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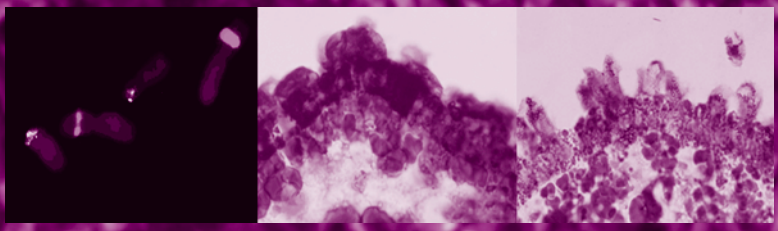
In Situ Hybridization Protocols

THIRD EDITION

Edited by

Ian A. Darby

Tim D. Hewitson



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Third Edition

Edited by

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Preface

The technique of *in situ* hybridization, in its various forms, has been used routinely in many laboratories for a number of years. In the post-genome era, gene arrays and proteomics have allowed us to identify hitherto unknown unrecognized pathways and mechanisms. However, rather than diminish the importance of *in situ* hybridization, the now widespread use of screening technologies has increased the need to temporally and spatially localize the distribution of mRNA expression.

Our intention, in In Situ *Hybridization Protocols* is to provide ample information for novices planning to set up the *in situ* hybridization technique and use it in their laboratory for the first time, as well as giving updates of recent developments for those laboratories where *in situ* hybridization techniques are already in use.

Despite its widespread significance, *in situ* hybridization has retained a reputation as one of the more difficult and capricious molecular biological techniques. This may in part be because of the hybrid nature of the technique, which often requires a mixture of molecular biological and histological skills. The two techniques are usually taught and acquired in different streams of biological science. The step-by-step and detailed protocols provided in In Situ *Hybridization Protocols* by researchers active in the field should make it possible for both the molecular biologist with little experience of histology and the histologist with little experience of molecular biology to use the technique successfully in their laboratories.

In the third edition of In Situ *Hybridization Protocols*, we have concentrated on *in situ* hybridization of cells and tissues. Detailed methods are presented for the preparation and tissue hybridization, and for a variety of detection methods from a number of groups working in diverse areas. In particular, developments in non-isotopic *in situ* hybridization and amplification techniques are constantly improving. Furthermore, as the technology has matured, a number of new applications have evolved. As well as the fundamentals, this edition covers a number of derivative techniques including identification of transplanted cells, histones, nick end labeling for apoptosis, the use of peptide nucleic acid probes, and *in situ* hybridization of plant specimens. Many of these were not included in the first two editions of In Situ *Hybridization Protocols*. We therefore hope that the third edition of In Situ *Hybridization Protocols* is far more than simply

an update of previous editions and will reach a new audience with new problems.

In our own laboratories we have used *in situ* hybridization on tissue sections and cultured cells for a number of years, and when we look back on results gained even a few years ago, there have clearly been continual improvements in the technique leading to better resolution and more sensitive detection of low-level gene expression. We trust that those who use this new edition will find it a valuable aid in setting up the technique or improving the sensitivity and scope of applications of *in situ* hybridization in their own laboratories.

Finally, we would like to acknowledge the contributions made by our coworkers in the laboratory, in particular Teresa Bisucci, and all of the authors who have contributed their protocols for this edition.

Ian A. Darby
Tim D. Hewitson

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1

Treatment of Tissue Sections for *In Situ* Hybridization

Gregory H. Tesch, Hui Y. Lan, and David J. Nikolic-Paterson

Summary

The treatment of tissue sections to enhance probe access to target mRNA is a critical step in the methodology of *in situ* hybridization. We have overcome some of the problems encountered in enzyme-based treatment of tissue sections by the application of microwave oven heating. Microwave treatment can (1) replace proteinase K digestion for frozen sections; (2) enhance proteinase K digestion in paraffin sections; (3) denature mRNA structure to enable better probe access; (4) preserve tissue architecture; and (5) inactivate endogenous alkaline phosphatase within tissue sections to reduce background with immunohistochemistry-based probe detection.

