDTA, and PBS in a 37°C water bath.
2. Clean a laminar flow hood with 70% ethanol before and after use. Sterilize with flame each component in contact with the cells (aspirator, pipets).
3. Remove used medium from a confluent dish (100 mm) with an aspirator and wash off any remaining media using 5 mL of PBS.
4. Harvest the cells by adding 1 mL of trypsin/EDTA and leave the cells 2 to 3 min to trypsinize in the dish at 37°C in the incubator.
5. Shake the dish and monitor the extent of detachment using a light microscope.
6. Once the cells are detached, add 5 mL of media to the cells and transfer to a 15-mL centrifuge tube. The media contains serum, and this will neutralize the action of trypsin.
7. Centrifuge the cells at 200g for 5 min; remove the supernatant using the aspirator.
8. Resuspend the cell pellet gently using 5 mL of medium. At this point a cell count may be carried out using a hemocytometer.
9. Depending on the extent of subculture (1:10 for transformed cells), split the cell suspension in a new dish with a total volume of 10 mL.
10. Some experiments require the seeding of an exact number of cells; these can be counted with a hemocytometer (*see Note 2*).

3.1.2. Storage

Liquid nitrogen (N_2) is used to preserve tissue culture cells. Freezing can be lethal to cells owing to the effects of damage by ice crystals, alterations in the concentration of electrolytes, and changes in pH. To minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent that lowers the freezing point, such as DMSO, is added. It is best to use healthy cells that are growing in log phase. Also, the cells are slowly cooled from room temperature to -70°C to allow the water to move out of the cells before it freezes.

1. To stock your cell lines prepare freezing medium and a 1-mL cryovial ampule (clearly labeled with date of freezing, passage number, name of investigator, and name of the cell line).
2. Harvest and pellet the cells as above.
3. Resuspend the cells in 1 mL of freezing medium and fill in the ampule.
4. Place the cryovial in a polystyrene box and place it overnight in a -70°C freezer. Alternatively, place the ampule in a freezing chamber filled with isopropanol at room temperature and place it in a -70°C freezer. The effect of the isopropanol is to allow the tubes to slowly equilibrate to the temperature of the freezer.
5. On the following day transfer the ampule to an N_2 storage vessel.
6. To resuscitate the cells from their storage in N_2 , prewarm the medium normally used to culture the cells in a 37°C water bath.
7. Wipe the ampule with a tissue soaked with 70% ethanol and gently warm the ampule in the water bath. Remove the content of the ampule and add it to a 15-mL tube containing warm medium.
8. Centrifuge at 200g for 5 min. Remove the supernatant and resuspend the pellet in fresh warm medium. Transfer the cell suspension to a new dish.

3.2. Establishing Primary Fibroblast Cell Line From Mouse Skin

As mentioned in **Heading 1**, the altered period of the biological clock of a *mCry* knockout mouse can be visualized in vitro using cultured fibroblasts derived from those animals. It is therefore important to establish a simple protocol to obtain primary fibroblast cell lines from the skin of mice, particularly because the number of clock-gene knockout mice available is increasing very quickly. Moreover, a complex set of functional interactions (nuclear import, transcription inhibition, stabilization, phosphorylation) occurs between core-clock proteins and it would be extremely interesting to test the functionality of a protein in absence of its specific partner.

1. Kill an adult mouse by cervical dislocation and pull off the hairs from the back. Alternatively, the animal can be anesthetized and a piece of the ear used instead of back skin (in this case more chondrocytes will grow in the culture).

2. Wash the exposed area with 70% ethanol and cut a small piece of skin (10 mm²).
3. Disinfect the tissue with 70% ethanol and wash in PBS.
4. In a small dish, cut the skin into small pieces with a razor blade.
5. Collect the tissue in an Eppendorf tube and incubate it for 3 to 4 h in TCH mix at 37°C in a cell incubator. Mix every 30 min to allow the enzyme to reach all tissues.
6. Add 2 mL of fibro-medium and spin down the dissociated cells at 200g for 5 min.
7. Remove the supernatant and resuspend the cell pellet in 2 mL of fibro-medium. Plate the cells in a 35-mm dish.
8. On the next day carefully replace the old medium with new one. After some days the cells will reach confluence and ready to be expanded. Culturing in a low-oxygen incubator (5–10% CO₂ and 3% O₂) will tremendously enhance the chance of success (*see Note 3*).

3.3. Transient Transfection of DNA in Adherent Cells

The efficiency of expression after transient transfection of plasmid DNA is dependent on the number of cells that incorporate DNA, the gene copy number, and the expression level per gene determined by the strength of the promoter in the plasmid. For several transformed cell lines (COS, HEK293, NIH3T3) it is possible to directly introduce plasmid DNA into 10 to 50% of the cells in the population. In contrast, primary fibroblasts and MEFs are extremely difficult to transfect and they require particular protocols (*see following sub-heading*). Transient expression offers a convenient means to compare different vectors and ensure that an expression plasmid is functional before using it to establish a stably transfected expressing cell line. A large variety of expression vectors for transient expression are described in the literature. Most useful vectors contain multiple elements that include (1) a simian virus 40 (SV40) origin of replication for amplification to high copy number in COS monkey kidney cells and HEK293; (2) an efficient, constitutive promoter element for transcription initiation (cytomegalovirus immediate early promoter, SV40 early promoter); (3) mRNA processing signals with polyadenylation sequences; and (4) selectable markers that can be used to select cells that have stably integrated the plasmid DNA into their genome.

3.3.1. Transfection of Immortalized Cells

The most widely used and convenient system for expression of a foreign gene is to introduce DNA into COS, HEK-293T, or NIH3T3 cells. COS cells are particularly used in the clock field because the endogenous expression of clock proteins is virtually absent in those cells, therefore allowing a better assessment about the functionality of a certain clock protein domain in cellular trafficking and protein–protein interaction. In contrast, NIH3T3 cells express endogenously all clock genes, thereby displaying a functional clock oscillation

upon serum shock induction. Functional studies in transient transfected NIH3T3 cells (nuclear import, etc.) must therefore take in account the possibility of functional interaction between the exogenous and endogenous clock proteins. Because of the high capacity of DNA incorporation of these transformed cell lines, different transfection reagents can be used (calcium phosphate, DEAE dextran, electroporation, cationic phospholipids) without affecting the end results too much. The following protocol is for transient transfection with Fugene6, which can be used for all the cell types mentioned above. Fugene6 is a nonliposomal reagent that produces high levels of transfection with minimal cytotoxicity for many eukaryotic cell lines.

1. COS cells are grown in DMEM medium supplemented with 10% FCS. They are usually subcultured twice per week at 1:8 split ratio.
2. Subculture the cells into a dish at 12 to 24 h before transfection. The cells should be 60 to 80% confluent at the time of transfection. For most cell lines, plate 1 to 3×10^5 cells in 2 mL in a 35-mm dish (6-well plate) to achieve this density.
3. On the day of transfection add 8 μ L of Fugene6 to a sterile tube containing 200 μ L of serum-free medium (OPTI-MEM). Wait 5 min at room temperature.
4. Dropwise, add diluted Fugene6 to the tube containing concentrated DNA ($\sim 2 \mu$ g).
5. Mix and incubate at room temperature for 15 min.
6. Add the mixture to the cells and test expression after 24 to 48 h.

3.3.2. Transfection of Primary Fibroblasts

Primary human dermal fibroblasts and MEFs are the most difficult adherent cells to transfect, and with the above method (Fugene6) a very low efficiency of transfection (1%) is normally achieved. The human lentivirus-based vector is one of the most utilized systems to obtain stable DNA integration and expression in these cells. As the particles are pseudotyped with the envelope of the vesicular stomatitis virus, the vector can serve to introduce genes in a broad range of tissues (human and mouse) and it can also transduce nondividing cells such as neurons. The design of a viral vector system relies on the segregation in the viral genome of *cis*-acting sequences encoding the viral proteins. To assemble the prototype vector particle the viral proteins are expressed from separate constructs (for safety reasons) stripped of all *cis*-acting sequences. These sequences are instead used to frame the expression cassette for the gene of interest (transgene) driven by an heterologous promoter (cytomegalovirus) but without polyadenylation signal. As the particle will transfer only the transgene construct to the genome, the infection process is limited to a single round without spreading. We will report briefly the major steps for the production of such transgene delivery system.

1. The primary construct is obtained by cloning the gene of interest driven by the CMV promoter in a self-inactivating lentiviral vector plasmid.

2. The additional plasmids required for the virus production are those expressing the human lentivirus-1 *gag*, *pol*, and *rev* proteins, and the plasmid expressing the envelope G-glycoprotein of vesicular stomatitis virus. All individual plasmids are prepared by using the Qiagen Endotoxin-Free Maxi Kit.
3. Grow the packaging cell line HEK-293T to approx 80% confluence in a 100-mm dish with DMEM and 10% FCS (7×10^6 cells/dish). Alternatively, for larger virus production, 24 × 24 cm dishes (7×10^7 cells/dish) may be used after scaling up the reagents to take into account the larger surface area.
4. The following day dilute the PEI stock solution to 1 mg/mL in water. To prepare the transfection mixture for one 100-mm dish, 20 µg of each of the four endotoxin-free plasmids (80 µg in total) are added to 1 mL of HBS solution.
5. DNA is added to 1 mL of PEI/HBS solution (240 µL of 1 mg/mL PEI in 760 µL HBS) dropwise during with vortexing. For optimal results, the PEI:DNA weight ratio should be titrated. The optimal ratio is usually 2 to 3 µg PEI per µg plasmid DNA.
6. Wash the cells with PBS and add DMEM without serum prior to transfection.
7. Add the freshly prepared transfection mixture dropwise while gently swirling the dish. Incubate the dish for 3 h at 37°C in the incubator. Then change the medium with fresh DMEM with 10% FCS and incubate overnight.
8. Harvest the virus by collecting the cell culture medium after 24, 48, and 72 h. Following each harvest filter the medium through a 0.45-µm cellulose acetate filter.
9. Store the collected medium at 4°C. After the final harvest concentrate the viral particles by pelleting with ultracentrifugation at 50,000g for 2 h at 20°C.
10. Aspirate the supernatant and resuspend the pellet in Hanks' buffer in 0.1% volume of the final volume of the medium collected. Aliquot and store at -80°C until further use.
11. After virus titration, the virus can be used to transfect primary fibroblasts by adding it directly into the medium. With this technique, up to 90% of the cells will stably express the gene of interest.

Infection with viral vectors (adenovirus, lentivirus) carrying the gene of interest is the most common technique to deliver DNA into primary fibroblast; however, this requires special knowledge of virus manipulation and expensive laboratory settings. A most convenient method has been recently introduced by Amaxa Biosystems. The Human Dermal Fibroblast Nucleofector™ Kit from Amaxa represents the first nonviral transfection method for human dermal fibroblasts that permits high gene transfer efficiencies and reproducibility. In neonatal normal human dermal fibroblasts (NHDFs), for example, transfection efficiencies of up to 90% can be routinely achieved. As the DNA is delivered directly into the nucleus during nucleofection, high transgene expression can already be detected just 5 h after transfection. Microscopic analysis reveals that neonatal NHDFs maintain their normal morphology after nucleofection.

As adult and neonatal NHDFs differ in terms of biological function and tissue source, two separate optimized protocols for the transfection of these cell types have been established and are included in the Amaxa kit. For instance, neonatal dermal fibroblasts can be used up to passage number 15, whereas adult dermal fibroblasts are suitable only up to passage number 10. Between 2×10^5 and 2×10^6 cells can be nucleofected per experiment using very low amounts (2 μ g) of plasmid DNA.

3.4. Establishing Stable Transfected Cell Line

Many proteins mislocalize when overexpressed, making the use of stable cell lines expressing physiologic levels of the protein of interest a more time-consuming but ultimately a more reliable method. There are many different approaches for establishing stable cell lines, depending on the type of expression in which you are interested (inducible, constitutive, endogenous) and the plasmid that you are incorporating (carrying selection for puromycin, G-418, hygromycin B). Prior to transfection, it is recommended that you linearize your gene construct. Linearizing will decrease the likelihood of the vector integrating into the genome in a way that disrupts the gene of interest or other elements required for protein expression. Furthermore, as each cell line has different sensitivity to G-418, hygromycin, or puromycin, you should determine by titration the optimal concentration of drug for selection. Then use the lowest concentration of drug that begins to give massive cell death in 3 d and kills all the cells within 2 wk.

We will present here a protocol for stable transfection of HeLa cells selected with G-418 (*see Note 4*). The protocol for stable MEFs is similar but these cells are usually selected with hygromycin B (200 μ g/mL), as they are derived from transgenic and knockout mice carrying in their genomes G-418-resistant targeting constructs.

1. Grow HeLa cells to approx 80% confluence in complete medium and transfect your plasmid with appropriate methods for transformed adherent cells; for example, Fugene6 is a good method.
2. After 24 to 48 h from transfection, cells are split to 1:10, 1:20, or 1:50 into a 100-mm dish containing 10 mL of DMEM supplemented with 10% FCS and 400 μ g/mL G-418.
3. Observe cell growth and change the medium containing the selection drug every 2 d. Keep exchanging the medium every 2 d for 7 to 10 d. This is the time needed for G-418 to act on the nontransfected cells, which then detach and are washed away during the medium exchange. Once all cells have died in the dish of the negative control (nontransfected) and the colonies begin to appear on the transfected dish, you can proceed with the cloning.
4. To pick colonies place an inverted light microscope under the laminar flow hood.

5. Prepare 24-well dishes with 1 mL of selective medium. Rinse the 100-mm dish with PBS and then add warm PBS containing 5% trypsin (1 mL standard trypsin-EDTA plus 19 mL PBS).
6. Using a Gilson pipet with a sterile yellow tip, lower the tip to the surface of the colony of interest, and scrape and suck gently until you have pulled it into the tip.
7. Transfer into a well of a 24-well plate. Repeat with other colonies.
8. In a day or two, when the cells are confluent, rinse in PBS and trypsinize with 100 μ L of trypsin/EDTA. Split into a 6-well plate for passaging.
9. Store the cells and test for expression.

4. Notes

1. Transformed cell lines can be split and seeded at high dilutions (1:10, 1:50) and they will continue to grow and divide until the dish becomes completely confluent again after a few days. NIH3T3 and HeLa cells undergo contact inhibition and will not grow at full confluence. COS and HEK293 cells will continue to grow even after having reached full confluence. This stage (overgrowth) will become clear by the change in color of the medium from red to yellow. In contrast, human primary fibroblasts are very sensitive to cell density concentration and will stop growing and go into crisis if the culture is too diluted. For that reason you must split and pass human primary fibroblasts at 1:2 and not at higher dilutions.
2. The hemocytometer is a modified microscope slide consisting of two polished surfaces, each of which displays a precisely ruled, subdivided grid. The grid consists of nine primary squares and is limited by three closed spaced lines. These triple lines are used to determine if cells lie within or outside the grid. The middle of the triple lines separating each primary square is the boundary. Cells that touch the upper or left boundaries are included; those that touch the bottom or right boundaries are excluded. In brief, harvest and resuspend the pellet cells in an appropriate volume of medium ensuring a unicellular suspension. Place the square glass cover slip on top of the hemocytometer and gently load 50 μ L of the cell suspension within the two polished surfaces using a Gilson pipet. Use the $\times 20$ objective of the microscope to count the cells in 10 primary squares. This count gives the number of cells within 1 mm^3 or 1×10^{-3} mL. The total cell concentration in the original suspension (in cells/mL) is then: total count $\times$ 1000 $\times$ dilution factor cells/mL.
3. The effects of providing low oxygen tension in the gas phase of a primary cell culture have been investigated. The growth of cloned primary mouse dermal fibroblasts was improved markedly by incubation within a low oxygen tension gas phase (48 mmHg [3%]) instead of air (135 mmHg [20%]), as measured by an increase in the number of colony-forming cells and in the colony sizes. This is likely the result of the higher environmental stress and increased DNA damage from free oxidant radicals when culturing in the presence of high oxygen.
4. The cloning of the protein of interest into a enhanced green fluorescent protein vector will greatly facilitate the establishing of a stable cell line, as the express-

ing clones will be identified with an inverted fluorescence microscope. Use a black pencil to label the dish underneath the positive colonies before picking them under normal light.

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Reporter Assays

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Summary

Transcriptional feedback loops are at the core of the molecular clockworks. As single clock genes were cloned it was compelling to develop an assay that allowed simple and direct functional testing of putative activators or repressors of transcription. This chapter includes a general description and guidelines to carry out transcriptional assays in transiently transfected Schneider's cells.

Key Words: Transcriptional assays; firefly luciferase; *Renilla* luciferase; S2 cells.

1. Introduction

Reporter assays are widely used to study gene expression and other aspects of cellular function. Among the most common uses are the characterization of transcription factors ([1–3](#)) and the dissection of signaling pathways ([4,5](#)). Transcriptional assays usually employ two reporter genes to improve experimental accuracy. This implies simultaneous expression and measurement of two reporters within a single experimental set. One of them reports the effect of the specific experimental conditions that are under investigation; meanwhile, the second reporter provides an internal control that reflects the baseline response. The latter is usually under the control of a constitutive promoter that is anticipated not to be affected by the experimental condition. Determining the expression of both reporter genes allows normalization of the activity of the “experimental” reporter to the internal control, minimizing variability owing to differences in cell viability or transfection efficiency. Other sources of variability, such as differences in pipetting volumes, cell lysis efficiency, and assay efficiency, are also under control.

Normalization against other parameters such as total protein (*see Note 1*) takes into account the discrepancy arising from an imperfect technique, but does not reflect the variability stemming from differential transfection efficiencies.

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1.1. Reporters

The bacterial chloramphenicol acetyl transferase (CAT) enzyme transfers acetyl groups to chloramphenicol from acetyl coenzyme A. In a typical assay this reaction is monitored by ^{14}C -labeled chloramphenicol, where acetylated and nonacetylated forms can be separated by thin-layer chromatography and quantitated in a scintillation counter. Because CAT is very stable (a couple of days in culture), it is not the reporter of choice when temporal changes in gene expression are studied.

Bacterial β -galactosidase (β -Gal), encoded by the *LacZ* gene, breaks down lactose in β -D-galactose and β -D-glucose. Several chromogenic or fluorogenic substrates have been synthesized to exploit the enzymatic activity of β -Gal as a reporter system.

The *green fluorescent protein* (*gfp*) is encoded in the genome of the jellyfish *Aequorea victoria*. The intrinsic fluorescence of the GFP protein is the result of a unique covalently attached chromophore that is formed posttranslationally within the protein upon cyclization and oxidation of residues 65–67, Ser–Tyr–Gly (6). GFP is a relatively small protein (~27 kDa) and diffuses freely within a cell. It is widely employed as an amino or carboxyterminal fusion to the protein of interest, to follow its destination within the cell as well as patterns of expression.

Firefly luciferase (LUC) catalyzes a bioluminescent reaction in which its substrate (luciferin) is oxidized, and in doing so it generates light as a byproduct. This light (or bioluminescence) can be quantitated with a luminometer or a scintillation counter (i.e., Packard TopCount). Bioluminescence has been measured in *Drosophila* tissue culture extracts (7) and in whole flies and isolated body parts (8–11). LUC has a relatively short half-life (about 4 h in flies as measured in ref. 11), which is an absolute requirement if it is to report in vivo rhythmic transcriptional oscillations.

1.2. How to Choose the Ideal Reporter Assay

Initially, CAT and β -Gal were the most commonly used reporters. Later on, GFP and LUC opened the possibilities further, as they allow in vivo monitoring of gene expression. The most appropriate reporter must be selected depending on the nature of the question. β -Gal and LUC are the reporters of choice when quantitation is an issue, as both fluorometric and colorimetric assays are available to monitor the expression of the former and, for the latter, bioluminescence is easily quantified across a broad range of emissions. On the other hand, GFP is by far the ideal one for in vivo spatial reporting. However, when it comes to reporting in vivo circadian expression, most reporters fail owing to protein stability issues (they stay around far too long). Only LUC and the unstable version of GFP are useful in this regard.

1.3. Transcriptional Assays

To analyze in depth the function of putative transcription factors, a reporter construct needs to be generated. Usually it takes the form of a full-length promoter or a portion therein where the major target sequences are included, driving the reporter gene selected (i.e., *luciferase*). This native regulatory region can also be replaced by a multimerized version of the core motifs (i.e., the E-box; [12](#)) flanked by the native sequences (from the *per* or *tim* promoters, [ref. 1](#); see [Note 2](#)). These constructs require the addition of a minimal promoter for basal transcription. Most of the circadian reporter constructs derive from the pGL3 basic vector (Promega), which carries the *luc* gene downstream a multiple cloning site (see [Note 3](#)). Likewise, most labs perform reporter assays in *Drosophila* Schneider's cells ([1,3,4,13](#)); when this is the case, the minimal promoter employed derives from the heat shock protein 70 (*hsp 70*) gene (see [Note 4](#)).

1.4. Expression Vectors

To test the ability of a putative transcription factor to activate or repress transcription in cultured cells, it must be subcloned into an expression vector such as pAct5C ([Fig. 1](#)), under the *actin* promoter ([14](#)). A more recent version of this vector (pAct5.1, available from Invitrogen) allows cloning in-frame with different tags (his, V5), simplifying the subsequent identification of the protein of interest (see [Note 5](#)). These type of assays benefit from constitutive and rather strong expression where the *actin* promoter is the one of choice.

1.5. Controls

When running transcriptional assays, a number of controls need to be included:

- Transfection efficiency: a second reporter not affected by the experimental condition. Originally β -Gal was employed, but given the disparity between the test conditions for the β -Gal and LUC assays, it is increasingly common to use a second LUC reporter (Renilla *luciferase* gene under a constitutive promoter such as *copia* or *actin 5C*) that can be assayed together with the firefly one (see Promega's dual-reporter assay system).
- Binding specificity: a mutated version of the regulatory region controlling reporter expression to highlight nonspecific binding of the putative transcription factor(s) under analysis.
- Baseline reporter activity: determined in the absence of any putative transcriptional regulator; usually an empty pAct5C vector is sufficient to determine the leakiness of the reporter system in use.

2. Materials

1. Schneider's S2 cells.
2. Serum-containing medium (SCM): Schneider's cells medium, 10% fetal calf serum (FCS) heat-inactivated at 56°C for 30 min, 50 U penicillin G, 50 μ g/mL streptomycin sulfate, filter-sterilized.

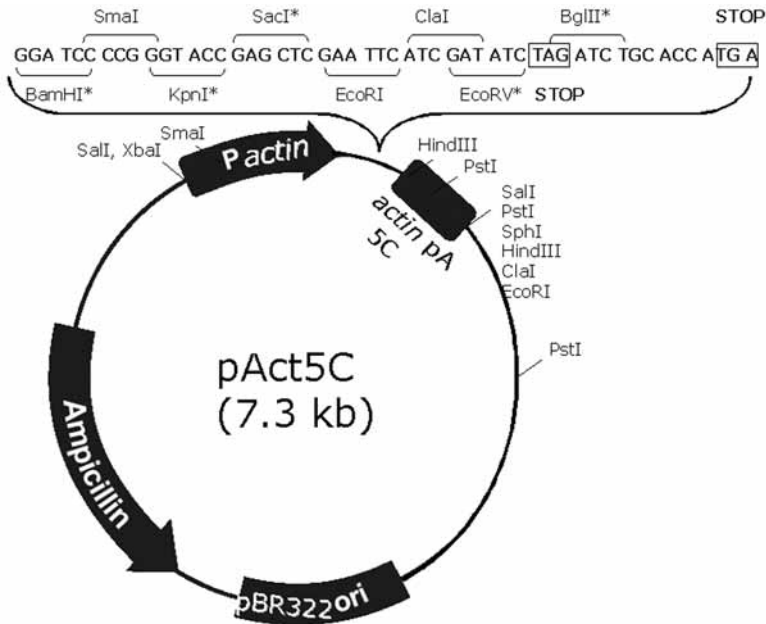


Fig. 1. Original version of pAct5C. Additional description: *pactin*, *actin* promoter region; *actin* pA, poly adenilation signals from the *actin* locus. *Ampicillin*, gene conferring ampicillin resistance in *Escherichia coli*; pBR322 ori, origin of replication in *E. coli* derived from plasmid pBR322. Arrows indicate the direction of transcription. Asterisks highlight single cutters.

3. Serum-free medium (SFM): Schneider's cells medium, 50 U penicillin G, 50 µg/mL streptomycin sulfate, filter-sterilized.
4. Sterile pipets and technique.
5. Laminar flow hood.
6. Quiet drawer or incubator: 22 to 25°C.
7. Plasmid DNA purification columns (such as Mini or Midipreps from Qiagen).
8. Lipid-based reagents: Lipofectin (Invitrogen) or other.
9. Tissue-culture-treated Corning (Costar) 6- and 12-well culture clusters.
10. 10X Phosphate-buffered saline (PBS) buffer: 11.5 g/L Na₂HPO₄, 2 g/L KH₂PO₄, 80 g/L NaCl, 2 g/L KCl, pH 7.4. Dilute to 1X PBS before use.
11. Passive lysis buffer (PLB; Promega).
12. Dual luciferase reporter assay kit (Promega, cat. no. E1910).
13. Luminometer.
14. Bicinchoninic acid (BCA) protein assay reagent kit (Pierce, cat no. 23227).
15. ELISA plate reader (or spectrophotometer).

3. Methods

A more detailed transfection protocol is provided in Chapter 33. Only a brief overview will be included here.

3.1. Transient Transfection Employing Lipofectin

1. Under the laminar flow hood, seed twice as many wells as needed in a 12-well culture cluster (*see Note 6*), adding 0.8 mL of cells/well at a density of 1×10^6 cells/mL.
2. Let them sit for 12 to 24 h.
3. Clean up the plasmid DNAs with Qiagen columns (*see Note 7*).

The following steps will greatly vary depending on the lipid-reagent chosen; a protocol employing lipofectin (Invitrogen) will be described in more detail.

4. Right before transfection, activate the lipid reagent by diluting it in SFM. Use 8 μ L lipofectin per well, diluted 1:5 in SFM (40 μ L in total). Let it sit for 30 to 45 min at room temperature inside the hood.
5. Prepare the DNA solutions in an equal volume of SFM (40 μ L). It is convenient to prepare a DNA mix for each of the reporter vectors to be tested mixed with the internal control (i.e., Promega's pRL). Prepare 10 to 20% extra of solution to account for pipetting errors. Reporter and control vectors should be used at a 10:1 (to even 50:1) ratio to help ensure independent genetic expression. Typical concentrations for reporter vectors are 0.1 to 0.2 μ g/well, for pRLcopia controls 10 to 25 ng/well (*see Note 8*). It is important to keep the total amount of DNA/well constant (*see Note 9*).
6. Combine the lipofectin and plasmid solution(s) and let it stand for 10 min at room temperature. In the meantime get a sterile cotton-plugged Pasteur pipet ready in an aspirator. Because lipofectin is inhibited by serum, the SCM in each well needs to be removed prior to transfection (*see Note 10*). Keep in mind that the S2 cells do not adhere tightly to the plastic surface, so be careful to avoid discarding some of them in the process.
7. Dilute the lipid-DNA complexes up to 400 μ L/well in SFM and quickly add to each well dropwise.
8. Cover with Parafilm. Place in an incubator (or quiet drawer) at room temperature (22–25°C).
9. Let the transfection proceed for a minimum of 8 h (typically overnight) and then add an equal volume (400 μ L/well) of SCM with 20% FCS.
10. Cover with Parafilm. Place in an incubator (or quiet drawer) at room temperature (22–25°C).
11. A time course is recommended to determine the optimal harvesting time. Usually the cells are collected 24 to 48 h after transfection.

3.2. Harvesting S2 Cells

1. Resuspend the cells by gently pipetting up and down with a P1000 micropipet, and transfer to a 1.5 mL microcentrifuge tube. If too many cells were left in the well, add 1X PBS and repeat the operation.
2. Spin down at 100g for 2 min.
3. Remove the supernatant and add an equal volume of 1X PBS to wash out residual SCM. Resuspend the cells carefully, so as not to break them open.
4. Spin down at 100g for 2 min and remove most of the 1X PBS.
5. Add 250 μ L of freshly diluted PLB, gently resuspend the cells, and keep them for 15 min at room temperature before vortexing to break the cells open (*see Note 11*). The efficiency of this operation can be enhanced by a freeze–thaw cycle.
6. Take 1 μ L of each lysate to determine firefly and *Renilla* LUC activity according to the manufacturer's recommendations, and keep the remainder at -80°C . Subjecting cell lysates to more than two or three freeze–thaw cycles may result in gradual loss of LUC reporter enzyme activities.

3.3. Performing Firefly and Renilla LUC Assays

1. Add 10 μ L of prepared cell lysate to 50 μ L of Luciferase Assay Reagent II (from dual LUC reporter assay kit) predispensed into luminometer tubes. This reagent is light-sensitive, so prepare only as many tubes as needed; mix by pipetting up and down two or three times (avoid vortexing) and initiate reading.
2. Quantify firefly luciferase activity.
3. Add 50 μ L of Stop & Glo Reagent (from dual LUC reporter assay kit) and quickly mix (*see Note 12*).
4. Quantitate *Renilla* LUC activity.

3.4. Normalization Against Protein Content With BCA Reagent

1. Prepare dilutions of bovine serum albumin (provided in the BCA kit) in a 0 to 2000 $\mu\text{g/mL}$ range following the manufacturer's recommendation to build a calibration curve.
2. To perform protein quantitation in 96-well microtiter plates, dispense 250 μ L of BCA reagent per well. The reagent is light-sensitive, so prepare only as many wells as needed for the calibration curve and samples. A calibration curve is built for each set of measurement (within the same microtiter plate).
3. Add 10 μ L of the bovine serum albumin standards together with 5 μ L of PLB to the BCA reagent in each well used for the calibration curve (*see Notes 13 and 14*).
4. To determine the protein concentration in the transfected samples add 5 μ L of each lysate diluted in 10 μ L of deionized water to the BCA reagent in the remaining wells.
5. Take the microtiter plate to an ELISA reader, setting the filter to 562 nm (wavelengths between 540 and 590 nm can be used successfully).

4. Notes

1. In some instances the normalization reporter does not stay constant throughout the experiment; instead, it appears to follow the same trend as the experimental control. Under those circumstances normalization against total protein is recommended to avoid diluting the experimental effect, although it is advisable to resort to a different normalization reporter for future experiments. Total protein can be determined using a modified version of Bradford's reaction (*see Subheading 3.4.*).
2. Each multimer reporter consisted of four 18-bp elements containing the E-box from either the *per* or the *tim* promoters. The multimers were made by piecing together two 50-mers containing half of the multimer each. The core sequence included the 6-nt palindrome (GAGCTC) flanked on each side by the six naturally occurring nucleotides; novel sequences introduced (between each monomer, 6 nt) were chosen so that they would not match with the corresponding ones in the native promoters. Each fragment was first annealed to its complementary sequence, and then ligated to the other half. A novel restriction site was created in the hinge to simplify the screening process. Additionally, two different restriction sites on each end were introduced to allow for directional cloning into the reporter vector (pGL3hs).
3. To test for binding specificity, it is convenient to generate another reporter construct carrying mutations within the consensus site. The mutated E-box contained the central two nucleotides swapped (CG instead of GC).
4. When employing mammalian cells, conditions will vary greatly depending on whether they adhere or not to the plastic surface; follow manufacturer's recommendation with regard to the ideal plating density depending on the lipid reagent used. Other aspects worth mentioning are that mammalian cells grow at 37°C and 5% CO₂, and they require a more refined sterile practice. Mammalian tissue culture is carried out under a biosafety hood. Promega has different series of firefly and *Renilla* LUC vectors that may be used to cotransfect mammalian cells with any experimental and control reporter genes.
5. As biological observations may vary to a certain extent depending on the levels of expression accomplished (compare **refs. 1** and **16**) it is recommended to set up the conditions for the constructs in hand and perform dose-response curves at a number of concentrations (*see Note 8*).
6. Running transcriptional assays in duplicate is recommended, although this repetition should not be taken as a statistically meaningful one.
7. Plasmid DNA used for transfection should be of high quality. Impurities present in the DNA preparations may lower the transfection efficiency. DNA should be purified employing Qiagen columns or similar. Ideally, the final concentration should be higher than 0.1 to 0.2 µg/mL, to reduce the amount of impurities present during the transfection.
8. The extreme sensitivity of both firefly and *Renilla* LUC assays, and the very large linear range of luminometers (typically 5 to 6 orders of magnitude), allows

accurate measurement of both activities. The advantage is that it is possible to add relatively small quantities of a control vector that will aid greatly in suppressing the occurrence of *trans* effects between promoter elements. Expression vectors alone or in combination need to be tested at a range of concentrations. Potent transcriptional activators such as *dClock* (from a pAct-*dClk* construct) are included at a concentration of 0.5 to 1 ng/well. The cognate CLK partner (CYCLE/DBMAL) does not need to be included, as it was reported to be expressed in naïve Schneider cells (Wager-Smith and Kay, unpublished results). On the other hand, *timeless* or *period* are usually tested in a wider range of concentrations: 10 ng/well (**1**); 10, 100, and 600 ng/well (**3**); 10 and 50 ng/well (**16**).