Key Words: *In situ* hybridization; microwave; digoxigenin; immunohistochemistry; proteinase K.

1. Introduction

In situ hybridization is a powerful technique that enables the detection of specific mRNA species within individual cells in tissue sections and can provide invaluable insights into physiological processes and disease pathogenesis. However, *in situ* hybridization can be a relatively difficult technique to master because of the many steps involved in the procedure and because several of these steps need to be optimized for each tissue to be examined and each probe to be used. After design of the hybridization probe, the next most important issue for any *in situ* hybridization protocol is how to treat the tissue section to optimize probe access to the target mRNA.

Preservation of target mRNA within tissues requires the use of crosslinking fixatives, such as formalin. However, this process hinders probe access to the target sequence and, thus, tissue sections require treatment before the hybridization step to optimize the probe-based signal. In general, treatment of fixed

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tissue sections is a compromise between increasing the hybridization signal vs a loss of tissue architecture. The use of strong crosslinking fixatives (i.e., 10% formalin) and paraffin-embedding produces excellent morphology, but there is significant mRNA degradation because of the high temperatures used in the embedding process and a loss of probe access to the remaining mRNA target because of extensive crosslinking. In contrast, frozen sections provide only moderate morphology, but mRNA remains intact, and the use of weaker crosslinking fixatives (i.e., 2 or 4% paraformaldehyde [PFA]) provides better probe access to the target mRNA. This chapter describes the use of microwave oven heating in the treatment of tissue sections as a means to improve probe access to target mRNA and maintain tissue morphology in both frozen and paraffin embedded tissues.

1.1. Overview of Treatment Options

Frozen sections are highly susceptible to proteinase damage, which often leads to substantial loss of tissue architecture. To avoid this problem, we use microwave oven heating in place of proteinase K digestion for PFA-fixed cryostat tissue sections ***(1)***. Microwave oven heating denatures mRNA secondary and tertiary structure without breaking the crosslinks, thereby keeping mRNA within the tissue and enhancing probe access to the mRNA target. In addition, microwave treatment does not affect tissue morphology, and it also inactivates endogenous alkaline phosphatase ***(2)***, leading to lower background when detecting nonradiolabeled probes by immunohistochemistry. Furthermore, many protein antigens are susceptible to proteinase K digestion, so that replacing this step with microwave oven heating enables combined *in situ* hybridization and immunohistochemistry staining that otherwise may not have been possible.

Formalin-fixed paraffin sections usually require extensive treatment with acid, detergent, and proteinase K to detect target mRNA. The main difficulty with this protocol is the variability of proteinase K digestion. We have used microwave oven heating to improve the efficiency and reliability of this digestion step ***(3)***. Using a microwave denatures large macromolecular complexes within the nucleus, thereby enhancing proteinase K access to target proteins within the nucleus. In addition, it facilitates the use of shorter periods of enzyme digestion, resulting in more consistent results and better preservation of tissue architecture. The other benefits of using a microwave—denaturation of mRNA structure and inactivation of alkaline phosphatase—are also evident.

The following treatment protocols for tissue sections can be used for hybridization with all types of probes (oligonucleotides, complementary [c]RNA, complementary [c]DNA) and both radioactive and nonradioactive methods. A recent review of microwave-based methods of tissue treatments for *in situ* hybridization also is worth consulting ***(4)***.

2. Materials

2.1. General

1. Xylene, ethanol, sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), glycine, PFA, and sodium dodecyl sulfate (BDH, Poole, UK).
2. Diethylpyrocarbonate (DEPC), Triton X-100, proteinase K (RNase free; Sigma, St. Louis, MO).
3. Trisodium citrate.
4. Hydrophobic barrier pen.

2.2. Slides

Sections of frozen or paraffin-embedded tissues are placed onto 3'-aminopropyltriethoxysilane (silane)-coated glass slides. This treatment is necessary to ensure adherence of sections to the slides during the subsequent procedures.