9. Usually the total amount of DNA is equalized by adding empty expression vector (i.e., pAct5C).
10. To avoid excessive dissection that will be detrimental to cell viability, remove SCM from a maximum of four wells at a time.
11. Generally, it is unnecessary to clear lysates of residual cell debris prior to performing the LUC assays. However, if subsequent protein determinations are to be made, clear the lysate samples for 30 s by centrifugation in a refrigerated microcentrifuge. Transfer the cleared lysates to a fresh tube prior to reporter enzyme analyses.
12. Quenching of firefly LUC luminescence and concomitant activation of *Renilla* LUC are accomplished by adding this reagent to the sample tube immediately after quantitation of the firefly LUC reaction.
13. Certain chemicals present in the PLB may interfere with the colorimetric reaction in a concentration dependent manner. When performing this reaction in microtiter plates it is advisable to use a small volume (5 to 20 μ L) of cell lysate and to include an equivalent volume of PLB in the calibration curve.
14. In the microtiter plate format the BCA kit works in the range of 20 to 1500 μ g/mL.

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Use of Firefly Luciferase Activity Assays to Monitor Circadian Molecular Rhythms In Vivo and In Vitro

Wangjie Yu and Paul E. Hardin

Summary

Circadian rhythms in metabolic, physiological, and behavioral processes are regulated by biological clocks. Many of these rhythmic processes can be measured over many days or weeks using automated recording devices, thus making it possible to precisely calculate period, phase, and amplitude values. With the advent of luciferase reporter genes and machines capable of quantifying luciferase-generated bioluminescence over long time frames, it is now possible to precisely monitor the rhythms in gene expression that underlie circadian clock function. These assays can be used to monitor gene expression in large numbers of individual plants and animals, and/or various cultured tissues and cells. After acquiring bioluminescence data, rhythm analysis programs are used to calculate the period, phase, amplitude, and overall levels of gene expression for individuals or groups, and to measure their statistical significance. Here we will describe how luciferase assays are performed and analyzed to measure gene expression rhythms in *Drosophila*.

Key Words: Circadian rhythm; biological clock; luciferase reporter; *period*; *timeless*; bioluminescence; gene expression; *Drosophila*; cultured tissues.

1. Introduction

Circadian clocks regulate numerous metabolic, physiological, and behavioral processes in most eukaryotes and even some prokaryotes. These clocks are organized as a series of three components: an input pathway that transmits environmental time cues to set the phase of the oscillator, an oscillator that keeps circadian time and activates output pathways, and output pathways that activate metabolic, physiological, and behavioral rhythms at the appropriate time of day (*1*). Rhythmic outputs have long been used to acquire information about the period, phase, and amplitude of the circadian oscillator. Precise determination of these circadian parameters has largely been the result of the

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development of automated devices capable of continuously monitoring outputs over many days or weeks. Such measures of oscillator function are necessarily indirect, but it is now possible to directly measure oscillator function by monitoring the abundance of rhythmically expressed components of the circadian oscillator. Methods for directly measuring gene expression include Northern blots, RNase protection assays, quantitative real-time reverse transcription polymerase chain reaction and *in situ* hybridization for detecting mRNAs, Western blots and immunohistochemistry for detecting proteins, and *in vitro* enzyme activity assay for detecting reporter enzyme levels. Each of these methods requires the sacrifice of animals, plants, or microbes at different circadian times, is labor-intensive, and takes several days to complete. Because of these drawbacks, it is not practical to measure gene expression levels at short enough intervals (i.e., ≤ 1 h) and over long enough time frames (i.e., ≥ 5 d) to accurately measure the key parameters of oscillator function. A system for measuring gene expression using firefly luciferase (*luc*) is now available that overcomes these drawbacks. Firefly *luc* acts on its substrate luciferin to produce bioluminescence, which can be accurately measured using a luminometer. Although *luc* protein is relatively stable, the half-life of *luc* activity (as measured by bioluminescence levels) is quite short—on the order of 4 to 5 h (2). This dynamic feature of *luc* activity makes it possible to measure clock-controlled gene expression *in vivo*. When a clock-regulated promoter is used to drive *luc* expression, bioluminescence can be recorded in real time from live samples at ≤ 1 -h intervals over days or weeks to directly measure circadian gene expression. This system was first applied to the clocks field in plants, where *chlorophyll A/B binding protein* (*cab*) promoter-driven *luc* transgenes were used to monitor clock function (3,4). The *cab* reporter has been particularly useful as a screening tool for clock mutants, ultimately identifying key components of the circadian oscillator in plants (5). The use of *luc* reporters to monitor gene expression rhythms is now used in a number of organisms including cyanobacteria (6), insects (7), zebrafish (Kaneko and Cahill, unpublished data), and rodents (8,9).

Here we will review the methodology used to monitor *luc* rhythms in the fruit fly *Drosophila melanogaster*—the first animal system to employ clock gene-regulated *luc* reporters. This methodology has been used to screen for clock mutants (10), define clock-regulated promoters (11), and study oscillator autonomy in cultured tissues (12–15) in this species, thus illustrating its usefulness and scientific value. Transgenic flies containing a *per* or *tim* promoter driven firefly *luc* cDNA have been generated, and exhibit a similar cyclic expression pattern and tissue distribution as their endogenous counterparts (7,16). Luciferin can be easily administered to live flies by adding it to their food, or to isolated fly tissues by adding it directly to their culture media. The

resulting bioluminescence is then monitored in an automated Packard TopCount multiplate scintillation and luminescence counter. Of particular significance is the fact that a large number of individual flies or isolated tissues can be monitored simultaneously using this system, thus increasing the statistical significance of the results. We will initially describe *luc* activity assays in living flies as the main protocol, then use of *luc* activity assays in cultured tissues as an alternative protocol.

2. Materials

1. Transgenic flies containing a firefly *luc* reporter gene driven by a clock-regulated promoter. Several lines of *per* or *tim* driven luciferase transgenic flies were developed in Dr. Steve Kay's laboratory at University of Virginia/The Scripps Research Institute, Dr. Jeffrey Hall's laboratory at Brandeis University, and Dr. Ralf Stanewsky's laboratory at the University of Regensburg (7,10,16–18).
2. Packard TopCount Multiplate Scintillation and Luminescence Counter (PerkinElmer Life Sciences).
3. A constant temperature (typically 22°C) dark room to house the TopCount.
4. A light source controlled by a daily timer.
5. White 96-well microtiter plates (white Optiplate, PerkinElmer Life Sciences).
6. Clear 96-well microtiter plates popularly used in cell culture or tissue culture (clear plates).
7. 96-Well microplate press-on adhesive sealing film: TopSeal-A (PerkinElmer Life Sciences).
8. Clear MicroAmp caps (8 caps/strip; Applied Biosystems).
9. Firefly D-luciferin, potassium salt (Biosynth; see Note 1).
10. Luciferin-fortified fly food: 5% sucrose, 1% microbiology-grade agar, 15 mM luciferin.
11. Import and Analysis Macro Set software developed in the Kay laboratory at The Scripps Research Institute (www.scripps.edu/cb/kay/ianda/).
12. Fast Fourier transform–nonlinear least squares (FFT-NLLS) statistical analysis software developed by Marty Straume at the National Science Foundation Center for Biological Timing.
13. Schneider's *Drosophila* Media (Invitrogen).
14. Heat-inactivated fetal bovine serum (Invitrogen).
15. 100X Penicillin/streptomycin (Invitrogen).
16. Insulin (Sigma; see Note 2).

3. Methods

The methods described below outline the following (Fig. 1):

1. Preparation of domes.
2. Preparation of luciferin-fortified fly food.
3. Loading of flies.
4. Setting up the TopCount monitoring program.

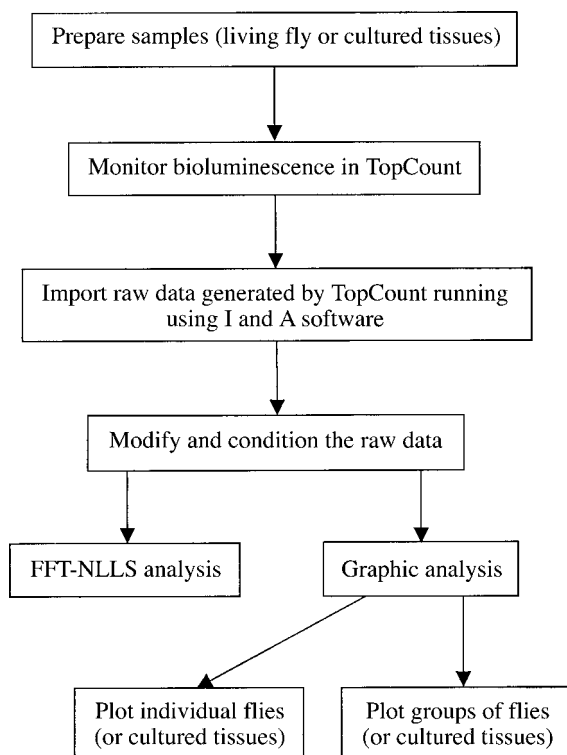


Fig. 1. Summary of the protocol for assaying *luc* activity in live flies and cultured tissues.

5. Running the TopCount.
6. Data analysis.
7. A protocol for monitoring cultured tissues.

3.1. Preparation of Domes

Poke two holes into each clear MicroAmp PCR cap using a 23G $\times$ 1" needle, then cut and trim the caps using a razor blade (*see* **Notes 3** and **4**).

3.2. Preparation of Luciferin-Fortified Fly Food

1. Add 3.0 g of sucrose and 0.6 g of microbiology grade agar to 50 mL of sterile MQ water in a 200-mL beaker.
2. Boil the mixture in a microwave until dissolved. Extended heating will cause the mixture to boil over, so short periods of heating followed by swirling the mixture is recommended.

3. Place the mixture in a 48°C water bath.
4. After the mixture cools to 48°C, put 5 mL into a plastic 50-mL centrifuge tube that is prewarmed to 48°C.
5. Vortex the defrosted luciferin stock solution to make sure the solution is well mixed, add 1 mL of the luciferin solution to 5 mL of warmed mixture, and mix thoroughly. This volume of luciferin-fortified fly food media is sufficient to fill 48 wells in a 96-well white Optiplate.
6. Set a micropipettor to a volume of 90 μ L, cut the end of a 200- μ L aerosol-barrier tip, and add 90 μ L of the media to each well. Fill only every other well (i.e., A1, C1, E1, G1, B2, D2, F2, H2, etc.) to avoid bioluminescence crosstalk between the wells during counting.
7. Let media solidify at room temperature for at least 15 min.

3.3. Loading Flies

1. Flies are aged 2 to 5 d and should be entrained to light–dark (LD) cycles.
2. Anesthetize the flies with CO₂, pick the fly up by the wing with forceps, and place the fly onto the food in a well.
3. Carefully cover the fly with a dome made in **Subheading 3.1.** (*see Note 3*).
4. Load flies onto the plate in column order (e.g., A1, C1, E1, G1, then B2, D2, F2, H2, etc.). Record the order, group name, and date (*see Note 5*).
5. Place TopSeal-A film on top of the white Optiplate and poke a hole through the film over each well using an 18G $\times$ 1 1/2" needle (*see Note 4*).

3.4. Setting Up TopCount Monitoring

Bioluminescence rhythms in *Drosophila* are counted using a TopCount. This instrument has an open (i.e., exposed to room conditions) microtiter plate stacker design. By intercalating empty clear plates between white Optiplates, samples contained in the white Optiplates can be exposed to external light while in the stacker, and then cycled into the light-tight counting chamber to measure bioluminescence. Readers should refer to the TopCount manual for details on setting up a TopCount monitoring program using Assay Wizard. Several points are mentioned here to meet the special requirements for in vivo luminescence counting and subsequent output analysis.

1. **Setting sample counting times.** In the Assay Wizard, adjust “How long would you like to count each well” in “Count Options,” “Delay before start of count” (3 to 10 min is recommended for whole flies; *see Note 6*) in “Advanced Count Options,” and the number of samples on maps for white Optiplates in “Sample Mapping” so that each sample is counted as close to once per hour as possible.
2. **Setting the run length.** Select “Number of times to count assay” in the “Advanced Count Options” window so that the machine will run continuously for more than 7 d. Entering 0 here instructs the instrument to run continuously until it is stopped manually.

3. **Organizing the raw data for export.** To meet the requirements for data import by the Import and Analysis (I&A) software, choose “ASCII File Output” in the “Report Definition” window. In the same window, select “Columnar” in “Data Layout,” “Excel Import” in “File format,” then choose “Do NOT Label Columns” and “Increment File Extension After Each Assay.”
4. **Selecting the luminescence counting mode.** Select CPS (count per second) as the unit for luminescence.
5. **Normalizing the TopCount for bioluminescence counting.** Before starting the run, the instrument is first normalized for counting bioluminescence using the Packard 96-well bioluminescence normalization plate that comes with the instrument. Set the “Number of times to count assay” in the “Advanced Count Options” window to 2 for normalization (i.e., samples are counted twice). If the counts are less than 40,000, you should contact Packard technical support for advice. Reset the “Number of times to count assay” for your sample counting as described in **step 2** after normalization is finished.
6. **Mapping samples in the bioluminescence assay plates.** A map of each plate is then made in the “Sample Mapping” menu. The first plate in the stacker, which is clear, will have a blank map, as it contains no samples. The clear plates allow light to reach samples in the white Optiplates; therefore, each white plate is preceded by a clear plate. The white Optiplates are each mapped by entering sample names according to the order of flies loaded into the wells. The order of samples is columnar; e.g., A1:UNK001, C1:UNK002, E1:UNK003, G1:UNK004, B2:UNK005, D2:UNK006, F2:UNK007, G2:UNK008, etc. This order of samples will be the same as that shown on the I&A imported data sheet (*see Note 5*). Once maps have been made for a pair of plates (i.e., a clear plate and a white Optiplate), these maps can be used for all clear plus white Optiplate pairs in the run. Only the mapped wells will be counted in the white Optiplates. Clear plates will be taken into the counting chamber and immediately removed to the output stacker. There will be no bioluminescence output data for the empty clear plates.

3.5. Running TopCount Monitoring Program

1. **Prepare the stop plate.** Place two identical bar codes that come with the instrument on one side of a clear plate. The TopCount recognizes this plate as the end of one cycle of counting and saves the output report automatically as one file with a unique extension. The instrument will automatically start counting the next cycle. Counting will continue for the number of times that was set in “Number of times to count assay” under the “Advanced Count Options” window.
2. **Setting up the stacker.**
 - a. In the input stacker, place an empty clear plate on the bottom, then place in the first sample containing white Optiplate in the stacker (make sure that it is in the correct orientation), then another clear plate followed by another white Optiplate, and so on. Place the stop plate made in **step 1** on top of the last white Optiplate and then place a weight (i.e., the metal plate having the

- dimensions of a microtiter plate that comes with the machine) on the top of the stop plate.
- b. Set the input stacker on the stacker deck closest to you when you face the machine.
 - c. Set the second stacker with a weight in it on the deck as an output stacker.
3. **Starting the run.** Select “Load from stacker” from the toolbar and choose “Start” on the TopCount main window to start the run. Turn off the monitor to avoid any effects from the emitted light.
 4. **Entrainment of flies for bioluminescence assays.** When the entire run is monitored during LD conditions, the phase of the LD cycle can be different than the entrained phase of the flies. When conducting a more standard constant darkness (DD) run, monitoring begins with 1 or 2 d of LD followed by 4 or 5 d of DD. In this case, flies should already be entrained to the same LD phase as the luc monitoring room, as one or two LD cycles may not be sufficient to effectively entrain the flies.
 5. **Stopping the run.** To end a 6-d TopCount run, select “End” on the TopCount main window, and copy the output reports to a portable disk for subsequent data analysis.

3.6. Data Analysis

The data analysis is described in **Subheadings 3.6.1.** through **3.6.4.**, and will focus on using the “standard” *luc* rhythm analysis package (**2,16**). Another software package has been developed for analyzing *luc* rhythms that runs in MatLab (**19**). We have not used this software package to analyze *luc* data, and thus will not be discussing this package further. Our description of *luc* data analysis includes raw data import by I&A software, raw data modification, FFT-NLLS analysis, and graphic analysis of output (**Fig. 1**). It is advisable to analyze the data on a separate Windows PC rather than in the TopCount Windows NT to accommodate the next run.

3.6.1. Raw Data Import

I&A software is an interface between Microsoft Excel and the raw data time series collected by the TopCount. I&A can import luminescence output from the TopCount into an Excel spreadsheet format, thus enabling the use of Excel analysis functions such as graphic and statistical analysis. I&A is also designed to interface with the FFT-NLLS software that is used to analyze rhythmic time series data. Readers should refer to the website of Steve Kay’s laboratory (www.scripps.edu/cb/kay/ianda/) for details on the use of I&A software. The order on the I&A data sheet can be tracked from the “Sample Map” that was defined when setting up the TopCount monitoring program (e.g., in a given plate, data in well No. 1 are from the sample UNK001; see **Note 5**).

3.6.2. Raw Data Modification

1. Discard data from any unhealthy flies. Unhealthy flies are defined as those having less than 1000 cps for *tim*-luc or less than 200 cps for *per*-luc on day 6 of the run.
2. Discard the data from the first partial LD cycle (i.e., the first 13 to 24 h of data, assuming sample loading was performed in the light) to allow for the inactivation of not recently synthesized luciferase (16).

After these steps, the modified raw data time series is now ready for analysis.

3.6.3. FFT-NLLS Analysis

FFT-NLLS is employed to extract rhythms from the modified raw data. Modified raw time series data are initially detrended via linear regression to produce data with a slope of zero and a mean of zero, which are then subjected to FFT. An FFT power spectrum is calculated, and the period and phase values of the most powerful spectral peak are used as a starting point for a sequential nonlinear least squares multicomponent cosine analysis (2).

1. Export data from I&A (Version 99.8.31) to the FFT-NLLS program. Only one plate can be exported and analyzed at a time. Designate the data to be analyzed by selecting the start and end time-points, and then export these data to the folder containing the FFT-NLLS program. Enter a unique file name for the exported data. After export, a file will be generated for each sample on the plate, and a ".in" file will be generated for the entire plate. All of the files generated in this step can be found in the FFT-NLLS folder.
2. Run FFT-NLLS analysis.
 - a. The FFT-NLLS software is run in DOS. To change the working environment to DOS, go to the "Start" menu and select "All Programs," on the list of choices select "Accessories," then on the next list of choices select "Command Prompt." Once you have a command prompt, change directory into the FFT-NLLS directory.
 - b. To perform FFT analysis, enter "`\four-anl < filename.in`". This analysis will generate a ".sum" file in the FFT-NLLS folder.
 - c. To generate theoretical curves for each sample, the bestplot.exe program is run by entering "`\fls-plot filename.sum filename.out 0 1`". This analysis produces ".the" files for each sample and a ".out" file for the entire plate in the FFT-NLLS folder.
 - d. To condense the data in the ".out" file into an easily readable form, run the condense program by entering "`\condense`". The program will show the prompt "FLS-PLOT output file to condense," which should be responded to by entering "filename.out". The next prompt to appear is "Name of condensed file to produce," which should be responded to by entering "filename.cnd". The next prompt to appear is "Choose lower, upper period to keep," which is

typically set to a range of between 10 and 50 h by entering “10,50”. After performing these steps, a “.cnd” file is generated in the FFT-NLLS folder.

3. To read the data analyzed by FFT-NLLS, they must be imported into the I&A program. To import the data, go to “Import” under the I&A menu and select “Condensed files.” In the dialog window that appears, choose a destination folder to place the “.cnd” files. Each “.cnd” file appears as a sheet within an Excel file that contains the relative amplitude (Rel-Amp), period, and phase values generated by FFT-NLLS for each fly. The theoretical curves for each sample can be imported via the same procedure as the condensed files. In this case, “THE files” are selected in “Import” under the I&A menu. In the dialog window that appears, choose a destination folder to place the “.the” files. Each “.the” file appears as a sheet within an Excel file that contains theoretical time series values generated by FFT-NLLS for each fly.

Rel-Amp is an indicator of rhythmicity. Theoretically, the range of Rel-Amp value is from 0.0 to 1.0. A value of 0.0 means a rhythm is infinitely precise and 1.0 means a rhythm is not statistically significant. By testing luciferase activity of *hsp-luc* flies, Stanewsky et al. determined that a Rel-Amp value below 0.7 can be considered rhythmic with 95% confidence (16).

3.6.4. Graphic Output

The following steps describe how graphic output is generated for individual flies and groups of flies (Fig. 1).

3.6.4.1. INDIVIDUAL PLOTTING

1. **Generating raw data plots.** You can generate graphs of raw data time series (Fig. 2A) for individual flies in either an I&A data sheet or in Excel. This is done by using the “Chart Wizard” to make a line graph from the selected samples.
2. **Butterworth filtered data plotting.** Both linear and nonlinear trends are common in time series data from luciferase assays. These trends are thought to be caused by the depletion of substrate from the medium over time. A Butterworth filter is employed to remove linear and nonlinear trends, and to filter out high-frequency (<3 h) and low-frequency (>72 h) noise (19). This filter takes a time series $x(t)$ and transforms it into another time series $y(t)$ by calculating the mean of present and past *luc* activity values (i.e., $X_t, X_{t-1}, \dots, X_{t-n}$) to generate a moving average. This transformation produces a phase shift in the data that is removed by running the filter twice, once in the forward direction and once in the reverse direction.
 - a. **3-h forward and reverse filtering.** The value of each time point in the modified time series is transformed to the mean of three points: a given time point and the two time points *preceding* that time point, i.e., $Y_t = (X_{t-2} + X_{t-1} + X_t)/3$. With this manipulation, a 3-h forward-filtered time series is generated. The value of each time point in the 3-h forward-filtered time series is then reverse-

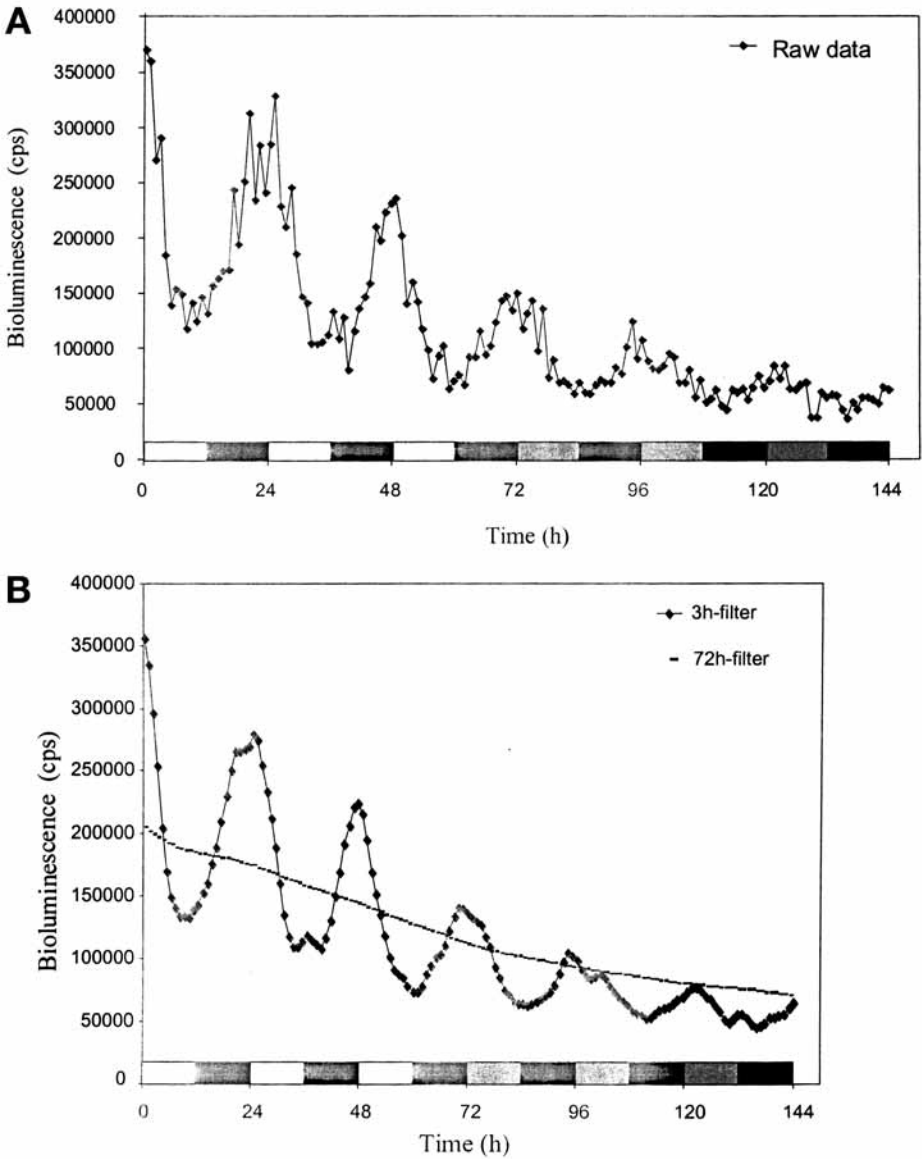


Fig. 2. Effects of filtering and normalization on bioluminescence time series data. Plots of a single *yw;tim-luc* fly monitored during light-dark (LD) and constant darkness (DD) conditions. Bioluminescence was measured in counts per second (cps). The x-axis indicates hours from the second LD cycle (data from the first partial LD cycle was discarded). Data from 0 to 72 h were collected during LD cycles, and data from 72 to 144 h were collected during DD. Open bars indicate lights on, hatched bars represent

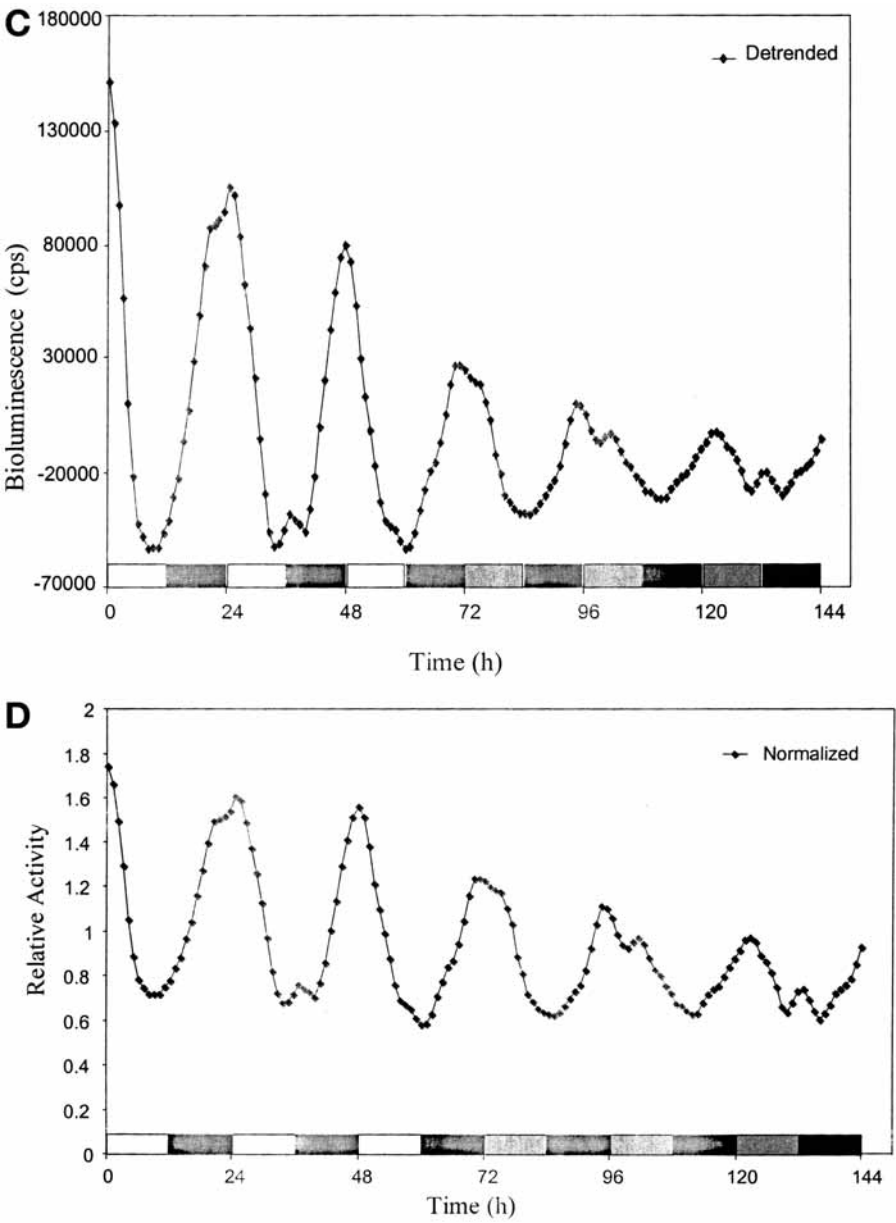


Fig 2. (*continued*) subjective lights on, and closed bars indicate either lights off (in LD cycles) or subjective lights off (in DD). (A) Raw time series bioluminescence data. (B) Data from panel A that has undergone 3-h or 72-h filtering. (C) Detrended time series data from panel A. (D) Normalized time series data from panel A.

filtered by transforming them to the mean of three points: a given time point and the two time points *following* that time point. The resulting 3-h filtered time series removes periodicities less than 3 h from the raw data time series (**Fig. 2B**).

- b. **72-h forward and reverse filtering.** The value of each time-point in the modified raw data time series is transformed to the mean of 72 points: a given time point and the 71 time points *preceding* that time point, i.e., $Y_t = (X_{t-71} \cdots + X_{t-1} + X_t)/72$, to generate a 72-h forward-filtered time series. Then the value of each time point in the 72-h forward-filtered time series is transformed to the mean of 72 points: a given time point and the 71 time points *following* that time point to generate a 72-h filtered time series. This 72-h filtered time series serves as a trend line (**Fig. 2B**).
- c. **Detrending the time series.** For each time point, subtract the value of the 72-h filtered time series from the value of the 3-h filtered time series. This manipulation removes the trend from the time series (**Fig. 2C**).
- d. **Normalizing the time series.** For each time point, divide the value of the 3-h filtered time series by the value of the 72-h filtered time series. This manipulation normalizes the time series, whereby the mean is adjusted to 1 (**Fig. 2D**). Normalizing the time series removes the units of measurement (cps) and emphasizes the relative rather than absolute value. This manipulation makes it easier to observe percent changes in values that are fluctuating above and below the trend line.

In an Excel sheet, you can generate the following:

- a. A trend curve for a given fly from the 72-h filtered time series that reflects linear and nonlinear trends (**Fig. 2B**).
 - b. The smoother-appearing time-course graph from the 3-h filtered time series (**Fig. 2B**).
 - c. The detrended time-course graph from the detrended time series that removes linear and nonlinear trends from the original time series (**Fig. 2C**).
 - d. The normalized time-course graph that increases the viewable amplitude of oscillations in the later cycles (**Fig. 2D**).
3. **FFT-NLLS-derived theoretical curve.** Using the imported “.the” files, an FFT-NLLS-derived theoretical curve can be generated for individual flies. This is useful to make comparisons to the filtered data from **steps 1** and **2** above, but is not required for analyzing *luc* rhythms *per se*.

3.6.4.2. GROUP AVERAGE PLOTTING

After individual plotting is finished, the group average can be plotted for raw data time series, 3-h filtered time series, and detrended and normalized time series. Alternatively, you can generate group average time series from the raw data and then carry out the detrending and normalization using the group average time series. By plotting the group average, the overall expression level, oscillation amplitude and waveform, and the phase can be evaluated for different genotypes.

3.7. Protocol for Monitoring Cultured Tissues

When one is interested in oscillator function in peripheral tissues (e.g., antennae), it is useful to measure clock gene or clock-controlled gene expression in an isolated tissue. Studies of isolated peripheral oscillators is important for understanding the overall structure of the circadian system, as a given peripheral oscillator may be affected by other oscillators (central oscillators or neighboring oscillators), and the phase of expression may be different between oscillators, especially in DD conditions. *Luc* activity assays on isolated tissues provide a powerful means to evaluate gene expression rhythms in peripheral tissues (13–15). As might be predicted, isolated tissues (e.g., single antenna) in culture give a much weaker signal than whole flies. However, one benefit of monitoring cultured tissues is that noise resulting from fly movement is eliminated. The following subheading describes the protocol used in our lab to monitor *luc* rhythms of isolated antennae in culture. However, this protocol can also be used to monitor other isolated tissues in culture. This description includes (1) preparation of luciferin-fortified culture media, (2) antennal dissection, (3) setting the background control, and (4) data analysis.

3.7.1. Preparation of Luciferin-Fortified Culture Media

- 1. Add the following to Schneider’s *Drosophila* media:

Component	Final concentration
Fetal bovine serum	12.0%
Penicillin/streptomycin, 100X	1X (100 U/mL)/(100 µg/mL)
Luciferin solution, 100 mM	1.5 mM
Insulin solution, 10 mg/mL	1 µg/mL

- 2. Filter-sterilize the media solution using a 25-mm filter having a 0.2-µm pore size.
- 3. Add 100 µL to each well of the white Optiplate. Fill every other well as described for the living fly protocol (see Subheading 3.2.7.).

3.7.2. Antennal Dissection

Flies are anesthetized with CO₂. Antennae are isolated under a dissecting microscope using a razor blade and no. 5F surgical forceps (13,14). Nonsterile isolated antennae are rinsed twice in tissue culture media before being placed in wells containing media.

3.7.3. Setting Background Control

Establish three background control wells on each white Optiplate using antennae from wild-type (without the luciferase transgene) flies (see Note 6). Place TopSeal-A film on top of the white Optiplate. Do not poke holes into the

film. Once prepared, cultured tissues are run on the TopCount using the same protocol described for living flies.

3.7.4. Data Analysis

As isolated tissues in culture give a relatively weak signal compared with whole flies, the background signal (i.e., signal from wells containing wild-type antennae) is subtracted from the raw data. Data-conditioning steps are used to remove the linear and nonlinear trends in the raw data (i.e., detrend), and to adjust the mean to 1 (i.e., normalize) to emphasize the relative rather than the absolute value. The data analysis steps for isolated cultures tissues include raw data modification, data conditioning using the Butterworth filter, and quantitative rhythm analysis.

3.7.4.1. RAW DATA MODIFICATION

1. Discard data for samples that do not have counts (background control level) between the start and 96 h, or if the signal drops to the background control level after 96 h.
2. Discard the data from the first partial LD cycle (*see Subheading 3.6.2.2.*)
3. Subtract background counts (i.e., the average of the three wells containing wild-type flies without *luc* transgenes) from each value (*see Note 6*).

By employing the steps above, the modified raw data are generated and will then undergo data conditioning.

3.7.4.2. DATA CONDITIONING USING BUTTERWORTH FILTER

1. Apply the 3-h forward and reverse filters to the modified time series to generate the 3-h filtered time series.
2. Apply the 72-h forward and reverse filters to the modified time series to generate the 72-h filtered time series.
3. Divide the value of the 3-h filtered time series by the value of the 72-h filtered time series to get the normalized time series. The normalized time series will then undergo quantitative rhythm analysis.

3.7.4.3. QUANTITATIVE RHYTHM ANALYSIS

The normalized time series is then subjected to FFT-NLLS analysis to generate the rhythmicity index (Rel-Amp), phase, and period for each sample. Alternatively, autocorrelation and maximum entropy spectral analysis can be used to evaluate the rhythmicity and calculate period and phase values ([19](#)). A cutoff value for rhythmicity has not been determined for cultured tissues (*see Note 6*). Whichever program is to be used to assess rhythmicity, keep in mind that genotypic controls give more information on the significance of the rhythm than quantitative rhythm analysis alone.

4. Notes

1. A 100 mM stock solution is made by dissolving firefly D-luciferin in sterile MQ water. Firefly D-luciferin is sensitive to direct light and freeze–thaw cycles, so aliquots of the stock solution should be made (we make 500- μ L aliquots to make food for testing live flies and 100- μ L aliquots for media to test cultured tissue) and stored at -70°C .
2. A 10 mg/mL insulin solution is made by dissolving insulin powder in acidified (pH ≤ 2.0) sterile deionized/reverse osmosis water. Acidified water is made by adding approx 0.1 mL of glacial acetic acid per 10 mL of deionized/reverse osmosis water.
3. The dome serves to reduce fly movement in the z-axis (toward and away from the detector). Without the dome, fly movement significantly increases the noise level (16).
4. The holes made in the domes and the sealing film prevents condensation in the process of monitoring. If this is not done, condensation will stick to the flies and kill them.
5. The “Sample Mapping” setting defines the well order on the I&A imported data sheet. The order of samples on the map should be recorded and dated. We recommend keeping the same order of sample wells from experiment to experiment so that the same map can be used for each experiment. By recording the order of the wells in the loading fly step and “Sample Mapping” setting step, you can track from which fly the time series on I&A imported data is derived. One may also track flies by row order, but either way, the order must be consistent and accurately recorded.
6. The background signal has not been more than 200 cps for us and others (16). This background level can be ignored in living fly assays, but should be subtracted in isolated tissue culture assays because the total number of counts is generally low. The background signal increases in samples counted during the light phase, but this difference in background can be eliminated by delaying sample counting by 3 to 10 min in whole flies (we use 5 min) and 7 to 10 minutes in isolated tissues in culture (we use 7 min) after they enter the TopCount. This parameter is set in “Advanced Count Options” menu under “Delay before start of count” (see **Subheading 3.4.**).

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Suprachiasmatic Nucleus Cultures That Maintain Rhythmic Properties In Vitro

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Summary

Brain slices prepared from early postnatal rodents can be maintained in culture from many weeks to months. In culture, brain slices retain their original characteristic cytoarchitecture (organotypic) and continue to differentiate and mature in vitro resembling the characteristics of the original tissue in vivo. Therefore, this fascinating approach allows us to investigate fundamental issues of structure, function, and development of the central nervous system. This chapter introduces two techniques for culturing slices of mammalian brain tissue that are most commonly used at present.

Key Words: Brain slices; tissue culture; SCN; luciferase; circadian rhythms.

1. Introduction

Culture systems have contributed to the development of biological studies. Neuronal cultures are especially widely used for neuroscience research. The most commonly used culture system is a dissociated cell culture. In this system, however, neuronal networks are rearranged after dissociation. Therefore, these networks are very often artificial and do not reflect the conditions observed in living tissue *in situ*. On the contrary, cultures of organs and tissues (slice cultures) considerably preserve their original characteristic cytoarchitecture (1–5). This is why slice cultures are so-called “organotypic;” this feature is the strongest advantage of this culture system. In addition, slice cultures have further benefits:

1. They keep healthy for a long time (several months) (3).
2. They are easily positioned under a microscope for electrophysiological and optical recording (1–6).
3. They progress through developmental changes very much like the in vivo situation (1,2).

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4. They can be used to coculture slices derived from brain regions that are anatomically remote but interconnected *in situ* (10–12).

Two general methods are currently used for long-term cultures of brain slices (Fig. 1): a “roller tube culture” modified and finally established by Gähwiler et al. (1) and a “porous membrane culture” introduced by Stoppini et al. (2). In the former, slices attach to a cover slip glass and obtain oxygen and nutrients while rotating in a rotor drum; they stay healthy for many weeks to months. The slices flatten to almost a monolayer during culturing (3), allowing us to observe individual living cells by phase contrast or Nomarski interference optics. In addition, slices cultured with this technique are easily amenable to electrophysiological and imaging approaches (10,13). With the latter technique, slices are cultured on a porous membrane and obtain oxygen from the air above and nutrients from the medium below the membrane. This technique is quite simple and imposes no limit on the size of the slice to be cultured. Slices cultured with this technique do not become flat, as do those cultured by the roller tube technique. Moreover, as there is no rotation, the status of the slices can be easily monitored in real time with a microscope equipped with a camera (14). Nevertheless, recent technical advances have made investigations using confocal microscopy, patch clamp, and optical imaging more easily performed than ever on both types of culture systems (15–17).

The suprachiasmatic nucleus (SCN) in the hypothalamus is the master pacemaker, which governs various physiological and behavioral circadian rhythms in mammals. The observation that SCN slice cultures maintain self-sustained rhythmic properties is powerful evidence to indicate this principle (3,18,19). We introduce SCN slice cultures by these two techniques.

2. Materials

1. Surgical tools (fine forceps, small dissecting scissor, spatulas).
2. Razor blade knives.
3. Disposable sterilized scalpels.
4. 60-mm Plastic culture dishes.
5. Tissue chopper (McIlwain) or other tissue slicer (vibratome, rotor slicer).
6. Plastic sheets (OHP sheet).
7. Whatman filter paper.
8. Pasteur pipets.
9. Stereomicroscopes.
10. Incubators.
11. Dissection solution (Gey’s balanced salt solution [BSS]): 138 mM NaCl, 4.9 mM KCl, 0.2 mM KH_2PO_4 , 0.8 mM Na_2HPO_4 , 36 mM D-glucose, 1.5 mM CaCl_2 , 1.0 mM MgCl_2 , 0.3 mM MgSO_4 , and 10 mM HEPES-NaOH, pH 7.4, at 4°C.

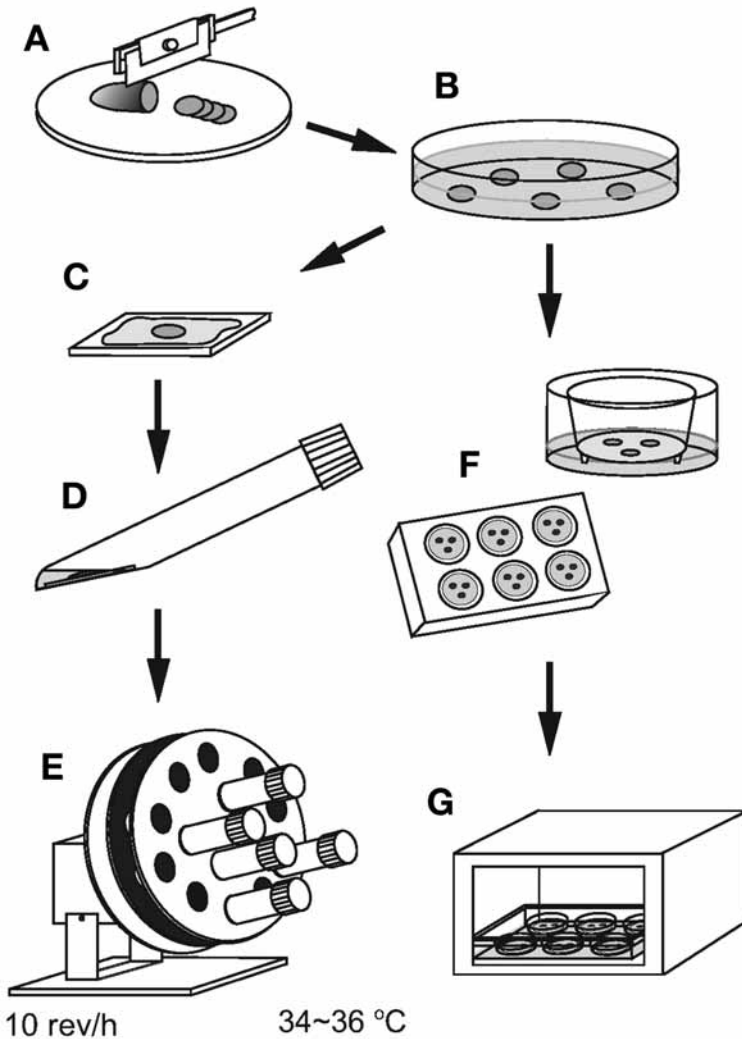


Fig. 1. Schematic illustration of the major steps in the production of organotypic slice cultures by a “roller tube technique”(steps A-B-C-D-E) or a “porous membrane technique”(steps A-B-F-G). Slices prepared with a tissue chopper (A) are maintained in Gey’s balanced salt solution for about 30 min (B). Then, in the case of the roller tube cultures, slices are mounted on cover slips with plasma clot (C). The cover slips are inserted into culture tubes containing medium (D), and the tubes are rotated at 10 revolutions per hour in dry air at 34 to 36°C (E). In the case of the porous membrane cultures, slices are placed on the membrane of well inserts and positioned in multiwell plates containing medium (F). The cultures are maintained in a humidified incubator at 34 to 36°C (G).

12. Minimum essential medium (Hanks' salt-based, Gibco), supplemented with 64 mM glucose and 20 mM HEPES-NaOH, pH 7.4, at 4°C.
13. Hanks' balanced salt solution (Gibco), at 4°C.
14. Horse serum (Gibco), at 4°C.
15. Laminar flow hood.

2.1. Roller Tube Culture

1. 100-mm Sterilized plastic dishes (not for tissue culture).
2. Chicken plasma (Cocalico Biologicals, Reamstown, PA).
3. Thrombin (Sigma, St. Louis, MO).
4. Culture tubes (flat-bottom, 16 × 110 mm; Nunc, cat. no. 156758)
5. Glass cover slips (12 × 24 mm).
6. Rotor drum.

2.2. Porous Membrane Culture

1. Well inserts (12- or 30-mm) for culture plates (Millicell-CM; Millipore, Bedford, MA).
2. Culture plates (24-well plate for 12-mm inserts, 6-well plate for 30-mm inserts).

3. Methods

Whichever technique you choose, success of slice cultures depends greatly on the appropriate preparation of slices. Handle slices carefully during all steps, and make sure that all processes are performed under sterile and clean conditions so that the slices do not become victims of bacteria and fungi.

3.1. Roller Tube Culture

3.1.1. Preparation of Materials

1. Sterilize all surgical tools and instruments that will come in contact with the slices. Also sterilize with 70% ethanol all surfaces of the tissue chopper (or other tissue slicer) coming in contact with the animal tissue.
2. Clean the cover slips through a series of solvents: xylene (24 h), acetone (24 h), and ethanol (24 h) followed by boiling in distilled water, to prevent their sticking together. Then sterilize them with dry heat (200°C for 2 h) in a glass Petri dish and cool to room temperature.
3. Autoclave the plastic sheets used on the stage of the tissue chopper.
4. All procedures should be performed under a laminar flow hood. If it is difficult to dissect and slice the brain under the hood because of space limitations, you may perform those steps outside it, but still under clean conditions.

3.1.2. Preparation of Dissection Solution and Culture Medium

1. When preparing the slices, before starting to culture them, use Gey's BSS. The original Gey's BSS for slice cultures has a low bicarbonate concentration (2.7 mM). However, we use Gey's BSS containing HEPES-NaOH (10 mM, pH 7.4) instead

of bicarbonate (*see Subheading 2.*). Sterilize Gey's BSS with a 0.20- μ m filter after adjusting the pH to 7.4. Just before starting dissection, transfer the necessary volume of Gey's BSS to a small glass bottle and bubble O₂ gas for about 10 min through a polyethylene tube sterilized in 70% ethanol.

2. The culture medium is serum-based and consists of 50% minimum essential medium 25% Hanks' BSS. and 25 % heat-inactivated horse serum, supplemented with 36 mM glucose and 10 mM HEPES-NaOH (pH 7.4). Sterilize all solutions using a 0.20- μ m filter before mixing with serum. Using this culture medium, we can maintain slice cultures in a humidified incubator without CO₂ control.

3.1.3. Animals

Slice cultures can be prepared from a variety of animals, but our experience is mainly with rats and mice. Brain slices of animals from 16 d after gestation to 9 d after birth have been cultured successfully; however, the best age for culturing depends on the region of interest and must be determined empirically. For example, we culture the SCN of the hypothalamus from 5- to 7-d neonates and hippocampus from 7- to 9-d neonates. We can culture hypothalamic tissue from adults, but maintenance of the culture is difficult, and neuronal survival tends to be low. Newborn animals should be used—especially for cerebellar cultures—because the age of animals seems to be quite crucial for good results of cultures (20).

3.1.4. Dissection

1. Anesthetize the animal, briefly immerse in 70% ethanol, and then aseptically decapitate using small dissecting scissors and forceps.
2. Open the skull and dissect out the brain. The technique for removal of the brain will vary depending on the particular brain region that will be cultured.
3. Transfer the brain into a 60-mm plastic Petri dish filled with cooled Gey's BSS. Wash the brain in the solution and cut a block of tissue including the region of interest.
4. Place the block on a plastic sheet positioned on the stage of a tissue chopper. Remove excess Gey's BSS on the plastic sheet blotting with autoclaved Whatman filter paper.
5. Cut the block into slices of 400- μ m thickness (*see Note 1*). Pour fresh cooled Gey's BSS on the slices (on the plastic sheet) with a Pasteur pipet. Trim further the slices around the region of interest with two scalpels. For roller tube cultures, slices of about 3 to 4 mm² seem to be maximal because larger slices are difficult to attach to a glass cover slip.
6. Transfer the slices into fresh, cooled Gey's BSS using a small flat spatula and maintain in the solution for about 30 min before proceeding to the next step. This process (curing) seems important to acclimatize the slices. It probably allows the removal of debris and the release of proteolytic enzymes and other substances, such as excitatory amino acids.

3.1.5. Mounting

Slices are mounted on cover slip glasses using chicken plasma and thrombin (*see* **Note 2**).

1. Prepare the chicken plasma solution, reconstituting the lyophilized powder with sterilized distilled water. This solution should be prepared just before use.
2. Prepare the bovine thrombin solution as 100 U/mL in Gey's BSS. This solution can be stored frozen for up to 1 mo.
3. Place the cover slips in a 100-mm sterile plastic Petri dish (not for tissue culture, as the cover slips would stick to the bottom of the dish) and arrange them so that they do not overlap.
4. Transfer the slices on top of the cover slips (*see* **Note 3**) with a small spatula. Remove the excess BSS with a pipet so as not to dilute the plasma clot (*see* following steps).
5. On each slice add a small drop (10 to 15 μ L) of plasma solution and spread it over the cover slip.
6. Add an equal volume of thrombin solution. Immediately stir, to mix the two solutions, and spread over the cover slip (*see* **Note 4**).
7. Arrange the position of each slice on the cover slip but be careful to avoid damaging the slices.
8. Keep in a humidified incubator for 10 to 15 min. The plasma forms a clot, allowing the slices to attach firmly to the cover slips.

3.1.6. Culturing

1. Insert each cover slip into a flat-bottom culture tube (*see* **Note 5**) containing 700 μ L of warm culture medium.
2. Place the culture tubes on a modified roller drum (**Fig. 1**), which, is tilted at an angle of about 5° with respect to the vertical axis. Set the rotation at about 10 revolutions per hour.
3. Incubate at 34 to 36°C. There is no need to control CO₂ levels or humidity.
4. Replace the medium once a week (*see* **Notes 6 and 7**).

3.2. Porous Membrane Culture

Porous membrane cultures are quite simple compared with those performed with the roller tube method. Slices are positioned on the membrane of well inserts of tissue-culture plates, and then culturing starts.

3.2.1. Preparation of Materials and Slices

The preparation of materials and slices is generally the same as described for the roller tube cultures (*see* **Subheadings 3.1.1.–3.1.4.**). However, this procedure does not need cover slips, plasma, and thrombin solutions, but requires instead culture plates and well inserts.

3.2.2. Culturing

1. Add culture medium to each well and add the inserts (*see Note 6*). Inserts are available in two sizes: 30 mm for 6-well plates and 12 mm for 12- or 24-well plates. Make sure you add an amount of medium that is just enough to touch the membrane.
2. After curing (*see Subheading 3.1.4.*) transfer the slices on the membranes with a small flat spatula.
3. Remove excess BSS with a pipet and carefully position the slices at the center of the membrane. This simplifies subsequent microscope analysis.
4. Put the culture plates in a humidified incubator without CO₂ control at 34 to 36°C. Alternatively, arrange the plates in a humidified airtight container and culture in a dry incubator.
5. Replace the medium once or twice a week according to size and number of slices on each insert (*see Note 6*).

3.3. Applications

During the first 2 wk in culture the slices become thin and stable. Thus, slices must be maintained *in vitro* for at least 1 wk before starting experiments.

We have successfully cultured slices of SCN that maintain rhythmic property *in vitro* with both the roller tube and the porous membrane methods. SCN slices cultured with the roller tube technique flatten to almost mono- or several layers and spread on cover slips but retain organotypic properties (*ref. 3; Fig. 2*). Using this approach, living cells can be individually visualized under a phase contrast microscope. Techniques such as immunohistochemistry and *in situ* hybridization can be directly applied without further sectioning and observed under a conventional microscope (*ref. 3; Fig. 3*). In addition, by monitoring the release of vasopressin, one of the peptides produced in the SCN, we demonstrated that this preparation maintains a self-sustained oscillator for more than 2 mo *in vitro* (*3,5*).

SCN slices cultured on porous membranes reduce from an initial 400- μ m thickness to about 150 to 100 μ m. As they are static, they are particularly suited for continuous, long-term observations. We produced SCN slice cultures from transgenic mice carrying a luciferase reporter gene under the control of the *mPer1* promoter (*14*). Using a microscope equipped with a two-dimensional charge-coupled device photon camera, we monitored the luminescence emanating from SCN cultures as a whole. We observed robust circadian oscillations with an amplitude comparable to that of the *mPer1* mRNA *in vivo* for at least five cycles without damping (*ref. 14; Fig. 4*). Moreover, exploiting the high resolution and sensitivity of a cooled charge-coupled device camera, we were able to observe luminescence at the level of single cells (*ref. 21; Fig. 5*),

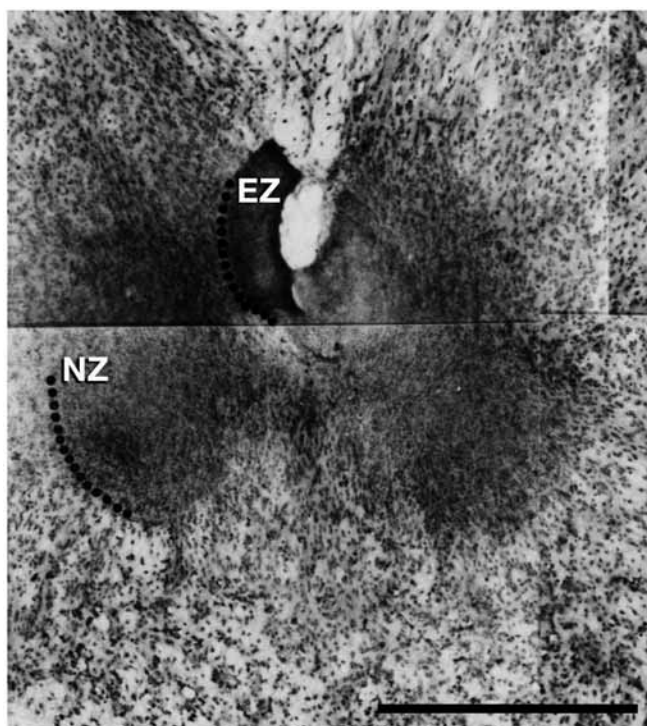


Fig. 2. Suprachiasmatic nucleus slice culture prepared by the roller tube technique and stained with cresyl violet after culturing for 3 wk. The slice is flattened from an initial thickness of 400 μm to a monolayer and spread. However, it maintained its topology, allowing morphological analysis without further sectioning. The dotted line delimits a border of the neuronal zone rich in neurons. Scale bar is 1 mm. Figure is adapted from [ref. 3](#).

although it is usually impossible to detect individual cells in 100- to 150- μm -thick slices under a conventional microscope. Single-cell analyses are also possible with a confocal fluorescent microscope ([16,17](#)); however, the repeated exposure to laser beams, which is needed for circadian recordings, might cause serious cell damage.

4. Notes

1. The thickness of slices can vary from 200 to 700 μm . Thicker slices are easier to handle, but thinner slices get better diffusion of oxygen and nutrients. Considering these points, 400 μm of thickness seems a good compromise in most cases.
2. Collagen gel can be used as an alternative to attach slices to cover slips. The cover slips are cleaned and sterilized as described above, then they are first coated with poly-D-lysine and finally with collagen.

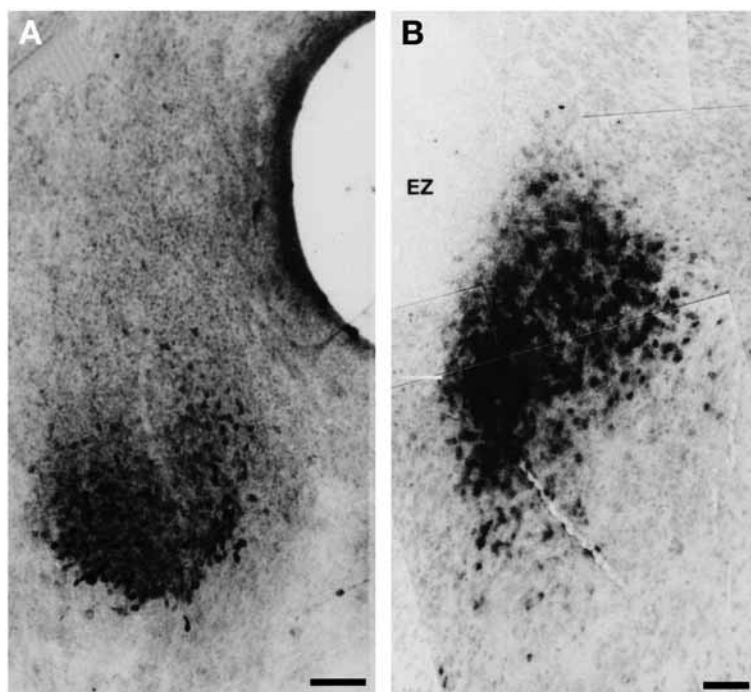


Fig. 3. Suprachiasmatic nucleus (SCN) slice retaining its original topology in culture as confirmed by immunohistochemistry and *in situ* hybridization. (A) Antibody staining shows that the neurons containing vasoactive intestinal peptide are localized in the ventrolateral part of the SCN slice in culture, as they are in vivo. (B) Moreover, the neurons expressing vasopressin mRNA are localized in the dorsomedial part of the slice, as in the SCN in vivo. Scale bar is 100 μ m. Figure is adapted from **ref. 3**.

- a. Apply poly-D-lysine solution (25 μ g/mL in water; Sigma, cat. no. P-7280, sterilized with a 0.45- μ m filter) to the surface of cover slips for 1 h.
 - b. Rinse the cover slips twice in distilled water and dry.
 - c. Apply a small amount of collagen solution (Cellmatrix Type I-A kit, Nittagelatin Inc., Japan) to the cover slips and spread over. Keep in a humidified incubator for 30 min at 37°C until the collagen solidifies.
 - d. Place each slice on a cover slip and add a small amount of collagen solution to cover the slice. Keep in a humidified incubator at 34°C for 1 to 4 h until the collagen solidifies.
3. Coating cover slips with 0.05 to 0.1% polyethylene imine (Sigma) prevents damage to the plasma clot during culturing. Apply the polyethylene imine solution to the surface of cover slips and keep them in a humidified incubator for 3 to 12 h. After the solution is removed, cover slips are washed in sterilized distilled water three times.

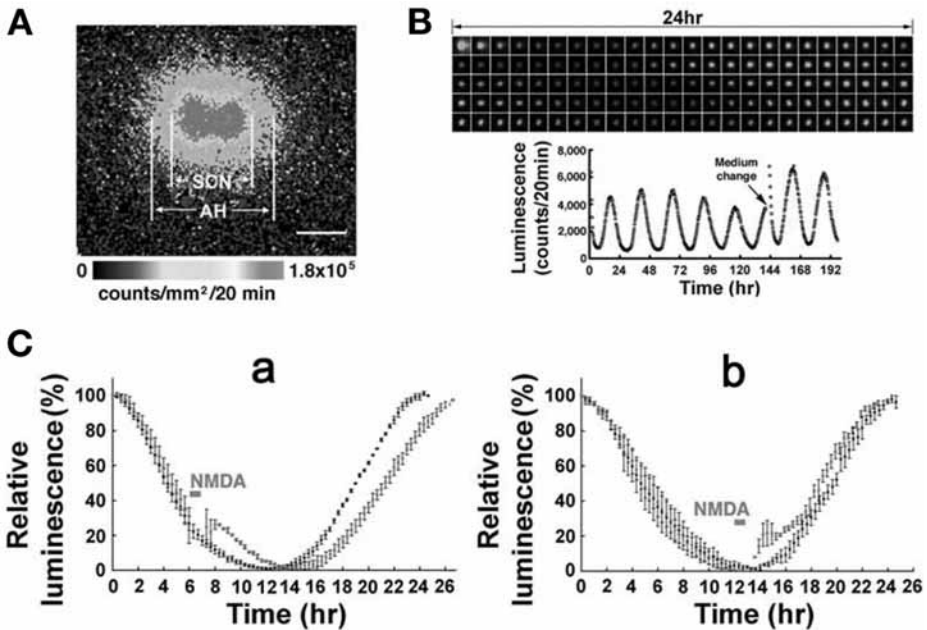


Fig. 4. Real-time recording of bioluminescence from suprachiasmatic nucleus (SCN) cultures of *mPer1-luc* transgenic mice. (A) High-power image of the bioluminescence of an SCN culture after addition of 1 mM luciferin to the medium. Note that the SCN occupies the majority of the slice, and the surrounding anterior hypothalamic area is thin. Scale bar = 500 μm . (B) Rhythmic change of bioluminescence in a SCN culture. The upper panels show the SCN culture imaged every 60 min for 5 d. In the lower graph, each circle represents the total intensity of luminescence counted for 20 min. Arrow indicates the change of the culture medium. (C) Waveform of the *N*-methyl-D-aspartate (NMDA)-treated cycle and the preceding cycle. Application of NMDA at 6 h after the peak induced a phase-delay (a), and at 12 h after the peak induced a phase-advance (b). Figures are adapted from **ref. 14**.