2.3. Hybridization

1. Glass cover slips (24 × 60 mm).
2. Digoxigenin (DIG)-labeled probes (cRNA, cDNA, oligonucleotides).
3. Hybridization buffer: 50% deionized formamide, 10% dextran sulfate, 4X standard saline citrate (SSC), 2.5X Denhardt's solution, 0.25 mg/mL salmon sperm DNA, 0.6 mg/mL yeast transfer RNA, 0.025% sodium dodecyl sulfate, and 0.1% DIG-block reagent (Roche Diagnostics, Mannheim, Germany).
4. Denhardt's solution (100X stock): 2% polyvinylchloride, 2% pyrrolidone, and 4% BSA in DEPC-treated distilled water).
5. Slide rack and container.
6. Humidified box.

2.4. Immunodetection of Hybridized DIG-Labeled Probes

1. Buffers: phosphate-buffered saline (PBS, pH 7.0), SSC (pH 7.0), and 0.1 *M* Tris-HCl (pH 9.5).
2. Sera: fetal calf serum (FCS), normal sheep serum (NShS), normal serum from same species as tissue.
3. Rotating platform shaker.
4. Alkaline phosphatase (AP)-conjugated sheep anti-DIG Fab fragments (Roche).
5. 5-Bromo, 4-chloro, 3-indolyphosphate/nitroblue tetrazolium AP substrate (Moss Inc, Pasadena, MD).
6. Aqueous mounting media (e.g., Glycergel, Sigma).
7. Histological counterstain (e.g., periodic acid Schiff's, methyl green).

3. Methods

3.1. RNase Treatment

RNase must be removed from all glass and nonsterile plasticware by washing in 0.1 *M* NaOH overnight and rinsed well in distilled water before use.

3.2. Tissue Processing

3.2.1. Snap-Frozen Tissue

Tissue slices are embedded in OCT compound (Tissue-Tek™, Miles Scientific, Naperville, IL) using an aluminum-foil boat, immediately frozen using liquid nitrogen or a dry-ice/ethanol slurry, and then stored at –80ºC.

3.2.2. Paraformaldehyde-Fixed Frozen Tissue

Tissues slices are immersed for 2–3 h in either 2% PFA-lysine-periodate (PLP) or 4% PFA in PBS, followed by immersion (3X) in DEPC-treated PBS containing 7% sucrose and 0.02% sodium azide at 4ºC for 24 h. Excess moisture is then removed using blotting paper and the tissue embedding in OCT compound as above and stored at –80ºC.

3.2.3. Formalin-Fixed Paraffin-Embedded Tissue

Tissue slices are immersed in 4 or 10% neutral-buffered formalin for 2–3 h, followed by overnight immersion in 70% ethanol at 4ºC, and then embedded in paraffin using routine methods. In animal-based studies, tissues can be perfusion fixed *in situ* if required for optimal morphology.

3.3. Pretreatment

3.3.1. Microwave-Based Treatment of Cryostat Tissue Sections (see **Note 1**)

1. Adhere sections (5 µm) of snap-frozen or PLP-fixed tissues to silane-coated slides and allow to air-dry. The snap-frozen tissue sections need to be fixed by immersion in PLP (20 min), 4% PFA (20 min), or formalin (10 min) at 4ºC.
2. Wash slides in freshly prepared 0.1% DEPC (600 µL of DEPC in 600 mL of dH_2O) for 1 h using stirrer in the fume hood (note that DEPC is toxic).
3. Transfer slides into a polypropylene container with 400 mL of DEPC-treated 10 m*M* sodium citrate buffer, pH 6.0, and cover with polyethylene plastic wrap (*see* **Note 2**).
4. Microwave for 10–12 min at 800–1000 W (i.e., on the "high" setting; *see* **Note 3**).
5. After microwave treatment, place container on ice for 10 min. Remove slides and place into chilled DEPC-treated 2X SSC buffer, pH 7.0, and then mark with a hydrophobic barrier pen

3.3.2. Combined Microwave and Proteinase K Treatment of Formalin-Fixed Paraffin Tissue Sections (see **Note 1**)

1. Adhere formalin-fixed paraffin sections (4 µm) to silanized slides in an oven at 60°C for 1 h.
2. Cool slides to room temperature, wash (2×) in 100% xylene (or equivalent hydrocarbon solvent) for 20 min, wash (2×) in 100% ethanol for 10 min, and hydrate through 90 and 70% ethanol (1 min each).

3. Place slides in freshly prepared 0.1% DEPC (600 µL of DEPC in 600 mL of distilled water) for 1 h using stirrer in the fume hood.
4. Transfer slides to a polypropylene container with 400 mL of DEPC-treated 10 m*M* sodium citrate buffer, pH 6.0, and cover with polyethylene plastic wrap (*see* **Note 2**). Microwave for 10–12 min at 800–1000 W.
5. After microwave treatment, place container on ice to cool for 10 min. Remove slides and mark with hydrophobic barrier pen.
6. Immerse slides in 0.2 *M* HCl for 20 min, then wash (2×) with DEPC-treated PBS.
7. Immerse slides in 0.3% Triton X-100 in DEPC-treated PBS for 10 min and wash (3X) with DEPC-treated PBS.
8. Apply pre-warmed (37°C) proteinase K (10 µg/mL in DEPC-treated PBS) to sections (*see* **Note 4**). Transfer slides into a humidified RNase free box and place in a 37°C incubator for 20 min. Stop the proteinase K reaction by a brief rinse with DEPC-treated PBS and immersion of slides in 0.2% glycine in DEPC-treated PBS for 5 min, followed by (1X) wash with DEPC-treated PBS.
9. Fix sections in 4% PFA in PBS for 10 min on ice (*see* **Note 5**). Then, wash slides three times with DEPC-treated PBS (*see* **Notes 6** and **7**).

3.4. Nonradioactive In Situ *Hybridization Using DIG-labeled Probes*

3.4.1. Hybridization Method

1. Put 50–100 µL of hybridization buffer on each section and cover slip, place slides into a sealed, humidified RNase free box, and incubate at 42–50°C for at least 30 min.
2. Dilute DIG-labeled probes 1:200 in hybridization buffer in a microfuge tube and heat to 90°C (cRNA probe) or 100°C (cDNA probe) for 5 min.
3. Place the probe on ice for 10 min.
4. Dilute the probe in hybridization buffer to the optimal concentration (*see* **Note 8**) and heat to 42°C.
5. After prehybridization, carefully remove cover slips and drain excess buffer onto filter paper.
6. Add 50–100 µL of probe to each section, then cover slip (avoiding air bubbles) and incubate overnight at 42°C in a sealed, humidified RNase free box.

3.4.2. Immunostaining Method

1. Gently wash cover slips off slides in a container of 2X SSC and place slides into a staining rack (*see* **Note 9**).
2. Wash in 2X SSC at room temperature for 20 min on rotating platform shaker.
3. Wash in 2X SSC at 42°C for 20 min in a shaking water bath.
4. Wash in 0.1X SSC at 42°C for 20 min in shaking water bath.
5. Wash in PBS at room temperature for 5 min on rotating platform shaker.
6. Incubate with 10% FCS and 10% NShS in PBS for 30 min and wash once with PBS.
7. Incubate with AP-conjugated sheep anti-DIG Fab fragments (1500) for 60 min.
8. The antibody is diluted with 10% normal serum (same species as tissue), 1% FCS, and 1% NShS in PBS (*see* **Note 10**).

9. Wash twice with PBS for 5 min.
10. Wash once with 0.1 *M* Tris-HCl (pH 9.5) for 2 min.
11. Initiate color reaction by incubating sections with 5-bromo, 4-chloro, 3-indolyphosphate/nitroblue tetrazolium AP substrate at room temperature. Store in darkness and observe development under microscope every hour. If signal is not sufficient after 3 h, leave overnight at 4°C or wash substrate from slides, leave in PBS, and reapply substrate the next day.
12. After development, sections can be counterstained with periodic acid Schiff's (PAS) reagent and/or methyl green and mounted in an aqueous medium (e.g., Glycergel, Sigma).