4. Mix plasma and thrombin solutions under neutral pH to obtain a firm plasma clot. Thrombin should be dissolved with Gey's BSS containing 10 mM HEPES.
5. There are two types of culture tubes: flat-bottom and round-bottom. We strongly recommend using flat-bottom tubes, as it is not necessary to take the cover slip outside the tube to observe the slice under a phase contrast microscope.
6. The volume of culture medium is a critical factor. In roller tube cultures, when replacing medium, aspirate the old medium and add 650 μL of fresh medium into each flat-bottom tube. Make sure that the slices are covered by medium for only half of the time during rotation. This allows optimal recovery of nutrients from the medium and oxygen from the air.

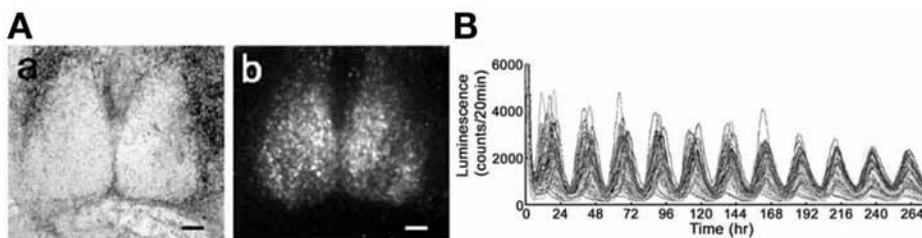


Fig. 5. (A) Suprachiasmatic nucleus (SCN) culture of *mPer1-luc* transgenic mice examined by light microscopy (a), and by cooled charge-coupled device (b). Scale bar = 100 μ m. (B) Temporal changes in bioluminescence signals from 100 SCN cells randomly chosen, were plotted and then superimposed. Figures are adapted from ref. 21.

For porous membrane cultures, at the beginning of culturing add 1 mL of fresh medium into each well of a 6-well plate. When replacing medium, aspirate the old medium and add 700 to 800 μ L of fresh medium into each well. Alternatively, move the well inserts to a new culture plate with 1 mL of fresh medium for each well. Make sure that the slices are not immersed in the medium; the surface of the medium must just touch the membrane.

7. It is reported that slices cultured by the roller tube method show more gliosis than those cultured on porous membranes (2,22). To prevent growth of macrophages, glia, and fibroblasts, it is recommended that a low concentration (10^{-6} to 10^{-5} M working solution) mixture of antimitotic drugs (mixture of uridine, cytosine- β -D-arabino-furanoside, and 5-fluorodeoxyuridine) is administered for no more than 24 h at days 2 to 4 in vitro.

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VII

MICROSCOPY ANALYSIS

RNA *In Situ* Hybridizations on *Drosophila* Whole Mounts

Corinna Wülbeck and Charlotte Helfrich-Förster

Summary

RNA *in situ* hybridization is a commonly used technique to achieve spatiotemporal detection of transcripts in tissues. This chapter gives an overview of novel techniques using fluorescent dyes, signal amplification methods, and confocal microscopy in regard to chronobiological applications on *Drosophila* adult brains.

Key Words: Transcript; DIG-UTP; hapten; *Drosophila*; riboprobe; RNA *in situ* hybridization; whole mounts; fixation; alkaline phosphatase; horseradish peroxidase; Dig-POD; primary antibody; secondary antibody; fluorescence; signal amplification.

1. Introduction

The original method of nonradioactive transcript detection was published in 1989 ([1](#)). In the last decades RNA *in situ* hybridization techniques have progressed considerably, with contributions by several investigators (for an excellent troubleshooting guide, *see* [ref. 2](#)). After being able to detect single RNA species in various animals ranging from invertebrates to mammals, a further breakpoint was the development of protocols to detect various RNAs or RNA–protein combinations simultaneously ([3,4](#)). However, the simultaneous detection of several transcripts originally depended on a single enzymatic reaction. Alkaline phosphatase (AP) can be used with various substrates, resulting in precipitates of different color in places of target gene expression ([3](#)). Besides the disadvantage of substrate-dependent variation in sensitivity, some precipitates, such as FAST RED, quickly faded in aqueous solutions and were absolutely not appropriate for permanent mounting of slides, as the precipitate is lost during ethanol-based dehydration series. Therefore, studying transcript colocalization turned out to be difficult. Furthermore, enzymatic reactions using colored precipitates made it nearly impossible to quantify the RNA content of the target cells, owing to more or less uncontrollable signal amplifi-

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cation. This point is very important in chronobiology, however, as transcriptional regulation is a key element of the circadian clock.

The development of fluorescent detection systems, together with advanced confocal microscopy, has overcome these difficulties and now it is possible to detect even weak signals, colocalize them, and measure their amounts.

In this chapter we present an overview of our favorite protocols covering dissection and fixation of samples, RNA probe preparation, and recent methods for transcript detection. We discuss possible limitations of these methods and provide a simple troubleshooting guide for each step of the procedure.

2. Materials

2.1. Solutions

2.1.1. Fixation

1. Diethylpyrocarbonate (DEPC; *see Note 1*).
2. Dimethylsulfoxide (DMSO), cell culture grade (Sigma, sterile-filtered, $\geq 99.7\%$; *see Note 2*).
3. 16% Formaldehyde solution, EM grade (Polysciences; *see Note 3*).
4. DEPC-treated H_2O (*see Note 4*).
5. 10X Phosphate-buffered saline (PBS)_a, pH 7.4, DEPC-treated (*see Note 5*).
6. 10X PBS_b, pH 7.4, DEPC-treated (*see Note 6*).
7. PBT: 1X PBS (either a or b) with 0.2% Triton-X100.
8. PFAT-DMSO: 4% paraformaldehyde in 1X PBS_a with 0.1% Triton X-100 and 5% DMSO.
9. Heptane.
10. 100% Ethanol.

2.1.2. RNA Transcription

1. 0.1 M Dithiothreitol (DTT; supplied with RNA polymerase, Promega).
2. 5X Transcription buffer: 200 mM Tris-HCl, pH 7.9, 30 mM Mg_2Cl , 10 mM spermidine, 50 mM NaCl (supplied with RNA polymerase, Promega).
3. T7-RNA polymerase (Promega, 80 U/ μL).
4. T3-RNA polymerase (Promega, 80 U/ μL).
5. SP6-RNA polymerase (Promega, 80 U/ μL).
6. RNase inhibitor: RNasin (Promega, 40 U/ μL).
7. 10X Digoxigenin-uracil triphosphate (DIG-UTP) RNA labeling mix (Roche: *see Note 7*).
8. 10X Biotin-UTP RNA labeling mix (Roche; *see Note 7*).
9. RNase-free DNase (Promega, 20 U/ μL).
10. STE buffer: 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA (with DEPC-treated H_2O).
11. Phenol/chloroform (*see Note 8*).
12. Chloroform (*see Note 8*).
13. 4 M LiCl, DEPC-treated.

14. 0.2 M EDTA, pH 8.0, DEPC-treated.
15. 75% Ethanol: ethanol diluted with DEPC-treated H₂O.
16. 3 M Sodium acetate, pH 5.2 (adjust pH with acetic acid), DEPC-treated.
17. NucTrap™ push columns (25 columns, Stratagene, cat. no. 400 701)
18. 7.8 M Ammonium acetate, DEPC-treated.

2.1.3. Proteinase K Treatment

1. Proteinase K solution, 10 mg/mL, polymerase chain reaction (PCR) grade (Roche; *see Note 9*).
2. Glycine stock solution, 200 mg/mL in DMSO (*see Notes 4 and 10*).
3. 4% Paraformaldehyde in PBS (*see Note 10*).
4. 25% Glutaraldehyde solution, EM grade (Polysciences).

2.1.4. RNA Hybridization

1. Formamide, recrystallized (*see Note 11*).
2. PBT_w: 1X PBS (either a or b) with 0.1% Tween-20.
3. 20X Saline sodium citrate (SSC): 3 M NaCl, 0.3 M sodium citrate, DEPC-treated.
4. 2X SSCTw: 2X SSC with 0.1% Tween-20 (*see Note 12*).
5. 0.2X SSCTw: 0.2X SSC with 0.1% Tween-20 (*see Note 12*).
6. Heparin: 10 mg/mL in Tris-HCl, pH 7.5 (*see Note 7*).
7. t-RNA: 10 mg/mL in Tris-HCl, pH 7.5 (*see Note 7*).
8. Salmon sperm DNA, sonicated, phenol extracted, 10 mg/mL in Tris-HCl, pH 7.5 (Sigma; *see Note 7*).
9. Sheep serum, heat-inactivated (*see Note 13*).
10. Hybridization buffer (HB): 50% formamide, 5X SSC-DEPC, 0.1% Tween-20, adjust pH to 6.0 with 1 N HCl (*see Note 14*).
11. Blocking buffer: HB, 100 µg/mL heparin, 100 µg/mL salmon sperm DNA, 500 µg/mL t-RNA.

2.2. Antibodies

1. Anti-DIG, AP-labeled (from sheep, Roche; *see Note 15*).
2. Anti-DIG, peroxidase-labeled (from sheep, Roche; *see Note 15*).
3. Anti-DIG (from mouse, Roche; *see Note 15*).
4. Antistreptavidin, horseradish peroxidase (HRP)-labeled (antistreptavidine HRP, supplied with the Tyramide Kit, Molecular Probes).

2.3. Detection

1. Diaminobenzidine tablets (Sigma).
2. Nitroblue tetrazolium (NBT): 75 mg/mL in 70% dimethylformamide (Boehringer).
3. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP): 50 mg/mL in 100% dimethylformamide (Boehringer).
4. AP detection buffer: 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM Mg₂Cl, 0.1% Tween-20.
5. Tyramide Detection Kit 488 nm (Molecular Probes).
6. Tyramide Detection Kit 568 nm (Molecular Probes).

2.4. Mounting

1. 80% Glycerol in 1X PBS with 0.1% Tween-20 (UltraPure Glycerol, $\geq 99.5\%$; Invitrogen).
2. Vecta Shield (Vector Laboratories).
3. Nail polish.

3. Methods

3.1. *In Vitro* Transcription

3.1.1. DNA Preparation

The detection of gene expression by *in situ* hybridization is carried out with RNA probes. Efficient RNA transcription requires the subcloning of the DNA fragment of interest into a vector. The multiple cloning site is flanked by phage–RNA–polymerase promoters—for example, T3-, T7- or SP6-RNA polymerase, such as pBluescript II (Stratagene) or, for polymerase chain reaction products, pGEMTeasy, respectively (Promega; [Fig. 1A,B](#)). The size of the cloned fragment should range between 0.5 and 3.0 kb. RNAs transcribed from templates larger than 3 kb may be too big to penetrate the tissue, resulting in no signal at all. Conversely, fragments that are too small may cause unspecific background staining. The following procedure explains how to prepare the DNA template used in transcription reactions. All solutions have been either treated with DEPC or prepared with DEPC-treated H₂O.

1. Restrict the DNA for 2 to 3 h with an appropriate enzyme cutting the multiple cloning site opposite to the transcription start site (e.g., T7 or T3) used later for *in vitro* RNA transcription (*see* **Note 16**).
2. Extract the restricted DNA with phenol/chloroform. Spin for 5 min in a microcentrifuge and transfer the upper phase to a new Eppendorf tube. Avoid touching the interphase.
3. Extract with chloroform to remove any residual phenol. Spin for 5 min and transfer the top layer to a new Eppendorf tube.
4. Precipitate the DNA with 0.1 vol of 3 M sodium acetate, pH 5.2, and 2.5 vol of 100% ethanol (-20°C) for 30 min at -80°C .
5. Spin for 20 min at maximum speed at 4°C .
6. Remove the supernatant and wash the pellet with -20°C 70% ethanol.
7. Spin for 5 min at 4°C , remove the ethanol, and air-dry the pellet.
8. Resuspend the pellet in DEPC-treated H₂O to a final concentration of 1 $\mu\text{g}/\mu\text{L}$. Store the DNA at -20°C ; it is stable for at least 1 yr.

3.1.2. RNA Transcription

We perform the transcription reaction, following the instructions of the manufacturer of the DIG labeling mix (Roche), except that we use an amount of template equivalent to 1 μg of insert rather than to 1 μg of total DNA (vector

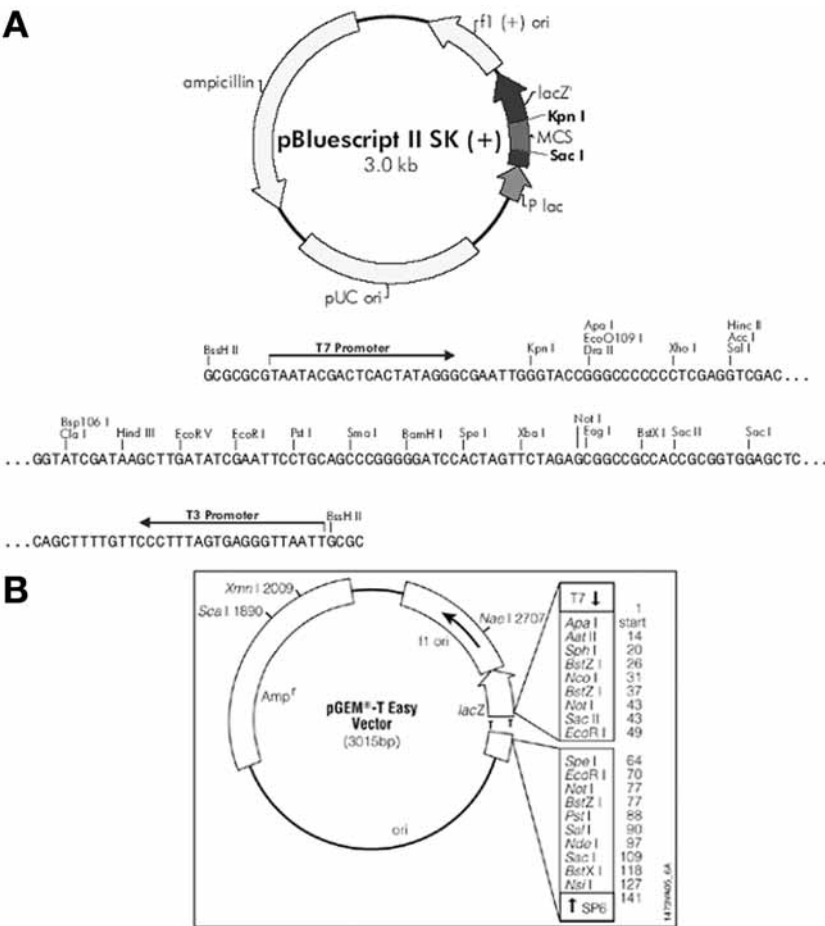


Fig. 1. Cloning vectors suitable for in vitro transcription. (A) pBluescript II KS (Stratagene). (B) pGEMT-easy (Promega).

+ insert). This improves the yield of the transcription, and up to 10 µg of RNA can be generated in a standard reaction.

1. Set up the 20-µL transcription reaction in a sterile Eppendorf tube on ice by adding the following components:
 - a. x µL of linear DNA, equivalent to 1 µg of insert.
 - b. 4 µL of 5X transcription buffer.
 - c. 2 µL of 0.1 M DTT.
 - d. 1 µL of RNasin.
 - e. 2 µL of 10X DIG or Biotin UTP RNA labeling mix.

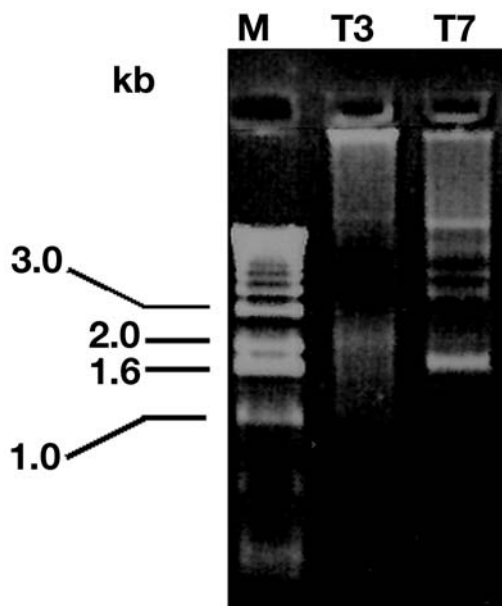


Fig. 2. Gel electrophoresis of in vitro transcribed sense and antisense riboprobes for the *Drosophila melanogaster u-shaped* gene. 1% agarose gel in TAE-buffer. Lane M: 1-kb ladder. Lane T3: digoxigenin (DIG)-labeled sense probe transcribed with T3-RNA polymerase; note the smear. Lane T7: DIG-labeled antisense probe transcribed with T7-RNA polymerase; note the strong band at about 1.6 kb. The clone of *u-shaped* cDNA was kindly provided by Marc Haenlin.

- f. 40 U of T7-, T3- or SP6-RNA polymerase (see **Note 17**).
- g. DEPC-treated H₂O to a final volume of 20 μ L.
2. Mix and incubate for 2 h at 37°C (see **Note 18**).
3. Add 1 μ L of RNase-free DNase and incubate for additional 30 min at 37°C to remove template DNA.
4. Stop the reaction by adding 2 μ L of 0.2 M EDTA, pH 8.0.
5. Add 2.5 μ L of 4 M LiCl and 75 μ L of -20°C 100% ethanol, mix well, and precipitate for 30 min at -80°C or for 2 h at -20°C.
6. Spin in a microcentrifuge at maximum speed for 15 min at 4°C.
7. Remove the supernatant and wash the pellet with -20°C 75% ethanol.
8. Spin for 5 min at maximum speed at 4°C, remove the ethanol, and air-dry.
9. Resuspend the pellet in a small amount of DEPC-treated H₂O in case you want to perform a gel electrophoresis (**step 10**), or in 100 μ L of HB. If the pellet does not dissolve, incubate at 56°C (see **Note 19**).
10. Check amount, quality, and dimension of RNA transcripts on a standard agarose gel (see **Fig. 2** and **Note 20**).

Although ethanol precipitation is usually the method of choice for cleaning and concentrating riboprobes, sometimes it is not efficient in removing unused labeled nucleotides, which consequently cause high background. The protocol below employs NucTrap push columns (Stratagene; *see also* **ref. 4**) to specifically remove these contaminating nucleotides by gel filtration.

1. At the end of template digestion (**Subheading 3.1.2., step 4**) add 40 μL of DEPC-treated H_2O to the reaction.
2. Equilibrate the column with 80 μL of STE buffer.
3. Apply the riboprobe solution (60 μL) to the column.
4. Elute the riboprobe with additional 70 μL of STE buffer; the eluted volume is about 110 μL .
5. Precipitate the riboprobe with 0.5 vol of 7.8 *M* ammonium acetate and 3 vol of 100% ethanol at -20°C for 30 min.
6. Spin in a microcentrifuge at maximum speed for 15 min at 4°C .
7. Wash the pellet with -20°C 75% ethanol.
8. Spin for 5 min at maximum speed at 4°C , remove the ethanol, and air-dry.
9. Dissolve the pellet in 100 μL of HB.

3.2. Dissection and Fixation

There are many protocols for the dissection and fixation of tissue. Key to the choice of the right protocol is the identification of the most important limiting factors. These could be paucity of material and/or time available for dissection, sensitivity, and reliability of the procedure. In chronobiology, material and time are generally the most crucial limiting factors, so the most convenient protocols are those that allow quick fixation and storage of samples.

3.2.1. Dissection Buffer and Sample Collection

RNA degrades quickly; therefore, the choice of buffer and the method of sample collection play an important role in RNA transcript detection. For instance, the concentration of sodium chloride in the dissection buffer has to be adjusted to take into account the nature of the tissue (*see* **Notes 5 and 6**). An incorrect saline concentration can disrupt tissue structure by water invasion, as for imaginal discs with a standard PBS buffer (personal observation). In circadian studies we must avoid not only RNA degradation via cell death, but also light-induced RNA transcription and/or degradation, and special care has to be taken in collecting time points during the actual or subjective night time. Animals should be shielded from light (i.e., wrapping *Drosophila* vials in aluminium foil) and anesthetized under darkness or red light. All physiological pathways slow down at low temperature; hence, if dissection precedes fixation, it is good practice to keep the samples on ice. For a detailed description of whole-mount dissection of adult and larval brain, *see* Chapter 42.

3.2.2. Fixation Methods

The outcome of *in situ* hybridization procedures is strongly influenced by the fixative used and the pretreatment of the tissue. Quality requires conservation of tissue morphology; however, this must not compromise sensitivity, which is determined by the ability of the probe to penetrate the tissue and hybridize efficiently.

It is crucial to use high-grade formaldehyde during fixation in order to achieve high sensitivity. Under light formaldehyde quickly oxidizes into formic acid, a well-known inhibitor of nucleic acid hybridization. Soft tissues (larval brain and malpighian tubules) are preserved better by glutaraldehyde than by formaldehyde fixation. In principle, it is useful to add 0.2% glutaraldehyde to the fixative if hybridization is to be carried out at high temperature. However, glutaraldehyde causes a high autofluorescent background and must not be used for fluorescence detection.

For tissues such as larval brains, imaginal discs, and malpighian tubules, which can be dissected quickly, we recommend a modified version of a protocol developed in 1992 (5). It allows the prolonged storage of dissected material at -20°C in 100% methanol without signal loss. Actually, the methanol treatment significantly reduces background staining of the trachea. In the hands of one author (C. W.), the following protocol has worked for various insect species and tissues (6,7 and C. W., unpublished observations). Perform the following steps in Eppendorf tubes. All following steps are performed with DEPC-treated solutions or buffers that have been diluted with DEPC-treated H_2O .

1. Dissect 10 to 15 larval brains in less than 15 min and store them on ice in PBS.
2. Remove the PBS and replace it with 325 μL of fresh PBT solution, 75 μL of 16% formaldehyde, and 500 μL of heptane (see **Note 21**).
3. Shake the mixture by hand for 30 to 45 s.
4. Let the foam sit, then remove the upper heptane phase and most of the aqueous phase, leaving just enough to cover the tissue.
5. Add 610 μL of fresh PBT, 150 μL of 16% formaldehyde, and 40 μL of DMSO and fix for 20 min at room temperature on a rocking table (see **Notes 2, 22**).
6. Wash two times for 5 min each in 100% methanol.
7. Store in methanol at -20°C .

The dissection of adult brains requires a long time; hence, three additional steps precede the protocol above.

1. Pre-fix whole adult flies for 2 h in PFAT-DMSO at room temperature (see **Notes 2, 22**). Flies should be completely drowned in the fixing solution.
2. Wash three times for 5 min with PBT.
3. Dissect the brains in PBT and store them on ice in PBT.

Continue with the previous protocol (**step 2**).

3.3. RNA In Situ Hybridization

3.3.1. Rehydration

1. Rehydrate the samples in a downgrading 90%, 70%, 50%, 30% methanol/PBT_w series. Carry out each step for 5 min.
2. Wash for 5 min in PBT_w. Repeat the wash four more times.

3.3.2. Proteinase K Treatment

Probe penetration can be a critical factor, especially for densely packed tissues as ovaries, imaginal discs, and adult brains. Strong crosslinking of proteins after fixation impairs probe penetration; a partial digestion with proteinase K provides a solution to this problem (*see Note 23*). Generally this procedure is compatible with a combined RNA–protein detection (8), although the proteinase K treatment must be strictly controlled to avoid loss of antigens. In case of low-abundance proteins, permeabilization can be achieved without proteinase K by treating tissues with acetone (9).

1. Dilute 1.25 μ L of proteinase K solution in 500 μ L of PBT_w and add to the sample in an Eppendorf tube (*see Note 24*).
2. Place the tube horizontally without shaking (*see Note 25*) and incubate for 1 to 5 min at room temperature. Do not exceed 25°C (*see Note 26*).
3. Stop the reaction by adding 10 μ L of glycine stock solution.
4. Rinse three times with 2 mg/mL glycine in PBT_w (1:100 dilution of the stock solution). This will dilute any residual proteinase K.
5. Wash two times for 5 min each with PBT_w. Place the tube horizontally without shaking.
6. Fix with 4% paraformaldehyde in PBT_w for 20 min at room temperature. Place the tube horizontally without shaking. This step completely inhibits proteinase K and increases the stability of the sample.
7. Wash five times for 5 min each with PBT_w on a rocking table.

3.3.3. Hybridization

RNA hybridization (*see Note 27*) is generally performed at temperatures $\geq 56^\circ\text{C}$. Although the perfect temperature might need to be determined empirically, in our hands 60 to 65°C works well for riboprobes we have tested so far (*tim* and *cry*). Both temperature and salt concentration are important in determining background. At high temperature, nonmatching DIG-RNA/mRNA hybrids will not bind together. The selectivity is also increased by reducing the amount of stabilizing positive charges provided by the Na⁺ ions in the SSC solution.

1. Incubate the samples for 5 min in a 1:1 mixture of PBT_w:HB at room temperature.
2. Replace the mix with HB and incubate for 5 min at 65°C in a water bath.
3. Replace with 200 μ L of blocking buffer and prehybridize for at least 2 h at 65°C.

4. Dilute the riboprobe to the optimal concentration (*see Note 28*) in blocking buffer, heat for 5 min at 70°C to remove RNA secondary structures, and then put on ice immediately.
5. Remove the prehybridization buffer and add approx 50 µL of the riboprobe solution.
6. Incubate overnight in a 65°C water bath with lid. Seal Eppendorf tube with Parafilm (*see Note 29*).

3.3.4. Washes

Our protocol combines two types of washes to reduce background because of both hybridization and detection steps. Initially, to wash away nonhybridized probe, we use solutions with decreasing HB:2X SSCTw ratio, at 65°C (*see Note 30*). The fine adjustment of washing conditions is achieved with low-salt SSCTw dilutions. Finally, to equilibrate the tissue before incubation with the antibody, we use solutions with decreasing SSCTw:PBTw ratio at room temperature.

1. Wash two times for 15 min each with HB at 65°C.
2. Wash for 15 min each with a 3:2, 1:1, 2:3 ratio of HB:2X SSCTw at 65°C.
3. Wash once with 2X SSCTw for 15 min at 65°C.
4. Wash two times for 15 min each with 0.2X SSCTw at 65°C.
5. Wash for 10 min each with a 3:2, 1:1, 2:3 ratio of 0.2X SSCT:PBTw at room temperature.
6. Wash three times for 5 min each with PBTw at room temperature.

3.4. Detection

After hybridization, riboprobes are recognized by hapten-specific antibodies (*see Note 31*). The detection is indirect, either through fluorescently labeled secondary antibodies or via enzymes coupled to the primary antibodies.

3.4.1. Detection With Fluorescent Secondary Antibodies

It is possible to detect simultaneously several RNAs or RNA–protein combinations by labeling the riboprobe with different haptens (such as DIG-UTP, biotin-UTP, fluorescein-UTP, or dinitrophenyl-UTP) and using different fluorescent secondary antibodies (8). However, to guarantee the specificity of fluorescent signals, all primary antibodies must originate from different host species, the secondary antibodies must be highly species-specific, and the coupled fluorescent dyes must not overlap in their adsorption and emission spectra. **Table 1** gives an overview of suitable primary and secondary antibody combinations.

1. Block the sample in 200 µL of 5 to 10% sheep serum (*see Note 32*) in PBT for 2 h at room temperature.
2. Dilute the appropriate primary antibody (*see Table 1*) in 5 to 10% sheep serum in PBT.

Table 1
Suggested Hapten/Antibody Combinations for Riboprobe Detection With
Fluorescent Secondary Antibodies

Riboprobe label	Primary detection	Recommended dilution range	Quality	Fluorescent conjugate
Digoxigenin	Mouse anti-DIG	1:300–1:500	++	Alexa 647 goat anti-mouse
Digoxigenin	Sheep anti-DIG	1:200–1:400	+	Alexa 488 donkey anti-sheep
Digoxigenin	Sheep anti-DIG-POD	1:300–1:500	++	Alexa 568 Tyramide
Biotin	Mouse anti-BIO	1:400–1:800	+++	Alexa 555 goat anti-mouse
Biotin	Goat anti-BIO	1:200–1:400	+	Alexa 488 donkey anti-goat
Biotin	Streptavidin HRP	1:200–1:300	++	Alexa 350 Tyramide
Fluorescein	Mouse anti-FITC	1:400–1:800	++	Alexa 488 goat anti-mouse
Fluorescein	Goat anti-FITC	1:200–1:400	+	Alexa 488 donkey anti-goat
Fluorescein	Rabbit anti-FITC	1:300–1:500	+	Alexa 488 goat anti-rabbit
Fluorescein	Rabbit anti-FITC-HRP	1:400–1:600	++	Alexa 350 Tyramide
Dinitrophenyl	Rabbit anti-DNP	1:400–1:800	+++	Alexa 647 chicken anti-rabbit

FITC, fluorescein isothiocyanate; BIO, biotin; DNP, dinitrophenyl.

From www-biology.ucsd.edu/~davek/reagents.html.

3. Remove the blocking solution and add 200 μ L of the primary antibody dilution. Incubate overnight at 4°C.
4. Wash with PBT five times for 20 min each at room temperature.
5. Dilute (typically 1:300) the appropriate secondary fluorescent antibody (*see Table 1*) in PBT.
6. Add 200 μ L of the secondary antibody dilution. Incubate for 4 h at room temperature.
7. Wash with PBT three times for 20 min each at room temperature.
8. Mount in Vectashield, sealing the cover slip with nail polish.

3.4.2. Visible Enzymatic Detection

Detection of rare transcripts might require amplification of the signal. This can be achieved with enzyme-labeled antibodies. The enzyme moiety catalyzes reactions, forming either visible or fluorescent precipitates in proximity to the antibody binding sites (3,8,10–12).

The visible detection system uses, in general, AP-labeled primary antibodies and NBT/BCIP as a substrate, as it is the most sensitive one. The optimal incubation time for the detection reaction is determined by direct observation under a stereo microscope. Hence this is the method of choice for testing quality and sensitivity of a new riboprobe (*see Note 33*). If the riboprobe proves to be satisfactory, enzymatic fluorescent detection might follow (*see Note 34*).

1. Block the sample in 200 μ L of 5 to 10% sheep serum (*see Note 32*) in PBT for 2 h at room temperature.
2. Dilute the anti DIG-AP primary antibody (*see Note 31*) 1:1000 in 5 to 10% sheep serum in PBT.
3. Remove the blocking solution and add 200 μ L of the primary antibody dilution. Incubate overnight at 4°C.
4. Wash with PBT five times for 20 min each at room temperature.
5. To equilibrate tissue before transcript detection reaction, wash with AP-detection buffer three times for 5 min, each at room temperature.
6. Prepare the staining buffer by adding 4.5 μ L of NBT and 3.5 μ L of BCIP to 1 mL of AP-detection buffer.
7. Transfer the sample to a block dish. Add the staining solution and let the reaction develop in darkness (light-sensitive) initially for about 30 min and up to 6 h, depending on the probe.
8. From time to time check the level of staining under a stereo microscope. When a purple precipitate becomes clearly visible, stop the reaction by rinsing twice with PBT.
9. To remove unspecific staining, incubate two times for 5 min each in 100% ethanol. The purple precipitate will become dark blue.
10. Replace the ethanol with PBT.
11. Incubate the sample in a raising series of glycerol/PBT (the sample will sink to the bottom) to the final concentration of 80% glycerol/PBT. Mount in 80% glycerol/PBT, sealing the cover slip with nail polish.

3.4.3. Fluorescent Enzymatic Detection

This detection method exploits the catalytic activity of the enzyme horseradish peroxidase (HRP) to cleave tyramide molecules coupled to a fluorescent dye ([11,12](#)). After cleavage, tyramide is transformed into a highly reactive compound that covalently binds nearby proteins, thus providing localized signal amplification ([11,12](#)). It is also possible to detect triple RNAs ([12](#)) and possibly RNA and proteins simultaneously using this novel tyramide-based amplification system.