3.5. Additional Technical Considerations

1. These microwave-treatment protocols have been used to obtain excellent *in situ* hybridization detection in a variety of normal and diseased tissues (kidney, lung, spleen, gut, liver, lymph node, and thymus) using cRNA and cDNA probes varying in length from 180 to 1756 bases. The target mRNAs detected with these techniques include: kallikrein, GAPDH, VCAM-1, IL-1β, TGF-β1, MCP-1, M-CSF, MIF, FGF-2, FGF-R, and osteopontin ***(1,3,5,6)***.
2. Tissues that have been fixed for long periods (i.e., days), as is sometimes the case with archived tissues, may require a longer period of microwave treatment or an extended digestion period with protease K to achieve adequate signal. However, longer treatments will also cause further degradation of tissue morphology.

4. Notes

1. Solutions used for tissue treatment and hybridization steps need to be RNase free. Therefore, solutions need to be treated with 0.1% DEPC for 1 h to destroy RNase and then autoclaved to degrade DEPC before use (exceptions are Tris-HCl and detergents, which need to be prepared in RNase-free glassware)
2. We routinely place as many as 20 slides in a portable polypropylene slide rack (Kartell Labware, Noviglio, del MI, Italy) when using the listed protocol.
3. The minimal time of microwaving required will be dependent on the maximum output of the microwave oven (10 min at 1000 W ≈ 12 min at 800 W).
4. Proteinase K stock of 1 mg/mL is made up in DEPC-treated PBS and stored at –20ºC.
5. 4% PFA is prepared by dissolving in DEPC-treated PBS with gentle heating (≤60ºC) and stored in 50-mL aliquots at –20ºC.
6. The postfixation (**step 9**) is optional (try with and without this step when establishing your method).
7. 4% PFA can be thawed-frozen up to five times for re-use.
8. Each new batch of labeled probe needs to be titrated to determine the optimal concentration for experiments. Probes can be effective in a wide concentration range (1–1000 ng/mL).
9. Solutions no longer need to be RNase free.
10. Antibody incubations can be performed using normal immunostaining equipment.

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2

Preparation of Template DNA and Labeling Techniques

Peter J. Roche

Summary

A variety of probes can be used for *in situ* hybridization, depending on the application and the labeling strategy. In general, RNA probes (riboprobes) are now more commonly used. However, some laboratories may still prefer to use DNA probes for *in situ* hybridization, and a number of techniques for this type of labeling have been described in this chapter. The preparation of plasmid DNA and the subsequent use of polymerase chain reaction products as labeling templates are discussed in detail. Both radioactive and nonradioactive labeling procedures are described and the latest nonradioactive detection methods are outlined.

Key Words: Random primed labeling; PCR template; oligonucleotide probe; DIG labeling; alkaline phosphatase; chemiluminescence.

1. Introduction

A variety of probes can be used for *in situ* hybridization, depending on the application and the labeling strategy. In general, RNA probes (riboprobes) are now used more commonly. However, some laboratories may still prefer to use DNA probes for *in situ* hybridization, and a number of techniques for this type of labeling are given in this chapter.

The two main procedures for labeling a double-stranded DNA fragment are random primed synthesis ***(1)*** and nick translation ***(2)***. The random primed method is the most commonly used protocol because of its simplicity and highly efficient labeling process. The random primed method is extremely robust and will generate probes with very high specific activity. Nick translation is used less commonly because several critical parameters need to be carefully optimized for successful labeling.

Random primed labeling involves the denaturation of the double-stranded DNA fragment and annealing of short oligonucleotide primers of random

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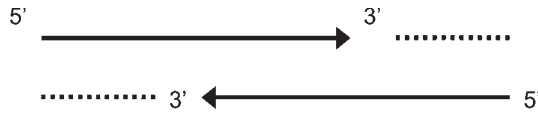

Fig. 1. Overlapping oligonucleotide probe. Two oligonucleotides of 30–35 bases (solid arrows) are designed to the gene of interest with the shown 5' to 3' orientation. The region of overlap is 10–15 bases. The dotted line indicates the fill-in reaction by Klenow fragment in the presence of [α-^{32}P] dCTP, which will generate a probe of 50–55 bases (*see* **Subheading 3.5.**).

sequence. These random sequence oligonucleotides are usually 8–10 bases long. The oligonucleotides prime the synthesis of a new DNA strand by the Klenow fragment of DNA polymerase I. The inclusion of [α-^{32}P] dCTP will result in uniformly labeled DNA on both strands.

Traditionally, the DNA fragment to be labeled has been a DNA insert from a recombinant plasmid, which is purified using agarose gel electrophoresis after restriction enzyme digestion. This multistep procedure is time-consuming, cumbersome, and routinely results in a DNA preparation containing impurities that reduce the efficiency of DNA labeling. This traditional source of template DNA should be replaced by a specific polymerase chain reaction (PCR) product generated from the recombinant plasmid.

A specific PCR product can be easily generated from a recombinant plasmid using primers to the polylinker region or gene specific primers. The PCR product can then be cleaned up using a commercial mini-column. The resulting template DNA is extremely clean and available in large amounts. This DNA is an excellent template for labeling reactions, which will result in a high specific activity probe (*see* **Note 1**). This type of DNA template also fully complies with Arthur Kornberg's fifth law of enzymology: "thou shalt not waste clean enzymes on dirty substrates" ***(3)***.

Another source of DNA template for labeling is two overlapping oligonucleotides (30–35 nucleotides long). These oligonucleotides can be designed to the gene of interest and they can generate a labeled double-stranded DNA probe of 50–55 bases long (*see* **Fig. 1**). This type of probe is particularly useful if a cloned DNA fragment is not available (*see* **Note 2**).

An alternative to radioactive labeling of DNA is nonradioactive labeling using digoxigenin (DIG)-labeled nucleotides. In the past, nonradioactive DNA probes have suffered from a lack of sensitivity compared with ^{32}P- or ^{33}P-labeled probes. However, recent advances in chemiluminescent and antibody/colorimetric detection methods have made this approach far more feasible. DIG-labeled DNA probes are safer and relatively stable, which means that a large

batch of probe can be synthesized and stored for at least 1 yr. This has advantages for reproducibility and efficiency of DNA detection. For these safety and practical issues, nonradioactive labeling of DNA should be seriously considered for each experimental application.

2. Materials

2.1. Preparation of Template DNA

1. Competent *Escherichia coli* cells (for example, DH5α strain) containing the plasmid with the insert to be labeled ***(4)***.
2. TB (terrific broth) ***(4)***: 12 g of Bacto tryptone, 24 g of Bacto yeast extract, 4 mL of glycerol, water to 900 mL. Autoclave and make up to 1 L with sterile phosphate buffer (0.17 *M* KH_2PO_4 and 0.72 *M* K_2HPO_4).
3. Plasmid purification kit: silica membrane kits are available from Qiagen (QIAfilter plasmid kit; Germantown, MD), Promega (Madison, WI) (PureYield™ Plasmid System), and Marligen Biosciences (Ijamsville, MD; Rapid Plasmid System). Anion-exchange kits include QIAGEN plasmid kit (Qiagen), and High Purity Plasmid System (Marligen Biosciences).
4. Taq PCR Master Mix Kit (Qiagen).
5. PCR clean-up kit; available from Promega (Wizard® PCR Prep DNA Purification System) or Qiagen (QIAquick PCR Purification Kit).

2.2. Radioactive Labeling of DNA

1. Random oligonucleotide primer; the original protocol used hexamers (6'mer); however, the efficiency of labeling is dramatically improved if a longer primer is used. Random oligonucleotides should be 10 bases long (10'mer). These can be ordered from any Custom Oligonucleotide manufacturer (*see* **Note 3**).
2. DNA polymerase I "Klenow fragment" (approx 5 U/µL), and 10X reaction buffer (provided by supplier).
3. [α-^{32}P] dCTP (3000 Ci/mmol, 10 µCi/µL).
4. dNTP stock solutions; 10 m*M* stocks of dATP, dGTP, and dTTP.
5. Sephadex G-25 Medium.
6. 1X TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* ethylene diamine tetra-acetic acid (EDTA).
7. Dry block heater.