3.4.3.1. SINGLE DETECTION PROTOCOL

1. Block the sample in 200 μ L of 5 to 10% sheep serum (*see Note 32*) in PBT for 2 h at room temperature.

2. Dilute the anti DIG-POD primary antibody (*see* **Note 31**) 1:400 in 5 to 10% sheep serum in PBT.
3. Remove the blocking solution and add 200 μ L of the primary antibody dilution. Incubate overnight at 4°C.
4. Wash with PBT five times for 20 min each at room temperature.
5. Wash with amplification buffer (supplied with the kit) two times for 5 min each at room temperature.
6. Replace the last wash with 100 μ L of fresh amplification buffer and then add 1 μ L of the tyramide solution. Preincubate the sample in the dark for 30 min. This step improves tissue penetration by the substrate.
7. Dilute the H_2O_2 stock 1:100 in amplification buffer. Start the detection reaction by adding 1 μ L of H_2O_2 dilution to the sample. Let the reaction develop for a minimum of 15 to 20 min depending on the activity of the riboprobe (*see* **Notes 34 and 35**).
8. Rinse with PBT three times at room temperature.
9. Wash with PBT five times for 15 min each at room temperature.
10. Mount tissue in Vectashield and seal the cover slip with nail polish.

3.4.3.2. SEQUENTIAL TYRAMIDE DETECTION

1. Perform the first tyramide reaction as described in **steps 1–7** of the single-detection protocol.
2. Inactivate HRP activity by washing in 1% H_2O_2 in PBT for 20 min at room temperature with rocking.
3. Rinse with PBT three times at room temperature.
4. Wash with PBT for 5 min at room temperature with rocking.
5. Incubate with another HRP-labeled primary antibody—or agent—directed against a different hapten (e.g., anti-fluorescein HRP or streptavidin HRP) overnight at 4°C.
6. Perform the second tyramide (coupled to a different fluorescent dye) reaction as in **steps 4–10** of the single-detection protocol.

4. Notes

1. DEPC is a carcinogen; work under a fume hood, wearing gloves and protective clothing. DEPC is inactivated by prolonged autoclaving.
2. DMSO is toxic and can cause female sterility; work under a fume hood, wearing gloves and protective clothing. It is very sensitive to oxidation; dispense in aliquots and store at –80°C.
3. Work under a fume hood, wearing gloves and protective clothing. Formaldehyde is light-sensitive. Wrap in aluminium foil and store at 4°C.
4. To treat H_2O or any other solution with DEPC, add 0.5 mL DEPC/L, shake, and incubate overnight at 37°C. Autoclave solution to inactivate DEPC. DEPC reacts with amino side groups; hence DEPC treatment is not suitable for solutions containing amino compounds, i.e., Tris-HCl and glycine. These solutions are pre-

pared by dissolving powder (molecular biology grade) from a new tab in DEPC-treated H₂O.

5. For 1 L: 3.21 g NaH₂PO₄·2H₂O; 20.7 g Na₂HPO₄·7H₂O; 90 g NaCl. Adjust pH with either H₃PO₄ or NaOH (recipe from Orié Shafer). Suitable for treating adult brains.
6. For 1 L: 200 g NaCl; 5 g KCl; 5 g KH₂PO₄; 27.8 g Na₂HPO₄·2H₂O. Adjust pH with either H₃PO₄ or NaOH. Suitable for treating imaginal discs, malpighian tubules, ovaries, and testes.
7. Store at −20°C; avoid multiple freeze–thaw cycles.
8. Phenol and chloroform are highly toxic. Work under a fume hood, wearing gloves and protective clothing. Dispose according to local regulation.
9. Stable at 4°C.
10. Aliquot and store at −20°C; stable for years.
11. Pour liquid formamide in a baked beaker placed in an ice container and stir overnight at 4°C. Discard the liquid layer (follow local regulation for disposal) and thaw the crystals at room temperature. Repeat the first step. Thaw the crystals, aliquot, and store at −80°C.
12. Dilute from 20X SSC stock with DEPC-treated H₂O; add Tween-20 to a final concentration of 0.1%.
13. Heat the serum for 2 to 3 h at 56°C, aliquot, and store at −80°C.
14. pH 6.0 improves tissue stability during high-temperature treatment (*see also* **ref. 2**).
15. Stable for 6 mo at 4°C.
16. The construct is linearized to avoid incorporation of sequences from the vector itself into the riboprobe, as this might lead to a weak hybridization signal. Digest at least 5 µg of DNA; this will provide enough material to repeat the transcription if needed. Check linearization of the construct by agarose gel electrophoresis before proceeding further.
17. The concentration of RNA polymerases vary between 20 and 80 U/µL depending on the enzyme and manufacturer. If possible, the more efficient T7- is preferable to T3- or SP6-RNA polymerases, especially for difficult or weak probes (C.W., personal observation; **ref. 4**).
18. A longer incubation time does not increase the yield. A first indication that the transcription reaction has been successful is a cloudiness of the reaction mixture.
19. It is often convenient to resuspend the RNA pellet in a small amount of DEPC-treated H₂O first and then bring to 100 µL with HB. RNA resuspended in HB cannot be run reliably on an agarose gel. However, RNA dissolved in a formamide-containing solution is more stable and can be stored for a couple of years at −20°C.
20. On a standard agarose gel, RNA transcripts are visible as a band (approximately half the size of the original insert size, e.g., 1 kb for an insert of about 2 kb) and a smear ranging from the size of the insert to 200 bp (**Fig. 2**).
21. Detergents such as Tween-20 or Triton X-100 improve penetration by the fixative, especially in densely packed tissues such as adult brains. However, they should never be added to the dissection buffer itself. In unfixed cells they cause cell death, ectopically switching on gene transcription. This provides misleading gene expression results.

22. DMSO improves the penetration of the fixative in densely packed tissues.
23. For delicate tissues such as larval brains or imaginal disks it might be beneficial to perform an additional fixation step with 4% PFAT for 20 min at room temperature before proteinase K treatment. The fixative is removed washing with PBT_w five times for 5 min each.
24. If you want to prepare proteinase K from powder, you should prepare a larger amount and dispense into small aliquots. These have to be stored at -20°C . Dissolve proteinase K powder in water and store in small aliquots at -20°C . Do not reuse frozen proteinase K aliquots; enzymatic activity drops in repeated thaw-freeze cycles. Nevertheless, it is better to purchase proteinase K as a solution, rather than to dissolve a powder. In the commercial solution, the enzyme is stabilized with a defined activity and can be used for a long time (4°C storage) without readjusting the incubation time.
25. Tissues become very fragile after proteinase K digestion; shaking might destroy them.
26. The incubation time for the proteinase K treatment must be tested individually for each type of tissue. Symptoms of overdigestion are samples that look degraded or become sticky after hybridization at high temperature. In our experience a 3- to 5-min digestion (depending on the batch of proteinase K) is optimal for adult brains. Laboratories without air conditioning might experience problems in summer, as the enzyme works more efficiently at higher temperatures. Under these conditions perform the reaction in a 25°C cooling water bath or equivalent.
27. Many protocols suggest hydrolyzing the probe before hybridization to improve tissue penetration. In our experience (*see also* **ref. 8**) this step is unnecessary and actually detrimental. After hydrolysis, the size of the RNA probe should have been reduced to 50 nucleotides. However, because the transcribed RNA is not homogenous in size, the accurate control of this reaction is difficult. Fragments smaller than 50 nucleotides are often obtained; these bind unspecifically, producing high background.
28. The optimal dilution depends on the nature of the probe and on the efficiency of the transcription reaction and must be determined empirically. Typical values are 1:100 to 1:200. Although multiple probes can be used simultaneously in the same hybridization mixture, the optimal concentration for each riboprobe must be adjusted independently. Too highly concentrated riboprobes in HB may cause unspecific background stain.
29. The hybridization temperature can be raised to 70°C for persisting background problems.
30. To save blocking agents, especially the expensive salmon sperm DNA, the washing steps are performed with HB rather than blocking buffer. There are no adverse effects.
31. It is important to preabsorb the primary antibodies with the same type of tissue used for the hybridization experiment to avoid unspecific binding and high background.
 - a. Dissect, fix, rehydrate, and pretreat 5 to 20 (depending on the amount of antibody needed) brains, as previously described in **Subheadings 3.2.2.–3.3.2.**
 - b. Block for 2 h at room temperature in 5 to 10% sheep serum in PBT (serum and antibody should originate from the same species).

- c. Dilute the antibody between 1:50 and 1:100 in 5 to 10% sheep serum in PBT.
 - d. Apply onto the brains and incubate overnight at 4°C.
 - e. Transfer the preabsorbed antibody stock to a fresh Eppendorf tube and store at 4°C. It is stable for at least 1 wk.
 - f. Test several dilutions from the stock to find the right one for signal detection.
32. Serum and antibody should originate from the same species. Alternatively, use the blocking solution available from Roche for Western blotting.
 33. In our hands this method is highly sensitive. For instance the *cry* riboprobe produces a strong signal in 30 min or less. In case of weak or no signal detection it might be helpful to transcribe the same probe using a different hapten for labeling, because of the different sensitivity of the antibodies available. In general, in multiple transcript detection the most abundant RNA is identified with the less sensitive hapten-antibody combination, and conversely, the rarest RNA with the most sensitive.
 34. Because fluorescent staining can be observed only after mounting, the optimal incubation time is determined empirically with a time-course experiment.
 35. To detect *cry* RNA, the reaction was carried out for 3 h with one replacement of the tyramide solution.

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***In Situ* Hybridization of Suprachiasmatic Nucleus Slices**

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Summary

The progress in the understanding of the molecular machinery of mammalian circadian clocks, in combination with the well-established role of the hypothalamic suprachiasmatic nucleus (SCN) as a master circadian clock, has provided an invaluable system for the study of the molecular basis of circadian rhythmicity. Using *in situ* hybridization (ISH) techniques that label specific clock-gene mRNAs within the SCN, researchers can now elucidate the core molecular oscillatory mechanisms underlying specific circadian physiological and behavioral phenotypes. In this chapter, two methods for ISH within the SCN are described. The first method is based on the fluorescent labeling of mRNA and is suitable for confocal microscopy analysis and double labeling techniques. The second method is based on the radioactive labeling of mRNA and is more sensitive and more adequate for the relative quantification of mRNA species.

Key Words: Suprachiasmatic nucleus; circadian; clock genes; fluorescent *in situ* hybridization; radioactive *in situ* hybridization.

1. Introduction

The hypothalamic suprachiasmatic nucleus (SCN) is the site of a circadian pacemaker that governs circadian rhythms in mammals (1). The SCN is constituted of multiple single-cell circadian oscillators (2), and several studies have identified the candidate regulatory molecules and biochemical processes that appear to constitute the basic intracellular oscillatory mechanism (3). Transcriptional and translational autoregulatory feedback loops involving so-called clock genes, and leading to the rhythmic expression of these genes, represent the basic mechanism by which SCN cells can behave as self-sustained oscillators. The identification of these genes and characterization of their temporal pattern of expression within the SCN has provided researchers with a powerful tool for the study of the neural basis of circadian rhythmicity. By analyzing the

expression patterns of these genes within a neuroanatomically discrete master circadian pacemaker such as the SCN, it is now possible to uncover core clock molecular processes underlying basic behavioral and physiological circadian processes (4). *In situ* hybridization (ISH) is a technique that has been pivotal for the progress of our current understanding of the molecular mechanisms by which the SCN orchestrates circadian rhythmicity of physiology and behavior. ISH is used to measure the steady-state levels of mRNA of a specific gene. The technique is based on the hybridization of labeled nucleic acid probes to the gene's specific mRNA. Unlike Northern blot hybridization of RNA, the hybridization of the probe to the mRNA takes place *in situ*—i.e., in the intact tissue, rather than in an RNA sample extracted from the tissue. Thus, ISH allows the neuroanatomically defined labeling of mRNAs of specific genes, which represents the main advantage of this technique. Using ISH it is possible to analyze the expression pattern of different clock genes within a small nucleus like the SCN, as well as within its regional subdivisions (4,5). ISH offers a less accurate method to quantify mRNA than other techniques such as Northern blot hybridization, RNase protection assays, or real-time reverse transcriptase PCR. Furthermore, ISH measures the levels of mRNA of a gene but not the rate of transcription of this gene. For some genes the levels of mRNA are regulated mainly by *de novo* transcription; the levels of mRNA represent a good estimate of the rate of transcription. For other genes, however, the levels of mRNA are regulated by changes in their half-life, and mRNA levels may represent an estimate of the rate of degradation rather than transcription. In this chapter two techniques of ISH are described. The first technique uses RNA probes that are tagged with digoxigenin (DIG), an antigen that can be easily detected with immunohistochemical techniques. The second technique uses radioactively labeled RNA probes and yields autoradiographic images of brain sections where the expression of specific genes can be visualized and easily quantified in neuroanatomically defined areas. DIG ISH allows mRNA labeling on the brain tissue, providing cellular resolution. In addition, DIG can be labeled with fluorescent antibodies, offering the possibility of labeling the mRNA of interest and a second specific mRNA or antigen with other fluorophores, as well as the possibility of analysis by confocal microscopy. On the other hand, radioactive ISH is a more sensitive method and allows detection of relatively low levels of mRNA. Furthermore, quantification of mRNA levels after radioactive ISH, by measuring optical densities on autoradiographs, is easier than counting cells after DIG ISH. Of note, the quantification is also more reliable because radioactive ISH is a *direct* method of labeling and does not rely on *indirect* amplification (enzymatic or other) methods, as DIG ISH does.

2 . Materials

2.1. Fluorescent ISH Using DIG-Labeled Probes

1. H₂O-Diethylpyrocarbonate (DEPC): DEPC-treated water (RNase-free water; *see* **Notes 1** and **2**).
2. 0.1 M Phosphate buffer (PB): 70 mM Na₂HPO₄, 30 mM NaH₂PO₄, pH 7.3.
3. 0.01 M Phosphate-buffered saline (PBS): 0.01 M PB, 0.15 M NaCl, pH 7.3.
4. Heparin-PBS: 30 U/mL in 0.01 M PBS. It can be stored for a week at 4°C.
5. PFA: 4% paraformaldehyde in 0.1 M PB. Prepared fresh or a day before use (*see* **Note 3**).
6. 20% Sucrose in 0.1 M PB.
7. TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
8. DNA template: 1 µg/µL in TE buffer or DEPC-H₂O (*see* **Note 4**).
9. SP6/T7 DIG RNA Labeling Kit (Roche).
10. T3 RNA polymerase (Ambion), if needed.
11. Positively charged nylon membrane (Ambion).
12. Corning Netwells™ (Corning Life Sciences) placed in 12-well cell culture microplates (Corning).
13. 24-Well cell culture microplates (Corning).
14. 20X Sodium citrate buffer (SSC): 3 M NaCl, 0.3 M sodium citrate, pH 7.0, prepared with H₂O-DEPC.
15. Triethanolamine hydrochloride (TEA-HCl)-acetic anhydride (*see* **Note 5**).
16. Proteinase K 2000X stock: 2 mg/mL in DEPC-treated water; aliquot and store at -20°C.
17. Proteinase K buffer: 0.1 M Tris-HCl, pH 7.5, 0.1 M EDTA in DEPC-treated water. It can be stored at room temperature for several months.
18. Hybridization buffer A (*see* **Note 6**).
19. 50% Formamide/50% 2X SSC (*see* **Note 7**).
20. RNase buffer: 0.5 M NaCl, 1 mM Tris-HCl, 1 mM EDTA, pH 8.0. It can be stored at room temperature for several months (*see* **Note 8**).
21. RNase A stock solution (Sigma, cat. no. R-4642): 32.5 mg/mL.
22. Buffer 1: 0.15 M NaCl, 0.1 M Tris-HCl, pH 8.0. This buffer can be stored at room temperature for approx 2 mo (*see* **Note 8**).
23. Buffer 2: 0.1 % Triton X-100 in buffer 1 (*see* **Note 8**).
24. Blocking solution: 1% Roche blocking reagent (Roche cat. no. 1096176) in buffer 1 (*see* **Notes 8** and **9**).
25. Peroxidase-conjugated anti-DIG antibody (Roche, cat. no. 1207733).
26. Cy3 TSA kit (PerkinElmer Life Sciences, Wellesley, MA, cat. no. NEL 744).
27. Antifade: Prolong® (Molecular Probes, Eugene, OR, cat. no. P-7481).

2.2. Radioactive In Situ Hybridization Using ³⁵S-Labeled Riboprobes

1. Styrofoam cup.
2. Stainless steel beaker.
3. -80°C Thermometer.

4. Two ice buckets with dry ice crushed into fine powder.
5. Methyl butane (*see Note 10*).
6. Embedding matrix (Shandon Lipshaw, Pittsburgh, PA, cat. no. 1310).
7. 1 mCi of ^{35}S -UTP.
8. MAXIsript[®] SP6/T7 Kit (Ambion, cat. no. 1320).
9. Yeast tRNA: 25 mg/mL.
10. Vectabond[™] (Vector Laboratories, Burlingame, CA, cat. no. SP-1800).
11. Ethanol, chloroform.
12. Hybridization buffer B (*see Note 11*).
13. Autoradiographic film (Kodak Biomax MR film, Eastman Kodak, Rochester, NY).

3 . Methods

3.1. Fluorescent ISH Using DIG-Labeled Probes

3.1.1. Tissue Preparation

For this ISH method, the whole procedure is carried out on free-floating sections obtained after perfusion fixation, postfixation, sucrose embedding, and cryosectioning of the brain (*see Note 12*).

3.1.1.1. PERFUSION

1. Anesthetize and perfuse with heparin–PBS followed by 4% PFA using a regular perfusion protocol.
2. Decapitate and remove brain carefully.
3. Postfix overnight (16–24 h) in 4% PFA at 4°C.

3.1.1.2. SUCROSE EMBEDDING

1. Drain PFA from the vessel containing the brain and fill it with 20% sucrose.
2. Store at 4°C for 48 h; this treatment will cryoprotect the sample.
3. Remove the brain from the vessel, blot the excess sucrose solution with a paper towel, wrap the brain in tape-labeled aluminum foil, and freeze the brain at –80°C until ready for sectioning. Brains can be stored at –80°C for several months.

3.1.2. In Vitro Transcription of DIG-Labeled Riboprobe

3.1.2.1. DNA TEMPLATE

As indicated in **Fig. 1**, two methods can be used to obtain DNA templates. The first method involves the synthesis of relatively large amounts of an in vitro transcription product from a plasmid vector containing an insert of the gene of interest (*see Note 13*). This is done by transformation of competent bacteria, followed by bacterial culturing and purification of plasmid DNA by mini-, midi-, or maxi-preparation. For general guidelines on how to perform these procedures refer to **ref. 6** and to the QIAGEN Plasmid Purification Hand-

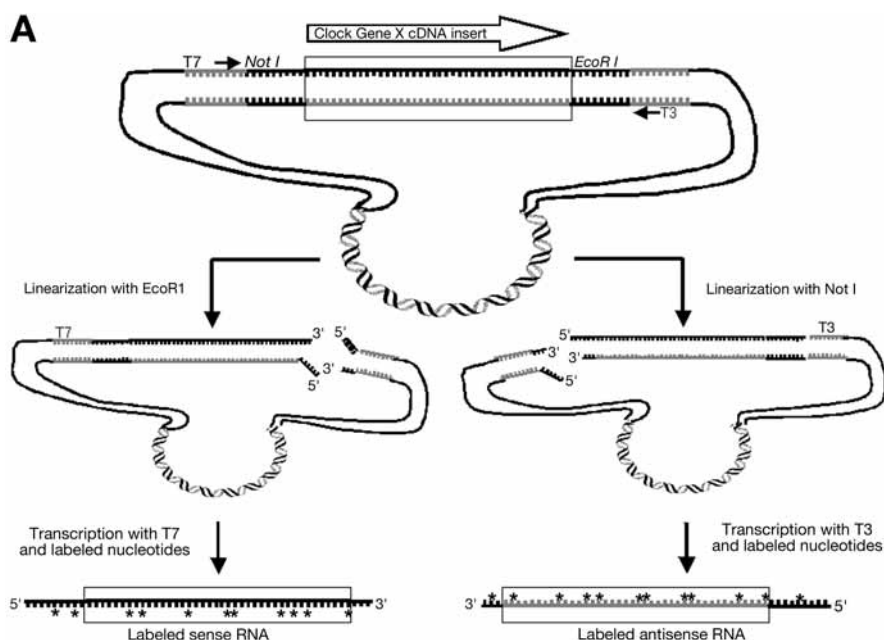


Fig. 1. Generation of DNA templates for riboprobe synthesis. (A) Schematic drawing of a generic transcription plasmid vector where a cDNA fragment of interest has been cloned (clock gene X cDNA insert, framed by white rectangle). The large white arrow indicates the original (in vivo) direction of transcription of the gene. In order to use the cDNA insert as a template for transcription of RNA the plasmid must be linearized with the appropriate restriction enzyme (specific sequence for the enzyme in black, flanking the insert) and transcribed with the appropriate RNA polymerase (T7 or T3, specific promoter sequences indicated in gray). In this case, digestion with *EcoRI* and transcription with T7 RNA polymerase and labeled nucleotides (*) will yield a sense RNA probe. Instead, digestion with *NotI* and transcription with T3 RNA polymerase will result in synthesis of an antisense RNA probe, which should hybridize and label the gene's mRNA in the tissue (*continued on next page*).

book (Qiagen). After purification, the plasmid should be resuspended in TE buffer or in DEPC-treated water at a concentration of $1\mu\text{g}/\mu\text{L}$ and stored at -20 or -80°C . Linearization of the plasmid with the appropriate restriction enzyme (see Fig. 1A and Note 14) can be done according to the enzyme manufacturer's instructions. Linearization should be confirmed by running a sample of the reaction product and the original nonlinearized plasmid in a 1% agarose gel. The nonlinearized plasmid should run slightly faster. Incomplete digests, which must be avoided, yield two bands. After the linearization reaction is completed,

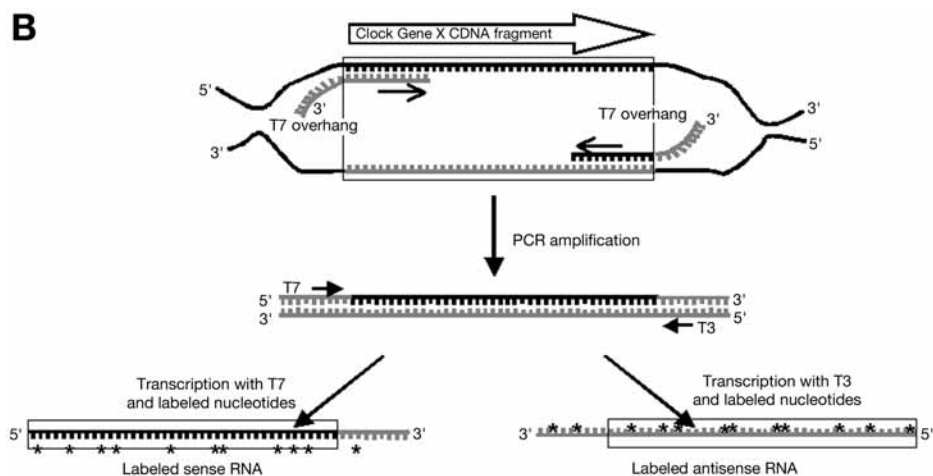
B

Fig. 1. (*continued*) **(B)** Schematic drawing of a double strand of DNA that contains a cDNA fragment of interest but no RNA polymerase promoter sequences flanking it. In this case the DNA template with the RNA polymerase promoter sequences is generated by polymerase chain reaction amplification using primers that flank the region of interest and have the RNA polymerase promoter sequences as overhangs (free “tails” in gray). Remaining symbols as in (A). Although this method to generate the template is different, in this example T7 and T3 RNA polymerization of the template will also yield antisense and sense RNA, respectively. Notice that with both methods, the labeled riboprobe will carry some labeled nucleotides that do not correspond to the DNA insert (outside white rectangles), but these nucleotides will not impair hybridization of the riboprobe to the specific mRNA.

the linearized plasmid DNA should be purified by phenol/chloroform purification and ethanol precipitation, or alternatively by using Quick Spin Columns (Roche). Purified DNA can be resuspended in TE buffer or DEPC-treated water and stored for at least 1 yr at or -80°C . See **ref. 6** for basic techniques.

The second method for obtaining DNA templates is by polymerase chain reaction (PCR) amplification of the DNA fragment of interest, using primers with RNA polymerase promoter sequences as overhangs (*see Fig. 1B* and **Note 15**). A sample of the PCR product should be run in a 1% agarose gel to confirm that a single band of the expected size is obtained. The amplified DNA can be purified and stored by the same methods used after linearization of plasmids.

3.1.2.2. RIBOPROBE SYNTHESIS

Labeling of RNA probes for fluorescent detection of specific mRNA is based on the tagging of specific RNA bases with DIG, and the subsequent fluores-

cent immunolabeling of the DIG molecules. The DIG-labeling kit uses DIG-labeled UTP as a source of uracils for the riboprobe.

1. Using 1 g of template DNA perform the labeling reaction according to the instructions of the DIG RNA labeling kit (Roche), which contains all the necessary components (*see Note 16*). Perform the optional DNase I treatment.
2. Purify the probe by ethanol precipitation. Do not use phenol/chloroform purification because the DIG-labeled RNA will partition into the organic phase.
3. Resuspend the riboprobe in 40 μ L of DEPC water and aliquot into smaller volumes, store at -80°C . Under this condition probes are stable for at least 1 yr if not repeatedly frozen and thawed.
4. To quantify the probe, make a dilution series of the probe and of the labeled control RNA as indicated in the kit instructions. Apply 1 μ L spot for each dilution of control and synthesized riboprobes on a nylon membrane. Air-dry the membrane and crosslink the RNA either by UV or baking, according to the manufacturer's instructions.
5. Incubate the dot blot for 1 h in blocking solution, then overnight in 1:500 dilution of peroxidase-conjugated anti-DIG antibody in buffer 1 (*see Subheading 3.1.5.* for details). Peroxidase staining is done with diaminobenzidine following the manufacturer's instructions.

Figure 2 shows an example of a dot blot where the labeled control RNA and two probes were applied after a 1:2 dilution series. Using the known concentration of the control RNA, the concentration of the DIG-labeled probe can be roughly estimated. When using a sense probe as control for ISH, it is important to use a similar concentration (based on the intensity of DIG staining in the dot blot) to that of the antisense probe (*see Note 17*).

3.1.3. Sectioning, Prehybridization, and Hybridization

3.1.3.1. SECTIONING

Tissue should be cut on the same day that the ISH is started. All buffers for prehybridization and hybridization should therefore be prepared before brain sectioning starts. Sections can be cut in either a freezing microtome or a cryostat. In both cases they will be kept free-floating throughout the procedure.

1. Remove the brains from -80°C and place them in dry ice.
2. Cut the brains into 40- to 50- μ m sections (*see Note 18*).
3. As you cut, transfer the sections to netwells placed in 12-well cell culture microplates containing chilled 2X SSC. Sections are kept at 4°C until the prehybridization is started. Leave every other well without a netwell for each set of sections.

3.1.3.2. PREHYBRIDIZATION

Unless indicated, the whole procedure is done by switching the netwells back and forward, after rinsing the well with 2X SSC and replacing with the

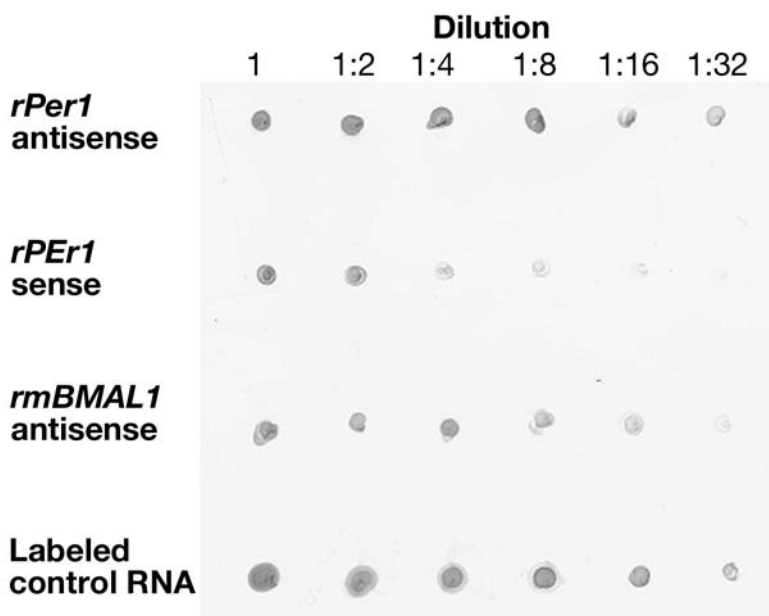


Fig. 2. Dot blot of digoxigenin (DIG)-labeled RNA. The membrane shows a blot of 1 μ L of a 1:2 dilution series of DIG riboprobes for *rPer1* sense and antisense, for *mBMAL1* antisense, and for the Roche RNA labeled control. After staining, the RNA concentration of each riboprobe solution can be roughly estimated based on the concentration of the labeled control RNA. Notice that the labeling efficiency is higher for *rPer1* antisense than for *rPer1* sense probe. If this sense probe is used as a control for antisense labeling by *in situ* hybridization, the same final concentration of sense and antisense probe, based on this dot blot estimation, should be used in the hybridization reaction.

appropriate solution. All washes should be done under gentle shaking, making sure that sections are moving freely within the netwell.

1. Pour 1.0 μ g/mL proteinase K solution into the empty wells and incubate at 37°C until temperature stabilizes.
2. Transfer the netwells with sections into the proteinase K wells and incubate at 37°C for 30 min.
3. Transfer the sections to 4% paraformaldehyde and incubate 5 min at room temperature.
4. Transfer the sections to 2X SSC and incubate 5 min at room temperature.
5. While the sections incubate, add 2.5 μ L of acetic anhydride per milliliter of TEA-HCl buffer.
6. Transfer the sections to TEA-HCl/acetic anhydride and incubate for 10 min at room temperature.

7. Transfer the sections to 2X SSC and incubate at least 10 min at room temperature.
8. Place plates at 4°C until hybridization begins (preferably on the same day).

3.1.3.3. HYBRIDIZATION

1. Prepare hybridization solution by diluting sense and antisense probes to an approximate concentration of labeled riboprobe of 500 ng/mL of hybridization buffer A (*see Note 17*). Approximately 500 μ L of solution per set of sections are needed.
2. Pipet 500 μ L of hybridization solution into each of the wells needed of a 24-well microplate.
3. Using an RNase-free glass rod or paintbrush, carefully transfer sections from 2X SSC to wells containing hybridization solution.
4. Cover the 24-well microplate with its lid and carefully seal with Parafilm. Make sure it is airtight.
5. Place the 24-well microplate inside a plastic container that has a DEPC-water-wet paper towel at the bottom, cover the plastic container with a hermetic lid, and incubate overnight on an orbital shaker in a high-humidity incubator at 60°C (*see Note 19*).

3.1.4. Post-Hybridization

1. Using an RNase-free glass rod or paintbrush, carefully transfer the sections from the hybridization mix in the 24-well microplate to the appropriate netwell in the 12-well microplate containing 50% formamide/50% 2X SSC at 60°C. Incubate 5 min at 60°C. When hybridizing with more than one probe, rinse the rod or the paintbrush with DEPC-treated water between probes.
2. Transfer the sections to a new well containing 50% formamide/50% 2X SSC and incubate 45 min at 60°C.
3. While this incubation takes place, warm up an appropriate volume of RNase buffer in a 37°C incubator. This should be done in the RNase area of the laboratory.
4. Transfer the sections to a new well containing 50% formamide/50% 2X SSC and incubate 15 min at 60°C.
5. Transfer the sections to 2X SSC and incubate 5 min at room temperature.
6. Transfer materials to the RNase area and add the appropriate amount of RNase A stock solution to the 37°C RNase buffer to a final concentration of 100 mg/L. Transfer the sections to the RNase solution and incubate 30 min at 37°C. Special care should be taken not to contaminate the RNase-free area of the lab after handling RNase A.
7. Transfer the sections to 50.0 % formamide/50% 2X SSC and incubate for 15 min at 60°C. Sections can be kept in 2X SSC at room temperature until temperature of incubator and formamide/2X SSC reach 60°C.
8. Transfer the sections to fresh 50.0% formamide/50% 2X SSC and incubate for 15 min at 60°C.

9. While incubation takes place, take advantage of the incubator temperature and prepare the blocking solution.
10. Transfer the sections to 0.4X SSC and incubate for 30 min at 60°C.
11. Transfer the section to buffer 1. Sections can be stored at 4°C overnight if necessary.

3.1.5. Immunohistochemical Detection of DIG

1. Wash sections in buffer 2 twice for 5 min each.
2. Transfer the sections to the blocking solution and incubate at room temperature for 1 h.
3. Transfer the sections to buffer 2 containing 1:500 peroxidase conjugated anti-DIG and incubate overnight at 4°C with gentle shaking. This step and the steps below are done in 24-well cell culture microplates.
4. Wash the sections twice for 5 min in buffer 2 and once for 5 min in buffer 1.
5. Incubate the sections for 8 min in 1:50 Cy3 TSA diluted in amplification buffer according to the manufacturer's instructions.
6. Wash the sections three times for 5 min in buffer 1.
7. Mount the sections on slides, let dry, rinse quickly with distilled water, let dry, and cover slip using a fluorescent dye anti-fade medium such as Prolong.
8. Cy3 labeling can be visualized under a fluorescence microscope with a Texas red filter or under a confocal microscope with the appropriate excitation wavelength (**Fig. 3**; see **Note 20**).

For double-labeling ISH combined with immunocytochemistry (**Fig. 3B,C**) or double-labeling ISH, see **Notes 21** and **22**, respectively.

3.2. Radioactive ISH Using ³⁵S-Labeled Riboprobes

3.2.1. Tissue Preparation

For this ISH method, the whole procedure is carried out on sections mounted on microscope slides after dissection, freezing, and cutting of the brain (see **Note 12**).

3.2.1.1. DISSECTION

1. Set up guillotine and surgery instruments for brain dissection.
2. Pour methyl butane into a stainless steel beaker and place in dry ice. Monitor temperature until it reaches -30 to -35°C. It is very important that temperature is kept within this range by placing the beaker back and forward in dry ice.

Fig. 3. (opposite page) Detection of *Per1* mRNA by *in situ* hybridization in the rat suprachiasmatic nucleus. **(A)** Film autoradiography of a coronal hypothalamic section of a rat sacrificed during the light phase of a light-dark cycle, and hybridized with ³⁵S-labeled *rPer1* antisense riboprobe. **(B)** Fluorescent confocal photomicrograph of a coronal hypothalamic section of a rat sacrificed during the light phase of a light-dark

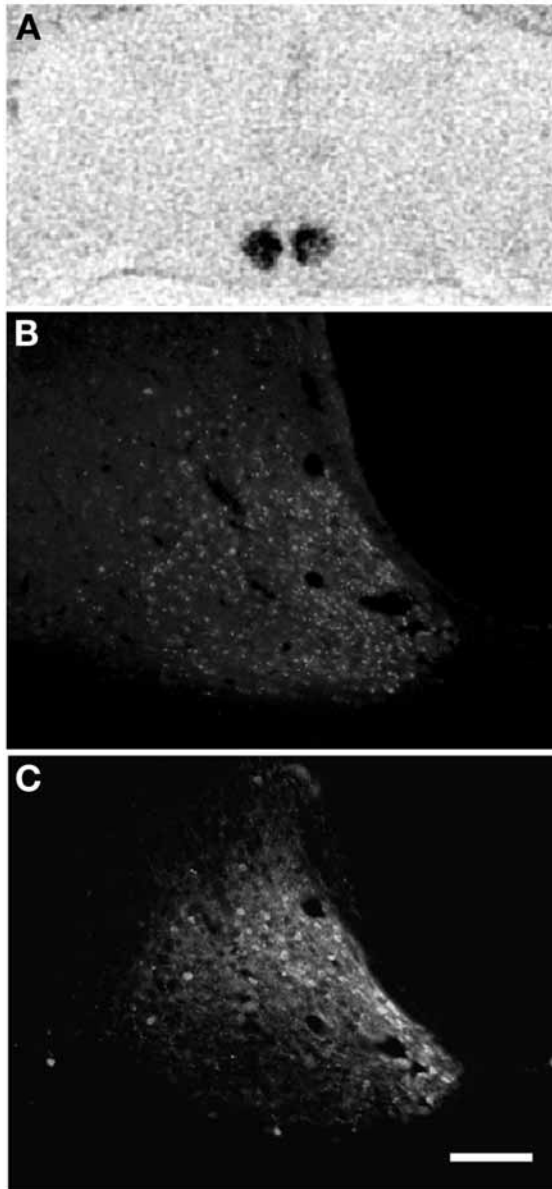


Fig. 3. (continued) cycle, and hybridized with digoxigenin-labeled *rPer1* antisense riboprobe, detected with Cy3 and visualized after excitation with 568-nm wavelength light. (C) The same section shown in (B) is double-labeled for arginine vasopressin by immunohistochemistry using PS 45 antibody (kindly provided by Dr. A. Gainer) and Alexa 488[®] goat anti-mouse secondary antibody (Molecular Probes), visualized after excitation with 488-nm wavelength light. Scale bar: 1 mm for A, 85 μ m for B and C.

3. Make a strainer with the Styrofoam cup by making holes at the base and cutting the wall so that only one third of the cup's wall is left. This strainer should fit comfortably in the stainless steel beaker.
4. Decapitate the animal. Depending on the mRNA species to label, decapitation without anesthesia may be recommended.
5. Carefully dissect the brain, taking special care to preserve the ventral surface, particularly at the optic chiasm region.
6. Place the brain on the base or the strainer, with the ventral side facing up. Brain should be symmetrically positioned on the strainer.
7. Dip the strainer in the methyl butane at -30 to -35°C and clamp the strainer so it stays in position for 3 to 5 min (*see Note 23*).
8. Place the brain in powdered dry ice. Leave the brain covered with dry ice for 5 min.
9. Remove the brain and shake all dry ice off its ventral surface. Put a drop or two of embedding matrix on your finger and rub it against the ventral surface of the brain. Embedding matrix should get in between all the grooves of the ventral surface of the brain. Return the brain immediately to dry ice and sprinkle powdered dry ice onto its ventral surface. Allow the embedding matrix to freeze and apply another coat of embedding matrix. There should be a 1- to 2-mm layer of embedding matrix on the ventral surface of the brain after two or three coats have been applied (*see Note 24*).
10. Wrap the brain in tape-labeled aluminum foil and transfer the brain from dry ice directly to the -80°C freezer until sectioning. Brains can be stored at -80°C for several months.

3.2.2. *In Vitro* Transcription of Radiolabeled Riboprobe

3.2.2.1. DNA TEMPLATE

Templates are prepared as indicated in **Subheading 3.1.2.1**.

3.2.2.2. RIBOPROBE SYNTHESIS

Radiolabeling of RNA probes is based on the tagging of specific RNA bases with a radioactive isotope, and the subsequent autoradiographic detection of the hybridized probe (*see Note 25*). In this protocol ^{35}S -UTP is used as a source of uracils for the riboprobe. RNA probes can be also labeled with other radioisotopes such as ^{33}P or ^{32}P , provided that the adequate radiolabeled nucleotide is used.

1. Using 1 μg of template DNA perform the labeling reaction according to the instructions of the MAXIscript Kit (Ambion), which contains all the necessary components with the exclusion of the radioactive nucleotide (*see Note 16*). Perform the optional DNase I treatment.
2. After DNase treatment, bring the reaction volume to 100 μL with DEPC-treated water. Take 1 μL of this final volume, dilute 1:100 in DEPC-treated water, and

count 2 μL of this dilution in the scintillation counter to obtain your initial counts per minute (cpm).

3. Use a phenol/chloroform extraction followed by ethanol precipitation to purify your probe out of the remaining 99 μL of each reaction product. Resuspend each reaction product in 100 μL of 50 μM dithiothreitol (DTT) in DEPC-treated water, and repeat the counting procedure to obtain your final cpm. The percentage of incorporation, calculated using the initial and final cpm and volumes, should be higher than 60 to 70%. The final cpm will also be used to calculate the amount of probe per volume of hybridization buffer (*see Subheading 3.2.3.3.*). ^{35}S -UTP-labeled riboprobes can be stored at -80°C for up to 2 mo. For other radioisotopes, this time will depend on the isotope half-life.

3.2.3. Tissue Sectioning, Prehybridization, and Hybridization

3.2.3.1. TISSUE SECTIONING

For this ISH protocol brain sections are mounted on microscope slides throughout the procedure. Before sectioning, microscope slides are coated with Vectabond™ according to the manufacturer's instructions. Brains are cut into 10- to 20- μm -thick sections in a cryostat and mounted immediately. Brains and sections are kept frozen all the time throughout the procedure. After cutting, the slides with sections are kept at -80°C in slice boxes within freezer bags with desiccant. Sections can be kept for several months at -80°C , although unnecessary long-term storage should be avoided. The integrity of the sections during cutting and mounting is critical for successful labeling of neuroanatomically defined areas.

3.2.3.2. PREHYBRIDIZATION

1. Remove slides from -80°C freezer and place them on slide racks.
2. Dip in 4% PFA for 5 min at room temperature.
3. Rinse 2 min in 2X SSC.
4. Incubate 10 min in TEA-HCl/acetic anhydride.
5. Quickly rinse in 2X SSC.
6. Dehydrate in ethanol series prepared with DEPC-treated water as follows: 1 min in 70%, 1 min in 80%, 2 min in 95%, 1 min in 100%.
7. Delipidate by incubating in chloroform for 5 min (*see Note 26*).
8. Wash 1 min in 100% ethanol followed by 1 min in 95% ethanol.
9. Let air dry in a clean area. Slides can be left for a couple of hours while drying. While slides dry, set a high-humidity incubator to 37°C .
10. Mix equal amounts of 4X SSC and deionized formamide and pipet approx 50 μL of this solution on each slide. Cover slip each slide with clean glass cover slips so that the whole surface of the tissue section is embedded in the solution. Place slides in a capped plastic container and place the container in the 37°C incubator. Incubate 20 to 60 min.

3.2.3.3. HYBRIDIZATION

1. Prepare the hybridization solution by diluting 10^8 cpm of probe per milliliter of hybridization buffer B with freshly added DTT.
2. Remove the plastic containers one at a time from the incubator. Remove the cover slips, carefully shake out any 50% 4X SSC/50% formamide left, and pipet approx 50 μ L of hybridization solution. Cover with a new glass cover slip (*see Note 27*).
3. Place a cap (i.e., from a conical tube) filled with water inside the plastic container and place the slides back into it. Alternatively, create a high-humidity chamber by placing on the bottom of the plastic container a piece of paper towel wetted with DEPC-treated water. Cover the plastic container, making sure it has a good seal. Otherwise, seal with Parafilm. It is extremely important that slides are in a high-humidity hermetic chamber; otherwise they will dry out.
4. Place the plastic container back into the incubator. Once every slide is covered with hybridization solution, and all plastic containers are back in the incubator, set the temperature of the incubator to 55°C and incubate overnight.

3.2.4. Post-Hybridization

1. Set an incubator or a water bath to 52°C and warm up enough 50% formamide/50% 2X SSC solution for two washes.
2. Set up three beakers with 1X SSC solution.
3. Remove the plastic containers from the incubator one at a time, remove each slide, and dip it in the first 1X SSC beaker. If the slides did not dry out after overnight hybridization, the cover slip should come loose easily. Rinse briefly in each of the two remaining 1X SSC beakers and incubate slides in slide racks in 1X SSC for 10 min. Transfer the slides to a second wash of 1X SSC for another 10 min. If many slides are processed, these two 10-min incubations can be done for a longer time. If hybridization was done with more than one probe, wash slides for each probe separately.
4. Incubate in 50% formamide/50% 2X SSC for 5 min at 52°C. For this incubation slides hybridized with different probes can be pooled together.
5. Transfer to fresh 50% formamide/50% 2X SSC and incubate for 20 min at 52°C.
6. While this incubation takes place, warm up an appropriate volume of RNase buffer in a 37°C incubator. This should be done in the RNase area of the laboratory.
7. Transfer materials to the RNase area and rinse two times in 2X SSC at room temperature for 1 min each (*see Note 28*).
8. Add RNase A to the RNase buffer to a final concentration of 100 mg/L and incubate the sections in RNase solution for 30 min at 37°C.
9. Remove the slide racks from the incubator and set it to 52°C.
10. While the incubator reaches 52°C, wash the slides two times in 2X SSC at room temperature for 5 min each, or longer if necessary.
11. Incubate in 50% formamide/50% 2X SSC for 5 min at 52°C.
12. Wash in ethanol series, diluted in 0.1X SSC instead of water, as follows: 3 min in 70%, 3 min in 80%, and 3 min in 95%.

13. Rinse the slides quickly in distilled water.
14. Wash in 70% ethanol diluted with distilled water, for 3 min.
15. Air-dry the slides and lay them out on autoradiographic cassettes.
16. In a dark room, place an autoradiographic film in each cassette, making sure that the emulsion side is against the sections. Cover the cassettes and place in a safe, dark area.
17. Develop the films after 2 d. Optimal exposure time must be determined empirically (*see Note 29*).
18. Autoradiographic images can be scanned and digitalized to estimate optical density. The optical density of the SCN can be normalized to the optical density of the surrounding hypothalamus (*see Note 30*).

4. Notes

1. Add 1 mL DEPC (Sigma-Aldrich, St. Louis, MO, cat. no. D-5758) per liter of distilled water to treat (DEPC is toxic and should be used only under a fume hood). Stir with a magnetic bar until in solution. Let sit overnight at room temperature. Boil for approx 2 h in the fume hood, and autoclave. Although some protocols prepare RNase-free water only by autoclaving water treated overnight with DEPC, boiling for 2 h before autoclaving assures that all DEPC is degraded. Autoclaved DEPC water can be stored indefinitely in capped containers at room temperature. All solutions used before the RNase treatment should be prepared with DEPC-treated water.
2. RNases are ubiquitous and hard to eliminate. Contamination with RNases may degrade both the mRNA that one is trying to label and the RNA probe used to label it with. RNases are usually found on human skin and hair, and the use of clean gloves in any ISH protocol is therefore highly recommended. The most important sources of RNase contamination, however, are contaminated buffers and laboratory equipment, and general precautions to avoid contamination (**6**) should be followed.
3. Paraformaldehyde is toxic and should be handled in a fume hood as much as possible.
4. DNA templates, either linearized or nonlinearized, are more stable in TE buffer than in water. Although some protocols recommend not to use templates diluted in TE, the volume of template used in this chapter's protocols are so low that they do not affect the transcription reaction.
5. Dissolve 18.56 g of TEA-HCl, 9 g of NaCl, and 20 pellets of NaOH in 800 mL DEPC-treated water. Adjust the pH to 8.0 and adjust the volume to 1 L. This solution can be stored for up to 2 wk at room temperature. Just before use add 2.5 μ L of acetic anhydride per mL of TEA HCl solution.
6. Add 3 g dextran sulfate to 5 mL DEPC-treated water in a 50-mL conical tube. Vortex and let stand 10 min, vortexing every now and then. Add 18 mL deionized formamide (Sigma, cat. no. F-9037), vortex, and heat to 37°C until in solution, vortexing now and then. Add 300 μ L 1 M Tris-HCl, pH 7.5, 3.6 mL 5 M NaCl, 60 μ L 0.5 M EDTA, pH 8.0, 0.75 mL 10% SDS, 600 μ L 50X Denhardt's

(Sigma, cat. no. D-2532), and 240 μL of 25 mg/mL yeast tRNA (Roche, cat. no. 0109495). Adjust volume to 30 mL with DEPC-treated water, vortex, and store at -20°C . Hybridization buffer A can be stored for up to 3 mo at -20°C . Formamide is toxic and should be handled under a fume hood as much as possible.

7. Mix equal volumes of formamide (not deionized; Sigma, cat. no. F-7503) and 2X SSC. Prepare enough for three washes in 12-well microplates. Prepare before use and place in incubator at 60°C .
8. This solution is prepared in a separate room where RNase work is done, and need not be prepared with DEPC-treated water. Both ISH protocols described here use a step in which RNase A is used to degrade nonhybridized riboprobe molecules. Every step prior to the RNase A treatment should be done in RNase-free conditions. It is highly recommended that the lab be divided into two areas, one for RNase-free work and the other where RNases or possible sources of RNases are explicitly used. No materials, including pipettors, tubes, flasks, chemicals, and buffers, should be transferred from the RNase area to the RNase-free area. Glassware to be used in RNase-free work should be baked at 200°C for 12 h or otherwise treated with inhibiting solutions such as RNase Away (Molecular Products, San Diego, CA). All solutions used for RNase-free work should be prepared with water treated with DEPC or with water that is freed of nucleases by some other method.
9. Add 1 g blocking reagent (Roche, cat. no. 1096176) and adjust volume to 100 mL with buffer 1. Heat to 60°C to assist dilution and vortex mildly every 5 to 10 min. It can be stored at 4°C for a couple of days.
10. Organic solvent; handle with care and under the fume hood as much as possible.
11. Add 2 g dextran sulfate to 4.5 mL DEPC water in 50 mL conical tube, vortex, and let stand 10 min, vortexing every now and then. Add 10 mL deionized formamide, vortex, and heat to 37°C until in solution, vortexing now and then. Add 2 mL of 20X SSC, 400 μL 50X Denhardt's, 400 μL of 25 mg/mL tRNA, and 800 μL sheared single-stranded DNA. Adjust volume to 20 mL with DEPC-treated water, vortex, and store at -20°C . Hybridization buffer B (without DTT; *see* below) can be stored for up to 3 mo at -20°C .

To shear DNA (salmon testes DNA; Sigma, cat. no. D-7656), draw up and down 12 times with a syringe with a 17- to 22-gage needle and store at -20°C . Heat 5 min in boiling water and chill quickly in wet ice just before adding it to the hybridization buffer.

Immediately before use add to hybridization buffer B 1 μL of 5M DTT (made in 0.01 M sodium acetate, pH 5.2) per 100 μL of buffer. Although 0.01 M sodium acetate can be prepared and kept at room temperature for several months, DTT must be added fresh just before the hybridization solution is prepared.

12. Tissue dissection, preparation, sectioning, and preservation represent the most critical steps in any ISH protocol. No matter how carefully designed an RNA labeling protocol is, it will never overcome poor dissection, sectioning, or improper storage and preservation of the tissue. Avoid unnecessary long-term storage of whole brains or sections.

13. DNA plasmid vectors for in vitro transcription of specific genes are constructed by standard recombinant DNA methods (6) that are beyond the scope of this chapter. In many cases these recombinant DNA clones are available from other laboratories and can be used to transform bacteria, which after growth can be used for mini- or maxi-preparations of plasmid DNA (6).
14. In vitro transcription vectors usually have promoter sequences for two different RNA polymerases flanking the gene insert. This allows antisense or sense transcription of the DNA insert. The most common promoter sequences correspond to T3, T7, and SP6 RNA polymerases. Figure 1 shows a schematic drawing of an in vitro transcription vector. Before transcription of riboprobes the plasmid must be linearized by digestion with the appropriate restriction enzyme to serve as a template for transcription.
15. Although this method requires a basic knowledge of PCR primer design, it has several advantages. First, it uses a very small amount of initial cDNA; second, the cDNA fragment of interest does not need to be cloned in a recombinant plasmid; and third, the template generated can be used for both sense and antisense RNA synthesis, depending on the RNA polymerase used.
16. Be sure to use the appropriate RNA polymerase. Use of the wrong polymerase could either yield no transcription, or (in the case where two different loose-end polymerase promoter sequences are flanking the DNA sequence of interest) lead to transcription of an undesired RNA probe (sense vs antisense).
17. Given the inaccuracy for the estimation of the DIG-labeled probe concentration, the optimal amount of synthesized probe per volume of hybridization buffer should be estimated empirically.
18. Sections thinner than 40 μm can be cut, but given that the protocol is rather long, they might fall apart. Furthermore, fluorescent labeling allows optical sectioning with confocal microscopy and there should be no need for extra-thin sections.
19. Hybridization temperatures for both fluorescent and radioactive ISH are standard for most mRNAs. However, decreasing stringency by decreasing the hybridization temperature is a possibility when no labeling is evident. Conversely, high nonspecific background labeling may be decreased by increasing the temperature.
20. Several controls for the specificity of hybridization signal can be used: first, a hybridization reaction in which no RNA probe is added; second, a reaction in which an unlabeled antisense RNA probe (synthesized with unlabeled UTP) is added; third, a reaction in which a labeled sense RNA probe is added. This third reaction is a key control, as it will reveal any nonspecific labeling emerging from the presence of labeled RNA.
21. Fluorescent ISH can be combined with fluorescent immunocytochemistry. For this purpose, coincubation with the specific primary antibody and the peroxidase-conjugated anti-DIG antibody is done. Detection of the primary antibody must be done with secondary antibody with a different fluorophore than Cy3. It is important to keep in mind that the proteinase K treatment may impair immunodetection of peptidergic antigens. Using the protocol described in this

chapter, a double-labeling protocol with ISH for *rPer1* and immunohistochemistry for vasopressin or vasoactive intestinal polypeptide did not show such impairment.

22. Double-labeling ISH can be performed with two riboprobes, one of them labeled with DIG and the other with biotin. Biotin labeling of riboprobes is also done with the Roche kit but using Biotin RNA Labeling Mix (Roche, cat. no. 1685597) instead of the NTP labeling mixture that comes with the kit. Hybridization is performed simultaneously with both the DIG- and the biotin-labeled probes. After immunohistochemical detection of the DIG probe, the tissue is incubated in 1% H₂O₂ (in buffer 1) for 10 min to block remaining peroxidase activity, and rinsed three times in buffer 1. The biotin-labeled probe is then detected by incubation in streptavidin-HRP and tiramide amplification, according to manufacturer's instructions (TSA™, fluorescein system, PerkinElmer Life Sciences, cat. no. NEL 701A). An alternative to biotin labeling of riboprobes is labeling with fluorescein (7).
23. Freezing of brains in methyl butane at temperatures lower than -35°C will cause the third ventricle to break open and the SCN to rupture.
24. Frozen embedding matrix on the ventral surface of the brain will protect the optic chiasm and the SCN region during cutting in the cryostat. For coronal sections, the direction of movement of the blade should be such that it reaches the ventral surface of the brain first.
25. Radiation safety protocols must be followed throughout this protocol to minimize exposure to radioactivity. Consult your institution's radiation safety office before carrying out this procedure.
26. Chloroform is toxic and should be handled in a fume hood.
27. Handling of slides and cover slips with hybridization solution will usually contaminate gloves with radioactivity. Monitor your gloves frequently and change them accordingly.
28. Up to this point all washes should be considered liquid radioactive waste and should be disposed according to radiation safety office recommendations.
29. Exposure time for the autoradiographic film is a critical variable that must be carefully controlled. Longer times of exposure may reveal labeling that is not evident with shorter times. Conversely, overexposing the films may increase non-specific labeling, as well as saturate the labeling of regions where the gene is highly expressed.
30. The optical density on the microscope slide but outside the brain section indicates nonspecific labeling. If this labeling is relatively high, it should be subtracted from both the SCN optical density and the hypothalamic optical density, before the ratio between them is calculated.

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Immunohistochemistry in *Drosophila*

Sections and Whole Mounts

Charlotte Helfrich-Förster

Summary

This chapter describes immunohistochemistry on *Drosophila* heads (brains) with respect to antigens involved in the circadian system. Two different methods have been successfully performed in several labs: immunolabeling on whole-mount brains and on cryostat sections of entire heads. Both methods are addressed here. The primary antisera can be detected by enzyme-labeled or fluorescence-labeled secondary antibodies. The advantages of the different methods are discussed.

Key Words: Antigen; antiserum; brain; circadian rhythms; compound eyes; cryostat sections; *Drosophila*; fluorescence; *period*; horseradish peroxidase; immunohistochemistry; peroxidase-antiperoxidase; secondary antibody; *timeless*; whole mounts.

1. Introduction

Specific antibodies against antigens involved in the circadian system have revealed the spatial distribution of clock cells in *Drosophila melanogaster*, shown the subcellular localization of clock molecules, and given an idea about their abundance at different time points during the day. Multilabeling has helped to compare the expression patterns of different clock molecules. The use of immunolabeling is, meanwhile, established as a standard method in most laboratories. However, it is clear that appropriate fixation, labeling, and detection can make a huge difference to the sensitivity and clarity of the result, and indeed whether the labeling actually gives a tolerably faithful representation of the *in vivo* distribution of the molecules. Several methods and recipes have been published. Selected staining procedures that give reliable results are described, and advantages and problems are discussed.

Immunolabeling of clock molecules has been successfully performed on whole mounts of the central nervous system and on cryostat sections of the head or body. Paraffin sections or vibratome sections have turned out to be less useful. The dehydration and embedding procedure for paraffin sections (employing ethanol, different intermedia, paraffin, heat, and xylene) appears to destroy or modify the antigens, so that the antibodies do not detect them anymore. In vibratome sections with the fixed tissue embedded in gelatin–albumin, antibody labeling is very good, but because of the solid cuticle a vibratome can barely cut whole flies. The tissue of interest (e.g., nervous system) must be dissected out first. Then, the relative thick vibratome sections (usually around 30 μm) provide little advantage to the whole-mount technique. Therefore, I will describe immunolabeling techniques on whole-mount brains and on cryostat sections of whole heads. The same techniques can principally be applied for any other part of the body. Secondary antibodies listed in **Heading 2.** go with primary antisera gained from rabbit (such as anti-period [PER]), but I will mention other relevant secondary antibodies (e.g., primary antibody raised in mouse, guinea pig, or rat) in **Heading 3.**

For the basic knowledge about immunocytochemistry and an overview on different immunohistochemical techniques the reader is referred to basic literature (**1–3**) and papers treating insect immunocytochemistry (**4**) or immunocytochemistry in *Drosophila* (**5,6**).

2. Materials

2.1. Whole Mounts

1. A pair of Dumont no. 5 forceps.
2. Sandpaper to sharpen the forceps (no. 1000 to shape the tip, no. 2000 for final polish).
3. Block dishes, preferably made of black glass (or transparent glass plus black paper).
4. Glass Pasteur pipets.

2.2. Cryosections

1. Cryostat.
2. 25% Sucrose in phosphate-buffered saline (PBS).
3. Liquid nitrogen.
4. Tissue Tec (Sakura, Zoeterwoude NL).
5. Subbed microscope slides.
6. Razor blades.
7. Fine and rough brushes.
8. Coarse long forceps.

2.3. Both Methods

1. 10X phosphate buffer (PB): 70 mM Na₂HPO₄, 30 mM NaH₂PO₄, pH 7.4.
2. PBS: 1X PB, 0.15 M NaCl, pH 7.4.
3. Triton X-100 (Merck).
4. PBT: 1X PBS with 0.5% Triton X-100.
5. PFA: 4% paraformaldehyde in 1X PB (*see Note 1*).
6. Normal goat serum (NGS; Sigma).
7. Blocking solution: 5% NGS in PBT.
8. Primary antibodies (i.e., anti-PER [7], anti-TIM [8], anti-DBT [9], anti-CRY [10], anti-PDH [11], anti-PDF [12]; not all are commercially available, *see Note 2*).
9. Secondary antibodies (for a primary antiserum from rabbit):
 - a. Horseradish peroxidase (HRP) labeled, e.g., peroxidase–antiperoxidase (PAP) complex developed in rabbit (Sigma).
 - b. Unlabeled, e.g., goat anti-rabbit IgG (GAR; Sigma) as bridge for PAP.
 - c. Fluorescence labeled, e.g., ALEXA 488-GAR, ALEXA 568-GAR, ALEXA 647-GAR (Molecular Probes).
10. Diaminobenzidine (DAB) tablets (Sigma).
11. 30% H₂O₂ (Sigma).
12. Vectashield mounting medium (Vector Laboratories, Burlingame, CA) or antifade glycerol: 0.25% *n*-propyl gallate, 50% glycerol in PBS, pH 8.0 to 8.6 (*see Note 3*).
13. Ethanol.
14. Methylbenzoate.
15. Entellan (Merck).

3. Methods

Immunolabeling on whole-mount brains has several advantages over labeling on sections:

1. The whole-mount preparation provides a better overview of the general staining pattern.
2. The stained structures can be easily related to known landmarks such as the antennal lobes, the optic lobes, the mushroom bodies, or the central complex.
3. Orientation of the brain is always similar (frontal).
4. No sections can be lost.
5. Projections of individual neurons can be followed up easily.

Only in brains with high background staining may details of the staining pattern be obscured in whole mounts. This is especially true if HRP-labeled secondary antibodies are used. With fluorescent secondary antibodies and the usage of a confocal microscope, a higher background is no problem at all, and whole-mount preparations are generally better than sections.

Nevertheless, whole-mount preparations are not adequate if structures outside the brain (e.g., the compound eyes) are in the focus of interest or if a horizontal view of the brain is necessary. In addition, good whole-mount preparations need some practice and are hard to achieve by beginners. The following section should help beginners through the main dissection steps.

3.1. Dissection and Fixation of Whole-Mount Brains

Good tools are most important. Newly bought forceps are usually not fine enough for good preparations. They need to be sharpened with the help of sandpaper under a dissecting microscope (**Fig. 1A**). The sharpened forceps should then be used only for the dissections (use other forceps to grasp flies, etc.). It is also necessary to resharpen the forceps from time to time after regular use.

Dissection is preferentially performed in a black block dish filled with PBS. The black background helps to distinguish the opaque white brain tissue from other structures during preparation. If no black-block dish is available, put a piece of black paper underneath a conventional dish. For illumination use a cold light source. Place sheets of tissue paper on both sides of the block dish to deposit pieces of cuticle, fat body, air sacs, tracheae, and the like during dissection, and to clean the forceps from debris once in a while.

3.1.1. Dissection of Adult Brains

1. Grab the fly with one forceps at the thorax and with another at the base of the proboscis.
2. Remove the head from the body by pulling slowly. Then remove the proboscis. Alternatively, remove the proboscis and then grab the head from below by inserting one forceps into the foramen of the mouth parts and remove the head by pulling.
3. Open up the head capsule from ventral to dorsal (*see Note 4*) by “cutting up” (i.e., carefully breaking the cuticle with the two forceps; **Fig. 1B**) the region between antennae, forehead, and the top of the head.
4. Gently pull apart the right and left halves of the head capsule to isolate the brain. It is important to pull extremely slowly to minimize mechanical stress; otherwise the optic lobes will separate from the central brain. Alternatively, make two further cuts through the cuticle of the optic lobes (**Fig. 1C,D**) and then remove the brain piece by piece; this is safer for the beginner.
5. Once the brain is isolated, remove the esophagus, air sacs (**Fig. 1E,F**), trachea, and fat body from the isolated brain (*see Note 5*).

3.1.2. Dissection of Larval Central Nervous System

1. Grab the larva with one pair of forceps at the mouth hooks and with another pair at its anterior-middle part (about one-fourth of the way from the head) and pull

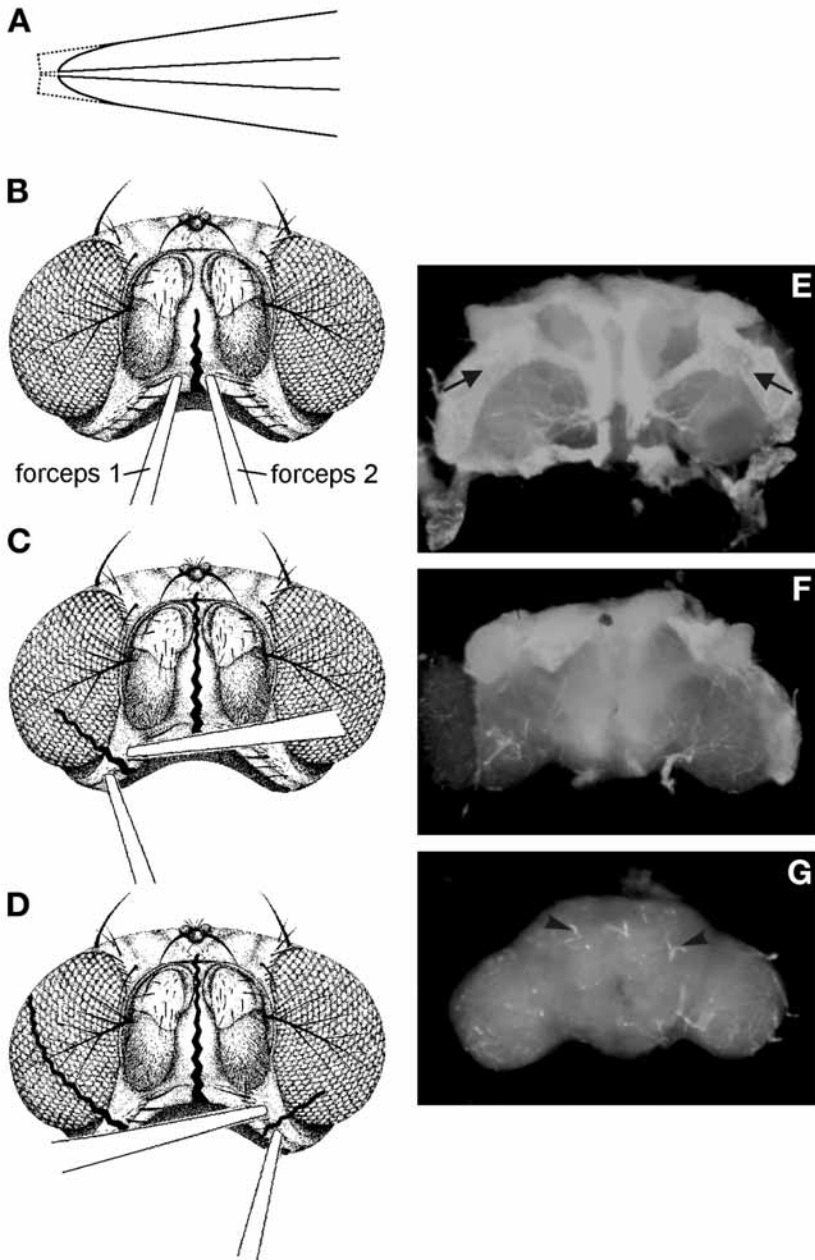


Fig. 1. Dissection of *Drosophila* brain. (A) Sharpening of a Dumont no. 5 forceps. (B–D) Opening the head capsule, main steps (*see Subheading 3.1.1.; steps 3 and 4 and Note 4*). (E) Isolated brain with air sacs (arrows), posterior view. (F) Brain with partly removed air sacs, anterior view. (G) Dissected brain with air sacs completely removed; only some tracheae are still present (arrowheads).

slowly. Usually, the larva breaks in the middle and internal organs spill out, whereby the central nervous system (CNS) remains attached to the mouth hooks.

2. Locate the CNS and remove other tissue (*see* **Note 6**).

3.1.3. Fixation of Whole Mounts

1. Using a glass Pasteur pipet (*see* **Note 7**) transfer the dissected brain together with a small volume of PBS to a second block dish filled with PFA (or other fixative; *see* **Note 8**). Keep dissecting for up to 20 min, pooling the brains in the second dish (5 to 20 brains, depending on your dissection speed).
2. Remove the diluted fixative and replace it with fresh fixative. Fix for another 40 min (*see* **Notes 9 and 10**).
3. Remove the fixative and wash the brains three times for 10 min in PBS and then 2 times for 10 min in PBT. Always remove the old buffer with a Pasteur pipet and immediately replace it with a fresh one; do not let the specimens dry in between (*see* **Note 11**).
4. Proceed with the immunostaining.

3.2. Cryostat Sections

Cryostat sections are the method of choice if the photoreceptor cells of the compound eyes, which strongly express the clock genes, are the focus of interest. The following sections describe the preparation of the flies for cryostat sections as well as the principal sectioning technique. Note that many cryostats are designed for cutting larger tissue from vertebrates. Therefore, the cryostat chuck is often too large for small insects. For *Drosophila* heads a cryostat chuck with a diameter of 5 to 7 mm is optimal, and it might be necessary to ask a workshop to make such a chuck.

3.2.1. Preparation and Fixation of Flies for Cryostat Sectioning

1. Remove head, proboscis, and air sacs of flies as described for the whole-mount preparation (*see* **Note 11**).
2. Fix the dissected heads in PFA for 3 h.
3. Wash three times for 10 min in PBS at room temperature.
4. Incubate the heads in 25% sucrose (in PBS) overnight at 4°C.
5. Put a drop of Tissue Tec on a slide and submerge one head in it; it is important that the whole head is bathed and that no air bubbles stick to the head.
6. Put a large drop of Tissue Tec on the cryostat chuck (no air bubbles). Using a long pair of forceps submerge the base of the cryostat chuck into liquid nitrogen. Only the base of the drop should freeze (white color); the top must stay fluid (**Fig. 2A**).
7. Remove the cryostat chuck from the nitrogen and place one head into the fluid Tissue Tec; orient it in the way shown in **Fig. 2A**.
8. Slowly submerge the cryostat chuck into nitrogen until the Tissue Tec drop is completely frozen.

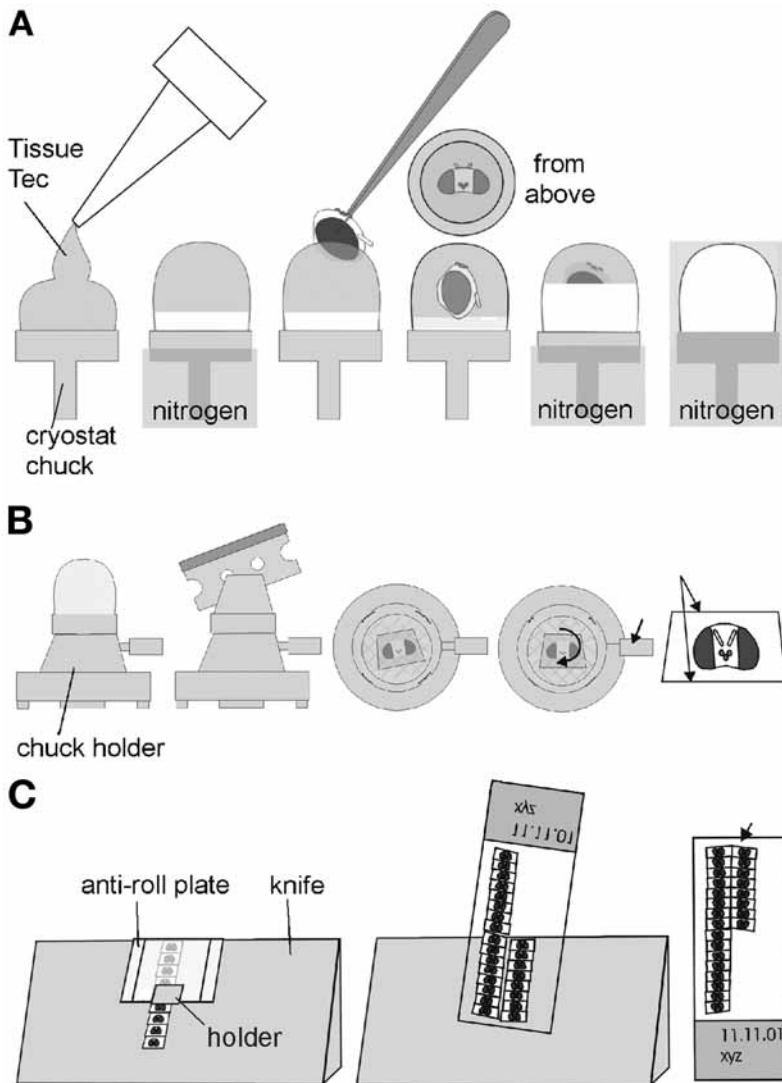


Fig. 2. Cryostat sectioning of *Drosophila* heads (horizontal sections). (A) Heads are embedded and oriented in Tissue Tec. (B) Trimming of the frozen block. (C) Sectioning and transferring of the sections to a subbed slide.

9. Put the cryostat chuck into the cryostat (-20°C), but wait some minutes before cutting as the block with the sample might be still too cold. Also put some subbed slides into the cryostat.

3.2.2. Sectioning With Cryostat

1. Cut the block with the microtome until the surface becomes flat. Trim the block with a razor blade to an oblong shape (**Fig. 2B**). It is important to make sure that the bottom edge and the vertical side of the block are, respectively, parallel and perpendicular to the knife edge.
2. Adjust the antiroll plate on the knife and cut 6- to 10- μ m sections; they should form a ribbon.
3. Remove the antiroll plate and pick up the ribbon with a cold slide (**Fig. 2C**).
4. Thaw the ribbon by touching the slide from below with a finger, and then keep the slide at -20°C for an additional 15 min.
5. Remove the slide from the cryostat and allow it to air-dry for 30 min (*see Note 12*).
6. Circle the sections with a wax pencil and arrange the slide(s) horizontally on two parallel glass bars in a humid chamber (*see Note 13*).
7. Proceed with the immunostaining.

3.3. Immunostaining

The immunostaining procedure for whole mounts and cryosections is essentially the same, with the difference that the incubation times are reduced for cryosections. The protocol below refers to whole mounts. For cryosections, blocking solution and secondary antibodies should be applied for 2 h, whereas the primary antibody should be incubated overnight. Washes can be reduced to 5 min for each step.

3.3.1. Blocking and Incubation With Primary Antiserum

1. Block the specimens for at least 3 h in blocking solution (*see Note 14*) at room temperature.
2. Dilute the primary antiserum in PBT with 2% NGS and 10 mM sodium azide (*see Notes 15 and 16*).
3. Remove the blocking solution and immediately (i.e., without washing) apply the antibody dilution.
4. Incubate at least for 24 h, either at 4°C or at room temperature depending on the fragility of the antigen and the antiserum (*see Note 17*).

3.3.2. Detection

The two main detection methods are immunoenzyme labeling and immunofluorescence labeling. Immunoenzyme labeling is predominantly based on HRP-labeled secondary antibodies, provides permanent preparations, and requires only a conventional light microscope. Most importantly, the bright field illumination allows the labeling to be correlated with the tissue morphology. Immunofluorescence gives a higher resolution and is the method of choice for double-labeling experiments, as it permits the simultaneous and independent detection of different antigens. However, the preparations tend to bleach under

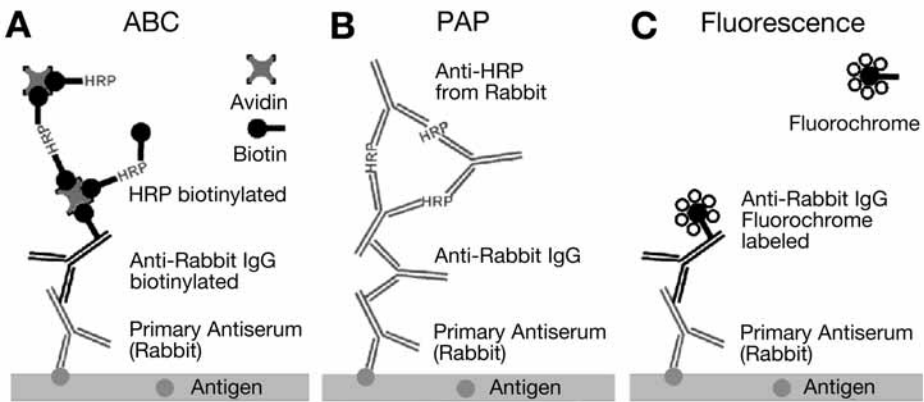


Fig. 3. Detection of a primary antiserum (from rabbit) bound to a relevant antigen by three different methods. (A) Avidin-biotin complex method. (B) Peroxidase-antiperoxidase method. (C) Fluorescence method.

observation and are not permanent. Furthermore, the resolution of internal brain structures is affected by out-of-focus fluorescence, which lowers the quality of the image, especially for whole mounts. Confocal microscopy solves this problem and produces very high-resolution images, but scanning is time-consuming and inappropriate to screen large numbers of labeled preparations. In many situations confocal immunofluorescence is most valuable for looking at details and subcellular localization, whereas immunoenzyme labeling is most suitable to get an overview.

I will describe only one method of HRP labeling—the PAP—which I find superior to the widely used avidin-biotin complex method (ABC kit, Vectastain, Vector Laboratories, Burlingame, CA; *see also* [ref. 13](#)). Both methods are schematically shown in [Fig. 3A,B](#).

The quality of immunofluorescence labeling ([Fig. 3C](#)) depends generally on the brightness and photostability of the fluorochromes. The most commonly used fluorochromes are fluorescein isothiocyanate, rhodamine isothiocyanate, and Texas Red. However, in the past few years new fluorochromes have been developed that are brighter and more photostable—for example, the Alexa series (Molecular Probes), with absorptions ranging from 350 nm to 680 nm.

3.3.2.1. HRP IMMUNOLABELING WITH THE PAP METHOD

1. Remove the primary antiserum (*see* [Note 18](#)) and wash three times for 10 min with 1 mL of PBT at room temperature.
2. Dilute the GAR serum 1:40 in PBST, 2% NGS (*see* [Note 19](#)).

3. Incubate the secondary antiserum either for 6 h at room temperature or overnight at 4°C.
4. Discard the secondary antiserum and wash three times for 10 min with 1 mL of PBT at room temperature.
5. Dilute the PAP complex developed in rabbit, 1:50 in PBT, 2% NGS (*see Note 20*).
6. Incubate for 48 h at 4°C.
7. Discard the PAP solution and wash three times for 10 min with 1 mL of PBT at room temperature.
8. Dissolve 1 tablet of DAB in 5 mL of distilled H₂O and add 2.5 µL of H₂O₂.
9. Immediately incubate the samples with the DAB solution. At intervals, observe the samples under a microscope to monitor the progress of the reaction. The tissue should turn slightly brown and the reaction completed after 5 to 20 min (*see Note 21*).
10. Stop the reaction by rinsing in distilled H₂O for 10 min.
11. Wash three times for 10 min with PB at room temperature.
12. Dehydrate through an ethanol series: 10 min in 30, 50, 70, 90, 95, twice in 100% ethanol.
13. Clear in methylbenzoate. For section: 2X 10 min; for wholemounts: add an overnight.
14. Embed in Entellan (Merck).

3.3.2.2. FLUORESCENT IMMUNOLABELING

1. Remove the primary antiserum (*see Note 18*) and wash three times for 10 min with 1 mL of PBT at room temperature.
2. Dilute the secondary antiserum directed against the primary antibody (e.g., Alexa Fluor 488 GAR) in PBST, 2% NGS (*see Notes 22 and 23*).
3. Incubate the secondary antiserum at room temperature for 4 h in the dark.
4. Discard the secondary antiserum and wash three times for 10 min with 1 mL of PBT at room temperature.
5. Mount in Vectashield embedding medium or 50% antifade glycerol. Seal the cover slip with nail polish.

3.3.2.3. MULTIPLE FLUORESCENT IMMUNOLABELING

It is often useful to see the expression of more than one protein or peptide in a single preparation (e.g., PER, TIM, and PDF). This can be achieved by using different fluorochromes. The simplest strategy uses primary antibodies raised in different species followed by their separate detection with species-specific secondary antibodies. For instance, anti-PER from rabbit ([7](#)), anti-TIM from rat ([8](#)), and anti-PDF-precursor from guinea pig ([14](#)) can be detected by Alexa Fluor 568 GAR, Alexa Fluor 488 Goat Anti-Rat IgG, and Alexa Fluor 694 Goat Anti-Guinea pig IgG.

However, in many cases the available primary antibodies are raised in the same species (e.g. anti-PDH [11] is raised in rabbit, as is anti-PER [7]; anti-PDF [12] is raised in rat as is anti-TIM; **ref. 8**). One solution for this problem is the visualization of one component with the help of the reporter green fluorescent protein (GFP). GFP absorbs light close to 488 nm and emits in the green, as does fluorescein isothiocyanate or ALEXA 488. GAL4 driver strains that could drive GFP in the relevant cells are available for PDF (12), PER, and TIM (15), although the latter two show expression in additional cells that express no native PER or TIM.

Other possibilities are the direct coupling of one primary antibody to one fluorochrome or its biotinylation, which allows detection with fluorescent-linked ABC (Vector Laboratories) reagents (16). This procedure requires an excellent primary antibody, however, which is available in high amounts and is therefore not perfectly suited for anti-PER or anti-TIM.

4. Notes

1. For 500 mL of fixative: dissolve 20 g paraformaldehyde in 350 mL of H₂O, heating to 60°C (~20 min); add drops of 1 N NaOH until the solution clears up. Dissolve 2 g NaH₂PO₄·H₂O and 4.075 g Na₂HPO₄·2H₂O in 100 mL H₂O. Mix both solutions, adjust the pH to 7.4 with either H₃PO₄ or NaOH, and bring to 500 mL with H₂O. Store in aliquots at -20°C and thaw shortly before use; do not use more than 24 h after thawing.
2. Store undiluted aliquots of antibodies at -20°C. Repeated freezing and thawing can damage them and should be avoided. For frequent use dilute the antibodies (e.g., 1:100) in PB with 10 mM sodium azide and store them at 4°C up to 1 yr.
3. For 10 mL of mounting medium: dissolve 24 mg *n*-propyl gallate in 5 mL PBS, add 5 mL glycerol, and adjust the pH to 8.0 to 8.6 with 1 N NaOH.
4. It is important to open the removed head from below (ventral), because here you can grip the head capsule without risk of damaging the brain, which is located in the dorsal part of the head. Sometimes it is possible to remove the large air sacs through the foramen of the mouth parts. If so, you may see the brain from below lying rather dorsal in the head capsule.
5. During dissection prior to fixation, speed is more important than perfection. Transfer the brain to the fixative as soon as possible. Small tracheae and fat bodies can be removed after fixation. It is important, however, to remove air sacs and large tracheae prior to fixation, because the air inside them makes the brain float on the surface and may prevent proper fixation. Gas inside the air sacs may also cause problems during dissection, because it causes the specimen to float as soon as it is released. Thus, it is necessary to hold the specimen all the time with at least one pair of forceps. The addition of 0.1% Triton X-100 to PBS can solve this problem. Most antigens do allow the use of Triton X-100 at this stage. However, be careful with fragile tissue such as the nervous system of first instar larva.

This tissue will be destroyed by detergents such as Triton X-100. Also never grab the brain itself with the forceps; grasp only things that you want to remove. Videos about brain dissections are available on the home page of Kei Ito (videos from Kei Ito at: <http://jfly.iam.u-tokyo.ac.jp/html/movie/index.html>).

6. At the beginning it might be hard to find the CNS among the other tissues, but once you know how it looks, it is much simpler to isolate the larval CNS than to dissect the adult brain.
7. A common problem with this procedure is that sometimes the brains stick to the glass inside the pipet. This can be avoided by spitting into the pipet before use; the saliva will cover the electrostatic charges of the glass.
8. PFA is the most universal fixative; it works with the great majority of antisera and should be tried first for any new antiserum. Most peptide antisera (e.g., anti-PDH), but also anti-serotonin, give better staining results when the specimens are fixed with Zamboni's fixative: 4% paraformaldehyde, 7.5% saturated picric acid in 0.1 M PB (prepare like PFA, but add 37.5 mL saturated picric acid before filling up with H₂O and adjusting the pH; the fixative can be stored at 4°C for years). However, Zamboni's fixative does not work for anti-TIM and anti-PER. Tris-buffered saline—0.3 M NaCl, 0.1 M Tris-HCl, pH 7.4—can be used as an alternative to PBS. In a direct comparison, I have found that Tris-buffered saline gave a little less background in anti-TIM and anti-PER labelings.
9. Fixation is a delicate balancing act. On the one hand it is necessary to preserve tissue morphology and allow permeabilization of cells but on the other hand, fixation can destroy antigenic determinants. The extent of fixation can be varied easily by changing the fixation time and temperature; this may have a dramatic effect on the antibody labeling. Many people recommend fixation at 4°C (then fixation time must be prolonged a little bit). I found that fixation at room temperature for 1 h works perfectly at least for anti-PER and anti-TIM antisera, but whenever difficulties in immunolabeling occur, varying fixation time and temperature can help.
10. Fixation and all following steps can be performed in the same block dish, which should be covered by a small plate of dark glass or PVC and placed in a humid chamber during longer incubation times. The necessary volume of fluid for all incubations with antisera is 200 μ L per block dish. Alternatively, the brains can be transferred to Eppendorf tubes after fixation and all steps can be performed there. However, I find the block dishes better for thoroughly rinsing the brains and for removing fluid without taking the risk of damaging the brains.
11. In chronobiology, frequently many brains must be fixed and dissected at exactly the same time of day, which is often at night (when the amount of PER- and TIM protein is maximal). In these cases an alternative dissection–fixation protocol must be followed. The flies must be fixed prior to dissection. For this purpose the flies are submerged as a whole in the fixative. To obtain a quick fixation, 0.1% Triton X-100 must be added to the fixative. Furthermore, fixation time must be prolonged to 2 to 3 h. The flies are then washed in PBT (3 $\times$ 15 minutes) and the procedures for either whole-mount preparations or cryostat sections are followed.

Note that fixed brains are more brittle than fresh ones. Therefore, the risk of fracturing the brain during whole mount dissection is higher. On the other hand it is easier to remove the tracheae of fixed brains than of fresh ones.

12. Several problems can arise during sectioning. The main ones and their possible cause are listed below:
 - a. Curling up of sections: the humidity is too high.
 - b. Wrinkling of sections: the temperature is too high.
 - c. Wrinkling of the ribbon: the knife is dirty; clean it with a rough brush.
 - d. Ribbon is not forming: the temperature is too low
 - e. Sections jump from knife: electrostatic load in the cryostat; the air is too dry.
 - f. Sections stick to the knife: the slide is too warm.

Points **a** through **c** may also be caused by the antiroll plate if not properly adjusted. Furthermore, there are unknown reasons that make sectioning difficult if not impossible on certain days. It is better not to spoil all preparations during such a day, but to spare them for the next day.

13. The exact horizontal arrangement of the slides in the humid chamber is very important. The immunoreaction is carried out with the minimum volume of antibody solution, which, as for whole mounts, corresponds to 200 μ L per slide. An uneven arrangement may result in part of the sample not to be covered or, in the worst case, in the antibody solution dripping off the slides. During washes, the slides are placed vertically in glass staining chambers filled with buffer. At every wash the slides are transferred to a new chamber with fresh buffer with the help of coarse forceps.
14. The serum used for the blocking solution should come from the same animal as the secondary antiserum. I usually use secondary antisera made in goat. If you use secondary antisera from donkey, you should use donkey normal serum for blocking.
15. A volume of 200 μ L is required per block dish and 50 to 100 μ L per Eppendorf tube. For the dilution of the primary antiserum, please see relevant publications or notes of the provider. In many cases a 1:1000 dilution is a good starting point; anti-PER (7) and anti-TIM (8) work perfectly at this dilution.
16. Nonaffinity purified PER antiserum (7) should be preabsorbed on dechorionated *per*⁰ embryos prior to use in order to reduce unspecific background staining. Dilute 4 μ L of anti-PER in 196 μ L of PBST, 5% NGS, 10 mM sodium azide. Add to 100 μ L of dechorionated *per*⁰ embryos and incubate overnight. When setting up the final dilution remember that the preabsorbed antibody has already been diluted 1:50.
17. Many protocols recommend performing all antibody labeling at 4°C. This is useful for instable antigens or antibodies and must be tested in individual cases. Generally, antibody labeling is slower but more specific at 4°C. Nevertheless, antibody binding is superior at higher temperatures (body temperature in nature). Therefore, I recommend transferring all specimens that were labeled at 4°C to 25°C at least 1 h prior to washing.

18. Recover used antibodies and keep them at 4°C. Used antibodies work better and can be reused for up to five staining processes.
19. The 1:40 dilution applies to the GAR antiserum from Sigma. Antisera from other manufacturers may require a different dilution. Note that if your primary antiserum is from mouse, guinea pig, or rat you must incubate in goat anti-mouse IgG, goat anti-guinea pig IgG, or goat anti-rat IgG sera, respectively.
20. The 1:50 dilution applies to the rabbit PAP system from Sigma. Rabbit PAP from other manufacturers may require a different dilution. Note that if your primary antiserum is from mouse, guinea pig, or rat you must incubate in PAP developed in mouse, guinea pig, or rat, respectively.
21. DAB is a carcinogen; it must be handled with gloves and manipulated with care. Solutions containing DAB must be treated with bleach (under a hood) for several hours to inactivate the chemical before disposal.
22. Dilute Alexa 488-GAR from Molecular Probes 1:300. Antisera from other manufacturers may require a different dilution. Note that if your primary antiserum is from mouse, guinea pig, or rat you must incubate in fluorescent-conjugated goat anti-mouse IgG, goat anti-guinea pig IgG, or goat anti-rat IgG sera, respectively.
23. In fluorescent labeled specimens, the red eye color of the flies can be disturbing, because the screening pigment has a strong autofluorescence. Solutions:
 - a. Use white-eyed flies if possible.
 - b. Remove the eyes as far as possible.
 - c. Wash the brains extensively in PBT (e.g., prolong the washing steps and keep the brains for 2 more days in PBT after the labeling is finished, replacing the PBT several times).
 - d. Use secondary antibodies with excitation in the red and emission in the infra-red (autofluorescence is the strongest under blue-green excitation).

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Immunocytochemistry on Suprachiasmatic Nucleus Slices

Marta Muñoz Llamosas

Summary

Immunocytochemistry (ICC) is a sensitive and powerful method that is used to localize and identify cells containing a particular antigen. This chapter is dedicated to ICC of suprachiasmatic nucleus (SCN) slices. After a brief introduction to the technique, the materials and methods sections describe two different methods to obtain SCN slices—the first one for fixed tissue, the second one for fresh frozen tissue—followed by the description of two methods of antibody detection: the indirect method and the avidin-biotin complex one. In addition, some remedies to the most common problems encountered while performing ICC and some alternative protocols are discussed.

Key Words: Immunocytochemistry; suprachiasmatic nucleus; brain tissue sections indirect method; avidin–biotin method.

1. Introduction

Immunocytochemistry (ICC) is a powerful technique that is used to localize and identify cells or subcellular structures containing a particular antigen. Owing to the wide range of specimen sources, antibody types, and methods of detection, there are many variations of the technique described in the literature; nonetheless, all ICC procedures include three different stages, as described in the three following sections.

1.1. Specimen Preparation

One of the first decisions to be taken is how to preserve the antigen of interest in a sample; this process is called fixation. If an antigen is not properly fixed it will be washed out of the specimen or degraded and no staining will result; on the other hand, overfixation can cause denaturation or modification of the antigen, making it unrecognizable for the antibody. Therefore a delicate balance must be reached to obtain good immunostaining results.

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A mild fixative that is widely used in ICC procedures is 4% buffered paraformaldehyde. It is a crosslinking fixative that forms bonds between reactive groups of adjacent proteins and is well tolerated by most tissues. The main problem regarding the use of 4% paraformaldehyde (or any other crosslinking fixative) is the formation of excess aldehyde linkages that can produce antigen masking. However, careful timing of the fixation process will prevent this problem.

Once the optimal fixation protocol is chosen, it is also important to consider the effects of any other processing used in the preparation of the sample, such as paraffin embedding, on the antigen of interest.

1.2. Antibody Staining

The usual procedure involves treatment of the sections with enzymes or heat if antigen retrieval is needed, blocking of the endogenous enzymatic activity if using an enzyme-conjugated antibody to visualize the primary antibody, incubation with normal (nonimmune) serum to avoid nonspecific staining, and incubation of the sample with diluted primary antibody in a humid chamber to prevent the sections becoming dry.

Nowadays the development of more sensitive methods of antibody detection in conjunction with the use of confocal microscopy allows applying this technique to a variety of other specimens, such as whole-mount embryos or organ cultures, thus increasing the number of applications of the technique. Although optimal conditions for antibody staining in this kind of specimens should be determined in each situation, the procedure employed will involve essentially the same steps as the one described above for staining of tissue sections.

1.3. Antibody Detection

There are several methods to detect antibodies bound to tissue; some will produce fast results, whereas others will detect the presence of rare antigens. Only the researchers taking in consideration the requirements of their work will be able to decide which one is the best for them.

The “indirect method” is the most commonly used method when fast results are needed. Briefly, sections of tissue are incubated with an unconjugated antibody that will bind to the antigen of interest. To localize this attachment a labeled secondary antibody is needed to bind to the first antibody. Secondary antibodies are generally labeled with fluorescent compounds or with an enzyme (such as horseradish peroxidase or alkaline phosphatase) that must react with a substrate and chromogen to produce a visible deposit. The main advantage of the indirect technique is its speed; however, this method might not be able to detect antigens present in low concentration in the sample.

In order to increase the sensitivity of the indirect method, several alternative methods have been developed, such as the avidin–biotin complex (ABC) method (1) and the peroxidase–antiperoxidase method (2).

In the ABC method the secondary antibody that has been conjugated to biotin is bound by a complex of avidin mixed with biotin that has been labeled with an appropriate enzyme, such as horseradish peroxidase or alkaline phosphatase, or with a fluorescent compound. This method is more sensitive than the indirect one because the final amount of label bound to the antigen is higher. However, the increased amount of time needed to complete the procedure and the increased cost of the reagents are two disadvantages that must be considered before choosing this method of detection.

This chapter is dedicated to ICC of SCN slices; however, the techniques described can be widely applied to a variety of other specimens. The first part of the chapter describes two different methods to prepare SCN slices. The first protocol, for paraformaldehyde-fixed tissue, will provide sections with excellent morphological detail and resolution. The second protocol, for frozen sections, allows excellent antigen preservation and offers the possibility of fixative optimization for different antigens in the same tissue sample. Next we will discuss two different ways of applying antibodies: the traditional one for tissue sections on slides and an alternative one for free-floating sections. Finally, two different methods of antibody detection are explained: the indirect method and the ABC method.

2. Materials

1. 0.01 M Phosphate-buffered saline (PBS), pH 7.2–7.4: Dissolve 9 g NaCl, 0.43 g KH_2PO_4 , and 1.48 g Na_2HPO_4 in 900 mL distilled water. Check pH and make up to 1 L.
2. Heparin–PBS (5 U/mL).
3. Formaldehyde fixative: add 4% (w/v) paraformaldehyde (*see Note 1*) to PBS and heat to 60°C, constantly stirring. Add 1 drop of 1 M NaOH for every 25 mL of fixative solution; the “milky” color of the fixative solution should clear totally in a couple of minutes. Cool to room temperature and filter-sterilize. Check the final pH.
4. Gelatin embedding medium: Mix 16 g of gelatin, 15 mL of glycerol, and 70 mL of distilled H_2O . Heat solution until totally dissolved. Keep at 37°C until use.
5. Liquid nitrogen.
6. Isopentane.
7. Optimum cutting temperature (OCT) medium.
8. 0.05 M Tris-buffered normal saline (TBS), pH 7.6: Dissolve 9 g NaCl and 6.01 g Tris(hydroxymethyl)methylamine in 900 mL of distilled water. Add concentrated HCl until pH reaches 7.6. Make up to 1 L.
9. H_2O_2 : 30% (v/v) solution.
10. Methanol.

11. Triton X-100.
12. DAB solution: Add 50 mg of diaminobenzidine (DAB) to 100 mL of TBS; stir until totally dissolved. Just before use add 100 μ L of 30% H_2O_2 to the DAB solution (final concentration 0.03%).
13. 0.1 M Acetate buffer, pH 5.2: Mix 210 mL of 0.1 N acetic acid and 790 mL of 0.1 M sodium acetate.
14. Antifade mountant: 3% (w/v) propyl gallate, 80% glycerol in PBS.
15. 0.5% acid alcohol: 0.5% HCl in 70% EtOH.
16. Harris' hematoxylin: commercially available from several manufacturers.
17. Xylene (*see Note 1*).
18. Synthetic mountant.
19. Gelatin solution for coating slides: Heat 350 mL of dH_2O at 50°C, add 1.5 g of gelatin and 1.5 g of potassium dichromate (*see Note 1*), and stir until totally dissolved. Let the mixture cool, filter-sterilize, and store at 4°C.
20. Poly-L-lysine.

3. Methods

3.1. Tissue Preparation and Sectioning

3.1.1. Paraformaldehyde Fixation

The SCN of the hypothalamus is a bilateral structure localized under both sides of the third ventricle, above the optic chiasm. Because of its position inside the brain, the best way for fixing this tissue is by transcardial perfusion. The following fixation protocol employs 4% buffered paraformaldehyde; it has been performed successfully with several commercially available antibodies. Alternative fixative solutions are described in **Heading 4** (*see Note 2*).

1. Open skin of thorax and abdomen of anesthetized animals, open the diaphragm, and lift the thoracic cage by cutting through the ribs carefully. It is important not to section any main blood vessel during this procedure to avoid loss of fixative through it. Make an incision in the right atrium of the heart to allow the blood to exit and insert the perfusion cannula into the left ventricle. Perfuse 30 mL of PBS-heparin, followed by 75 mL of 4% paraformaldehyde at pH 7.4. This procedure must be done with a peristaltic pump, taking care not to apply excessive pressure.
2. To dissect out the brain, cut and remove the surrounding cranial bones. Lift the brain carefully from the base of the cranium, and cut the optic nerves to detach the brain without pulling down the optic chiasm and SCN. After dissecting the brain, complete the fixation by immersion in the same fixative overnight at 4°C.
3. Wash tissue in PBS for 24 h at 4°C to eliminate excess fixative.

3.1.1.1. GELATIN EMBEDDING AND SECTIONING

1. Place the block of fixed tissue in PBS at 37°C for 30 min.
2. Leave tissue overnight in molds containing gelatin embedding medium at 37°C.

3. Take molds out of the oven and orient the brains for subsequent sectioning.
4. Cool the mixture at 4°C until totally solidified.
5. Cut 40- μ m-thick sections on a vibratome.
6. Place sections in PBS until further processing. Gelatin embedding medium will dissolve and wash out in PBS.

3.1.2. Isopentane Freezing

Although cryostat sections of paraformaldehyde-fixed tissue are used most widely in ICC protocols, greater signal intensities can be obtained using cryostat sections that are freshly cut from unfixed frozen tissue. The protocol described below has been used to freeze unfixed fresh brain tissue without suffering some of the usual problems associated with the use of other freezing protocols, such as poor morphological preservation.

1. Pour 500 mL of liquid nitrogen into an ice bucket (*see Note 3*).
2. Pour 30 mL of isopentane into a 50-mL beaker; hold the beaker in the liquid nitrogen until the bottom of the isopentane starts freezing.
3. Place one drop of OCT embedding medium on a cryostat chuck at room temperature and place the piece of brain tissue containing the SCN on top of the OCT. If coronal sections are intended, make sure that the brain is oriented with the nasal end facing up and the caudal end positioned toward the base.
4. Use forceps to immerse the chuck in the isopentane for 5 to 10 s; liquid isopentane should cover the tissue.
5. Immediately transfer the tissue to a cryostat chamber for sectioning or store at -80°C until use.

3.1.2.1. CUTTING FROZEN SECTIONS

One of the biggest problems encountered when cutting fresh frozen brain tissue in a cryostat is to establish the optimal temperature at which to cut the specimen. If the temperature of the chamber is too cold, sections will become brittle; if the temperature is too hot, sections will stick to the blade. I have found that the best temperature to cut fresh frozen brain tissue is between -20 and -22°C.

1. Cut sections 10 μ m thick on a cryostat and mount on gelatin-coated slides. Coating slides with gelatin will prevent sections from becoming loose and falling off owing to the frequent rinsing steps involved in any ICC procedure.
2. Air dry sections for 1 h at room temperature. If slides are to be stored, seal a batch of slides in an airtight box and place in a deep freezer. Before use, bring the box to room temperature to prevent condensation on the slides.

3.2. Antibody Staining

Tissue sections are usually mounted on glass slides to facilitate their handling during all the steps involved in ICC protocols. However, sometimes it

might be advisable to perform ICC on free-floating sections, such as thick vibratome sections, in order to make sure that all of the tissue section is exposed to the ICC reagents. Described below are both the general method for applying antibodies to tissue sections on slides and an alternative protocol for free-floating sections.

3.2.1. Frozen Sections on Slides

1. Bring slides to room temperature and fix for 10 min in freshly prepared 4% buffered paraformaldehyde (*see Note 4*).
2. Wash slides in PBS for 5 min.
3. Block endogenous peroxidase activity by immersing slides in 0.3% H₂O₂, 70% methanol in PBS, for 30 min (*see Note 5*).
4. Wash slides in PBS for 5 min.
5. Take slides from buffer, shake off excess buffer, and dry around the section area.
6. Place the slides on a rack in a humid box and cover each preparation with the appropriate blocking solution (*see Note 6*).
7. Incubate for 30 min at room temperature.
8. Drain off the normal serum, dry around the section area, and apply the primary antibody, making sure that the whole tissue section is covered (*see Note 7*).
9. Incubate at 4°C overnight (*see Note 8*).

3.2.2. Free-Floating Sections

Free-floating sections are easily handled with a paintbrush. It is also a good idea to perform all washing and incubation steps in multiwell plates in order to reduce the amount of reagents needed.

1. Immerse sections in PBS twice for 5 min each time.
2. Place sections in PBS containing 0.3% Triton X-100 for 30 min.
3. Quench endogenous peroxidase activity by incubation sections with 0.3% H₂O₂ in PBS, pH 7.4, for 30 min (*see Note 5*).
4. Rinse sections in PBS twice for 5 min each.
5. Place sections in 2% blocking solution overnight at 4°C (*see Note 6*).
6. Incubate sections in the presence of the primary antibody diluted in PBS containing 1% blocking solution for 72 h at 4°C (*see Notes 7 and 8*).

3.3. Antibody Detection

Once the tissue sections have been incubated with the primary antibody, several methods of detection can be applied. The indirect method described below provides a fast and simple way to localize the site of the attachment of the primary antibody. The secondary antibody employed in the procedure is labeled with a fluorescent compound; however, it is also possible to use a secondary antibody labeled with an appropriate enzyme, such as peroxidase or

alkaline phosphatase. If the antigen of interest is present in low quantities, a good alternative to the indirect method is the ABC method. In the protocol described below the secondary antibody is recognized by an ABC that has been labeled with peroxidase; however, it is also possible to use biotin labeled with another enzyme, such as alkaline phosphatase, or with a fluorescent compound.

3.3.1. Indirect Detection

1. Rinse slides in PBS three times for 5 min each.
2. Dry around the section area and apply the appropriate dilution in PBS of the fluorescence conjugated secondary antibody (*see Note 9*).
3. Incubate slides for 1 h at room temperature in a humid chamber.
4. Rinse slides in PBS three times for 5 min each.
5. Rinse slides in distilled water and mount in aqueous mounting medium (80% glycerol in PBS) containing antifade agents.

Steps 2 through **5** should be carried out in dimmed light to avoid photobleaching of the fluorochrome.

3.3.2. Avidin-Biotin-Peroxidase Method

1. Rinse slides in PBS three times for 5 min each.
2. Place slides in the appropriate biotinylated secondary antibody (*see Note 9*); incubate for 1 h at room temperature.
3. Rinse in PBS three times for 5 min each.
4. Place slides in the avidin-biotin mix and incubate for 45 min at room temperature.
5. Rinse in PBS three times for 5 min each.
6. Immerse slides for 10 min in the DAB solution (*see Notes 10 and 11*).
7. Transfer slides to buffer and examine under the microscope. The end product of the reaction is dark brown. The incubation time can be adjusted by returning the slides to the DAB solution until reaching optimal staining.
8. Rinse slides in distilled water (*see Note 12*).
9. Dehydrate slides through graded alcohols, clear in xylene, and mount in synthetic mountant.

3.4. General Protocols

It is a regular practice to counterstain tissue sections to reveal all the nuclei. When using a fluorescent label, counterstaining can be done with propidium iodide (red dye) or DAPI (blue dye), depending on the color of the fluorochrome used for immunostaining. If using an enzymatic label, the color of the chromogen will determine which substance can be used to counterstain the sections. If the product of the immunoreaction is red, black, or brown, slides can be counterstained with hematoxylin.

3.4.1. Counterstaining With Propidium Iodide

1. Immerse slides for a few seconds in 1.5 μM propidium iodide (*see Note 1*) in PBS.
2. Wash slides several times in PBS.
3. Mount slides with antifading agent.

3.4.2. Counterstaining With DAPI

1. Add 0.5 $\mu\text{g/mL}$ of DAPI (*see Note 1*) to the secondary antibody mix and proceed with the usual protocol of immunostaining.

3.4.3. Counterstaining With Harris' Hematoxylin

1. Rinse slides with water.
2. Incubate slides in Harris' hematoxylin for 5 min.
3. Wash slides in tap water for 2 min. This step must be performed with tap water owing to its alkaline pH.
4. Place slides in 0.5% acid alcohol for 10 s.
5. Wash slides in tap water until hematoxylin turns blue. This step must be performed with tap water owing to its alkaline pH.
6. Dehydrate in graded alcohols, clear in xylene, and mount in synthetic mountant.

Because of the numerous steps involved in any ICC procedure, sections may become loose and fall off. Coating slides with gelatin or poly-L-lysine will improve the adhesion of tissue sections to glass slides.

3.4.4. Coating Slides With Gelatin

1. Place slides in 1 M HCl for 30 min, rinse in distilled H_2O twice, leave in 96% ethanol overnight, and air-dry in a dust-free environment.
2. Immerse slides in gelatin solution for 2 min.
3. Dry slides at 37°C.
4. Repeat **steps 2 and 3** at least three times.

3.4.5. Coating Slides With Poly-L-Lysine

1. Place slides in 1 M HCl for 30 min, rinse in distilled H_2O twice, leave in 96% ethanol overnight, and air-dry in a dust-free environment.
2. Dissolve poly-L-lysine in water to make 0.1% (w/v) solution.
3. Immerse slides in poly-L-lysine solution for a few seconds.
4. Dry slides at room temperature and store.

3.5. Controls

Because of the possibility of nonspecific reactions caused by tissue/antibody factors, several controls should be included in all ICC procedures in order to ensure the accuracy of the results.

3.5.1. Control for Nonspecific Staining by Primary Antibody

When using a polyclonal antibody, an aliquot of serum can be absorbed with the antigen. Incubation with the preabsorbed serum, instead of the primary antibody, should reveal no staining.

3.5.2. Controls for Nonspecific Binding of Second and Third Reagents

Second and third reagents can produce unwanted binding, giving false-positive results. In order to rule out this possibility, each run should include a section where the primary antibody has been substituted by antibody diluents alone. If using the ABC method, a section should be incubated with the avidin-biotin reagent alone.

3.5.3. Positive Control

The lack of staining on the experimental sections might be produced by a handling error in the procedure. A section of tissue known to contain the antigen and processed identically to the experimental sections will rule out this possibility.

4. Notes

1. This chemical is toxic; it should be weighed and/or handled in a fume hood while wearing gloves, laboratory coat, and safety goggles.
2. The fixation step is crucial for immunostaining. Sometimes it is necessary to try several different fixatives before finding the one that will retain the chosen antigen while producing the minimal epitope masking. Some fixative recipes are given below with some indication of their use:
 - a. Formal saline: 10% commercial formalin, 0.9% sodium chloride. It is a common alternative to 4% buffered paraformaldehyde.
 - b. Ethanol: 70% ethanol in PBS at 4°C for 10 min. It is a mild fixative that may be used on frozen sections.
 - c. Bouin's fixative: 70% saturated picric acid, 10% commercial formalin, 5% acetic acid. This fluid is excellent for preserving the antigenicity of small peptides.
3. Liquid nitrogen temperature is -185°C; wear gloves and a face protector when working with it.
4. This protocol is also suitable for previously fixed tissue. If using paraffin-embedded sections, remove paraffin by immersing the sections twice in pure xylene for 5 min each time. Rehydrate the sections by immersing them in graded ethanols (100, 96, 80, and 70%) for 5 min each time and finally place them in distilled water. Proceed to **step 2**.
If using cryostat sections of fixed tissue, air-dry slides for 1 h at room temperature and proceed to **step 2**.

5. If the method of detection chosen to localize the primary antibody makes use of an enzyme as a label (e.g., peroxidase) it is important to block any endogenous peroxidase activity in the sample to avoid any positive staining from it. There are several ways to inhibit endogenous peroxidase; the standard method for paraffin sections is to immerse the sections in a solution of 0.3% H_2O_2 in PBS for 30 min. However if poor cellular morphology is observed after this treatment or if using cryostat sections, it is advisable to immerse sections in 0.3% H_2O_2 , 70% methanol in PBS.
6. It is usual to employ normal serum from the same species in which the secondary antibody has been made (diluted before use 1:20 in PBS) as blocking solution. However, sometimes chicken albumen or casein (diluted 1 to 2 % in buffer) solutions might also be used.
7. In general, protocols for commercially available antibodies recommend a wide range of working concentrations. In order to establish the optimum concentration, a series of dilutions of the primary antibody should be tested on positive control tissue known to contain the antigen.
8. Polyclonal antisera contain a mixture of antibodies with different affinities for the antigen. The use of long incubation times will allow low-affinity antibodies to bind the antigen, enabling the use of higher dilutions of the primary antisera.

Temperature also affects the binding rate of antibodies; at higher temperatures (e.g., 37°C) reaction time will be shorter than at lower temperatures (e.g., 4°C). However, antibody incubations at higher temperatures do increase the rate of nonspecific binding.

9. Whenever a secondary antibody is used, it must be generated against the immunoglobulins of the primary antibody source—e.g., if the primary antibody is raised in rabbit, the secondary antibody could be goat (or sheep, etc.) anti-rabbit immunoglobulin. The optimal dilution of the secondary antibody must be determined empirically starting from the dilution range suggested by the manufacturer.
10. Sometimes it may be desirable to obtain a darker reaction product than the one provided by the DAB alone. The following protocol will produce a dark blue-black precipitate:
 - a. Place slides in acetate buffer.
 - b. Dissolve 2.5 g nickel sulfate in 100 mL acetate buffer.
 - c. Add 50 mg of DAB to the nickel solution; stir until totally dissolved.
 - d. Just before use add 100 μL of 30% H_2O_2 to the DAB solution (final concentration 0.03%).
 - e. Transfer slides to DAB/nickel buffer for 10 min and then examine under the microscope. The end product of the reaction is gray/black. The length of the incubation time must be determined empirically.
 - f. Rinse slides in distilled water.
 - g. Dehydrate slides through graded alcohols, clear in xylene, and mount in synthetic mountant.
11. Alternative protocols to intensify DAB staining after the enzymatic development has taken place can be easily found in the literature (3,4). However, these proto-

cols are more likely to produce enhanced background staining than the ones used during the peroxidase reaction.

12. If performing ICC on free-floating sections, mount sections on gelatin-coated slides and air-dry overnight.

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Immunofluorescence Analysis of Circadian Protein Dynamics in Cultured Mammalian Cells

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Summary

The timing of both entry and permanence of core-clock proteins in the nucleus is critical to maintain the correct pace of the clock mechanism. Several such proteins, namely CRYPTOCHROMES (CRY), PERIODs (PER), and BMAL1, were recently shown to contain nuclear transport signals that facilitate their “shuttling” between the nucleus and the cytoplasm. This type of dynamic intracellular movement not only regulates protein localization, but also often affects functions by determining interactive partners and protein turnover. Because most clock genes have been identified by genetic screening in *Drosophila* and by gene knockdown in mammals, it is important to develop cellular techniques to study the structure–function and regulation of the corresponding proteins. Here we present working protocols for immunofluorescence studies of clock proteins in mammalian cultured cells. This technique allows the visualization in the cell of one or multiple proteins at the same time.

Key Words: Immunofluorescence; antibodies; clock proteins; mammalian; culture cells.

1. Introduction

The fingerprint of a protein is determined by its amino acid sequence composition. A sequence of 10 to 15 amino acids is sufficient to create a specific antigen recognizable by the immune system, which will later develop antibodies identifying those 10 to 15 amino acids. It follows that every cellular protein has its unique pattern of antigenicity with multiple antigen sites (and potential antibodies) scattered along its tertiary structure. Immunofluorescence reveals the presence, within the cell, of protein antigens by using antibodies labeled with different chromophores. A major goal of the technique is to determine the subcellular distribution of a known protein in tissues or cells, as the presence

of a protein, either in the cytoplasm or in the nucleus, is the first step in understanding its function. This is particularly true for clock proteins, as the timing of nuclear and/or cytoplasmic accumulation is critical for maintaining the correct pace of the clock (1,2).

This chapter focuses on protocols for the immunofluorescence analysis of cultured mammalian cells. Manipulations involved in all staining reactions are the ultimate in simplicity. Drops of labeled or unlabeled antibodies are pipetted onto slides containing the material to be stained, and these are then incubated for varying periods of time. Despite this simplicity, controls are necessary before staining results can be evaluated, such as specificity of the primary antibodies for a certain antigen, or the leakage of unwanted fluorescence signals from the secondary antibodies through the filters of the microscope. The choice of fixative is another variable and it is dependent on the adequate preservation of the cell and antigen and accessibility of the antibody to the antigen.

2. Materials

1. Phosphate-buffered saline (PBS): 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 0.15 M NaCl, pH 7.4.
2. Collagen solution: Vitrogen 100 (Cohesion Technologies). Dilute 1:10 in 12 mM HCl to a final concentration of 0.3 mg/mL.
3. Fibronectin solution: human fibronectin (Sigma, cat. no. F-0895). Prepare to a final concentration of 50 mg/mL in PBS.
4. Poly-L-lysine (molecular weight [MW] ~70–90 kDa) solution: prepare to a final concentration of 40 µg/mL in PBS.
5. Methanol.
6. Paraformaldehyde solution (4%): Dissolve 4 g of paraformaldehyde in about 80 mL of deionized H₂O by stirring at 70°C in a fume cupboard. Add a few drops of 1 N NaOH to depolymerize the paraformaldehyde. Adjust the pH to about 7.0 and check with pH paper. Cool down to room temperature and bring up to 100 mL. Filter through an 0.45-µm Millipore. Divide into convenient aliquots and store frozen at –20°C. Discard after thawing and freezing two or three times.
7. Permeabilization solution: 0.1% Triton-X-100 in PBS diluted from a stock solution of 10% Triton-X-100 in PBS.
8. Blocking solution: 0.5% bovine serum albumin (BSA), 0.15% glycine in PBS.
9. Mowiol mount: in a 50-mL Falcon tube, mix 6 mL of glycerol and 2.4 g of Mowiol 4-88 (Calbiochem, cat. no. 475904) and 6 mL of deionized H₂O. Vortex and then shake for 2 h. Add 12 mL of 200 mM Tris-HCl, pH 8.5, and incubate at 50°C with occasional vortexing until the Mowiol dissolves (~3 h). Filter through a 0.45-µm syringe filter and store in aliquots at 4°C. Just before use add 2.5% (w/v) 1,4-diazabicyclo-[2.2.2]octane (DABCO; Sigma, cat. no. D-2522) and vortex for about 30 s to dissolve it. This antifade reagent will prevent photobleaching of fluorescent signals.

10. DAPI/DABCO mount: Dissolve 1 g DABCO in 90 mL glycerol (may need to heat to 60°C). Add 10 mL of 1 M Tris-HCl, pH 7.5, and adjust to pH 8.0 with 5 M HCl. Cool to room temperature and add sodium azide to 0.02% final concentration. Add 15 μ L DAPI (Sigma, cat. no. D-9542, 1 mg/mL stock in water). Mix with Vectashield (Vector Laboratories) in 1:1 ratio. Store at 4°C in the dark.

3. Methods

3.1. Seeding Cells on Cover Slips

The first step consists in seeding and growing the cells of choice on glass cover slips, which will ease the subsequent manipulations. Almost all types of cultured mammalian cells used to study clock proteins (2–5), such as fibroblasts, COS, NIH3T3, and HeLa, will attach to glass cover slips as well as plastic. During plating the cover slips will become coated with vitronectin from the serum in the media. Cover slips should be previously acid-washed to remove spots of dirt or detergent, and sterilized to avoid bacterial/fungal contamination of the culture. The cover slips used for immunofluorescence experiments have a diameter of approx 13 mm and fit well in a 24-well plate.

1. Place the cover slips in a glass beaker and cover with concentrated nitric acid. Alternatively, use a mixture of 50% methanol and 50% diethyl ether.
2. Swirl for 10 min.
3. Discard the acid and rinse the cover slips with copious amounts of distilled water.
4. Use tweezers to place the cover slips on aluminium foil and dry them in a dust-free area. Transfer the cover slips into a glass Petri dish.
5. Sterilize by baking for 4 h at 80°C.
6. Use sterile tweezers to put a cover slip in each well of a 24-well plate. Add sterile PBS and press down the cover slip with a pipet (*see Note 1*).
7. Remove the PBS and seed the cells.

HEK-293T-cells, typically used in overexpression studies for their high transfection efficiency, attach very poorly to glass. In this case it is advisable to coat the cover slips with collagen, fibronectin, or poly-L-lysine before plating.

1. Collagen: Clean and sterilize the cover slips as above. Place them in a 100-mm dish and cover with collagen solution. Leave for about 2 h and then wash twice in PBS and once with water. Remove the cover slips and place them in the wells of a tissue-culture plate. Leave to air-dry under a laminar flow hood for about 2 h before plating.
2. Fibronectin: Place sterile cover slips in the wells of a tissue-culture plate. Cover with fibronectin solution and leave overnight at 4°C. Wash twice with PBS before seeding the cells. Do not allow to dry out.
3. Poly-L-lysine: Incubate sterile cover slips in poly-L-lysine solution for 1 h at room temperature. Wash the cover slips extensively in distilled water. Keep dry and dust-free until use.

3.2. Fixation

Depending on the stability or accessibility of the antigen, various fixation protocols can be used. Methanol is an easy-to-use fixative, which is used mainly for cytoskeletal components, as it works really well for microtubules and can sometimes uncover epitopes left hidden by other methods. However, it frequently solubilizes and removes membrane-bound antigens, because it acts both as a fixative and as a permeabilizing agent, and provides only low structural preservation of samples.

1. Rinse the cover slips containing cells with PBS.
2. Fix by incubating the cells in precooled 100% methanol at -20°C for 10 min.
3. Wash with PBS several times before proceeding to the incubation with primary antibodies.

Paraformaldehyde fixation is the classical protocol for immunofluorescence on cultured cells, because it preserves the antigenicity of all clock proteins tested so far, and membrane-associated components are not lost during the fixation procedure. Moreover, paraformaldehyde optimally preserves the cellular structures.

1. Rinse cells with PBS at room temperature.
2. Fix in 3 to 4 % paraformaldehyde in PBS for 15 min at room temperature.
3. Wash the cover slips in PBS several times and proceed to the incubation with primary antibodies. Alternatively, it is possible to store the cells in sterile PBS at 4°C for several days before antibody treatment.

3.3. Indirect Immunofluorescence

After fixation a permeabilization step allows the antibodies to penetrate and reach their target antigen within the cell. It is very important to keep the cover slips covered with solution at every step; otherwise the cells will dry out and the experiment will fail.

1. From the previous section the cover slips are in the wells of a 24-well plate and are covered with PBS.
2. Replace the PBS with 500 μL of permeabilization solution for each well.
3. Incubate for 10 min.
4. Remove the permeabilization solution and wash three or four times for 5 min each with PBS to remove the Triton X-100 in excess. The cover slips are now ready to be processed with the primary antibody.

3.3.1. Primary Antibodies

It is important to ascertain that the antibodies used bind only to the designated targets (*see Note 2*). However, aspecific binding can occur as a consequence of weak electrostatic interactions with many different proteins or

Table 1
Filters and Spectral Information

Fluorophore	Absorption peak (nm)	Emission peak (nm)
Alexa Fluor 350 (blue)	347	442
Cyan fluorescent protein (CFP, blue)	433	475
Green fluorescent protein (GFP, green)	488	507
FITC (green)	492	520
Alexa Fluor 488 (green)	495	519
Yellow fluorescent protein (YFP)	513	527
Cy3 (red)	550	570
TRITC (red)	550	570
Alexa Fluor 594 (red)	591	618
Texas Red (red)	596	620
Cy5 (purple)	650	670

cellular structures. This can be prevented by blocking those sites with albumin before addition of the primary antibody.

1. Replace the last PBS wash with 500 μ L of blocking solution for each well and incubate for 5 min. Repeat this step to saturate all nonspecific binding sites.
2. Prepare in an Eppendorf tube a suitable dilution of the primary antibody in blocking solution (*see Note 3*).
3. Use a curled needle and tweezers to recover the cover slips from the wells and place them (cell side up) on a Parafilm strip inside a box. Place wet paper inside the box to create a humid chamber to avoid evaporation.
4. For each cover slip add immediately 100 μ L of the primary antibody solution on the cells; this should form a stable round drop on the cover slip (*see Note 4*).
5. Close the box and incubate for 1 h at room temperature.
6. Wash the cover slips four times for 5 min with blocking solution.

3.3.2. Fluorescent Conjugated Secondary Antibodies

The choice of secondary antibody is dictated by the host on which the primary antibody has been raised. For example, to detect a primary antibody against mammalian CRY1 raised in rabbit, you must use a secondary antibody recognizing rabbit immunoglobulin (anti-rabbit IgG) that has been prepared in a different host, such as donkey or goat. For detection, the secondary antibody is conjugated to a fluorescent dye, which adsorbs and emits light at specific wavelengths and allows one to visualize in the cell the presence of the antigen under a fluorescent microscope (*see Table 1*).

1. After washing off the primary antibody, incubate each cover slip with 100 μ L of a fluorescent secondary antibody of choice (*see Note 2*).

2. Incubate in the dark in a humid chamber for 45 min at room temperature.
3. Wash the cover slips four times for 5 min each in blocking solution.
4. Rinse two times in PBS.

3.3.3. Mounting

1. Briefly dip a cover slip in water to remove the salts present in PBS.
2. Blot the edge on a piece of tissue to remove excess water and quickly mount in 10 μ L of Mowiol mount on a microscope slide.
3. Leave the slide at room temperature in the dark for about 2 h until the Mowiol has set. Store at 4°C (*see Note 5*).
4. Alternatively, if it is necessary to label the nucleus, mount the cells in 3 μ L of DAPI/DABCO mount. DAPI stains blue the DNA present in the nucleus.

3.4. Double and Triple Staining by Immunofluorescence

It is generally accepted that many mammalian clock proteins interact with each other to form regulatory complexes that are active in nuclear import and transcription (6). Therefore, it is very informative to study the subcellular localizations and activities of two clock proteins interacting in the same cell. Indirect double immunofluorescence allows visualization of two primary antibodies with specific secondary antibodies conjugated to contrasting fluorochromes. This technique is carried out just as the single labeling. For example, for a colocalization study of CRY1 (tagged with the epitope of influenza HemaAgglutinin [HA]) and PER2 (tagged with the FLAG peptide DYKDDK) (2), primary antibodies raised in different animals can be mixed and incubated as a cocktail (for instance, rabbit anti-HA and mouse anti-FLAG). The same is valid for the secondary antibodies, which, for example, will be goat anti-rabbit Alexa 594 conjugated (red) and goat anti-mouse fluorescein isothiocyanate conjugated (green). CRY1 is now visible in red using an excitation filter with a band pass of 515 to 560 nm and an emission filter of 580 nm, while PER2 is visible in green using an excitation filter of 460 to 500 nm and an emission filter of 510 to 560 nm (*see Note 6*).

It is also possible to visualize three different proteins in the same cell—for example, CRY1 tagged with HA, PER2 tagged with enhanced green fluorescent protein (EGFP), and TIM tagged with FLAG. The procedure is carried out just as the double labeling. However, in this example, PER2 is now tagged with EGFP and it is visible in the green channel without the use of antibodies, as EGFP is already a fluorescent tag. The staining of TIM-FLAG is detected with a mouse anti-FLAG (primary) followed by a goat anti-mouse Alexa Fluor 350 conjugated (secondary), which can be seen in blue using an excitation filter with a band pass of 340 to 380 nm and an emission filter with long pass of 430 nm. In this case no counterstaining of nuclei with DAPI (blue) must be performed.

4. Notes

1. This helps removing air bubbles that are present between the cover slip and the bottom of the dish. The cover slips will not float and the cells will be homogenously seeded on the glass.
2. The easiest way to ensure specificity of labeling is to express proteins that are tagged by the fusion of highly antigenic epitopes, such as HA, FLAG, V5, and Myc, which are not expressed in the cells investigated and against which are available very specific commercial antibodies. For primary antibodies against endogenous proteins the best control of specificity is to test the same immunofluorescent reaction in cells lacking the protein/antigen of interest. For example, in a specificity test for anti-CRY1 antibodies, tissue/cells derived from *Cry1^{-/-}* mice must give a negative result, because the CRY1 protein is absent. Knockout mice have been recently developed for several clock genes (7,8), providing a source for specificity tests. Several companies are now producing antibodies against mammalian clock proteins. Unfortunately, most of them are not very good when tested by immunofluorescence on fibroblasts (nonspecific signal), although the rabbit polyclonal anti-PER1 antibodies of Alpha Diagnostic are excellent for immunofluorescence studies.

Others specificity controls are performed by staining cells either with preimmune serum, (i.e., derived from the same animal before immunization), or with primary antibodies preabsorbed with the specific antigen. Specificity is demonstrated by lack of detectable staining. Finally cells should be stained only with the secondary antibody (used at the same concentration). This is an internal control and it is useful not only in determining the specificity of the antibody reaction, but also in monitoring cell preservation. Cells often show nonspecific nuclear staining when damaged.

3. Working dilutions must be tested by the investigator, but usually 1:100 or 1:1000 are the most common working concentrations for antibodies.
4. Do not exceed 100 to 200 μ L, as too much liquid (e.g., 300 μ L) will drop out of the cover slip, compromising the experiment.
5. Mowiol is a polyvinyl alcohol-based mount that sets as a hard resin. It has the advantage that cells under the cover slip do not dry out and the cover slips do not require sealing with nail polish.
6. In transfection studies the proteins of interest are often overexpressed at very high levels. For colocalization analysis it is very important to control that the intense fluorescent signal derived from the staining of one protein (for example, green) is not leaking on the channel used to detect the second protein (for example, red).

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