2.3. Nonradioactive Labeling of DNA

1. The materials for nonradioactive labeling are similar to radioactive labeling, except for the substitution of [α-^{32}P] dCTP with DIG-11-dUTP. Components can be bought separately or a DIG labeling kit can be purchased from commercial suppliers (Roche Applied Science, Basel, Switzerland).
2. 10X DIG labeling mix (1 m*M* dATP, 1 m*M* dCTP, 1 m*M* dGTP, 0.65 m*M* dTTP, and 0.35 m*M* DIG-11-dUTP).

2.4. Detection of DIG-Labeled DNA

1. For color detection; NBT/BCIP solution (Roche Applied Sciences).
2. For chemiluminscent detection; CDP-Star solution (available from both Applied Biosystems, Foster City, CA, and Roche Applied Science).

3. Methods

3.1. Plasmid and Template Preparation

3.1.1. Preparation of Plasmid DNA

1. An *E. coli* colony containing the plasmid DNA of interest should be placed in 2 mL of TB with appropriate antibiotic selection (for example: ampicillin at 100 µg/mL) and grown at 37°C for 16 h with shaking ***(4)***.
2. 0.5 mL of the overnight bacterial culture is then placed in 50 mL of TB (with antibiotic) and grown at 37°C with vigorous shaking to achieve high density (approx 4–5 h). The remainder of the overnight culture can be stored frozen as a glycerol stock. It is important that plasmid DNA for a preparative purpose (e.g., riboprobe template) is isolated from cultures that have just reached saturation, not from an overnight incubation.
3. Plasmid DNA should be isolated using a commercially available kit from a molecular biology supplier. There are two types of kit based on the purification method; silica membrane spin column (e.g., Qiagen, Promega, Marligen Biosciences) and anion-exchange chromatography (e.g., Qiagen, Marligen Biosciences; *see* **Note 4**).

3.1.2. Preparation of Template DNA for Random Primed Labeling

1. A PCR DNA fragment can be generated from plasmid DNA that contains the gene of interest. A 50 or 100-µL PCR should be performed according to the manufacturers protocol (Qiagen) with approx 1.0 pg of plasmid DNA. The PCR primers can be either specific to the gene of interest or designed to the polylinker region (e.g., nucleotide sequencing primers). The optimal size of the PCR fragment should be 200–500 bp.
2. The PCR product should then be purified using a PCR clean-up kit. This procedure will remove excess primers and nucleotides.
3. The PCR product is then analysed by agarose gel electrophoresis (1.5–2.0% agarose) with appropriate DNA MW markers ***(4)***. The PCR product should be the expected size, based on primer location, and free of any nonspecific DNA fragments.
4. Quantitation of the PCR fragment should be performed by measuring the absorbance at 260 nm (1 A_{260} unit = 50 µg/mL). Alternatively the PCR product can be quantitated by fluorescence using SYBR Green and a DNA standard curve. This fragment can then be used as a template for radioactive (*see* **Subheading 3.4.**) or DIG labeling (*see* **Subheading 3.7.**).

3.1.3. Preparation of Template DNA for Riboprobe Synthesis

1. Plasmid DNA (5–10 µg) containing the gene of the interest is digested with an appropriate restriction enzyme, which cuts at the 3' end of the gene ***(4)***.
2. Digested DNA should be analyzed on a 0.8% agarose gel with suitable DNA MW markers to confirm complete digestion. This is particularly important when a large amount of DNA is digested.
3. The linearized plasmid DNA should then be cleaned up with a PCR clean-up kit (e.g., QIAquick PCR Purification Kit; Qiagen). This kit will remove enzyme and result with the DNA being in a suitable solution for further reactions (10 m*M* Tris-HCl, pH 8.0). The use of phenol extractions and ethanol precipitations to clean up the DNA should be avoided at all cost. These methods usually result in poor efficiency and considerable inconsistency in subsequent reactions.
4. The purified linear plasmid DNA can then be used as a template for riboprobe synthesis. A detailed protocol for riboprobe synthesis after plasmid preparation can be found in this book (*see* Chapter 3).

3.2. Radioactive Probe Labeling

3.2.1. Random Primed Radioactive Labeling of a DNA Fragment

1. Prepare the DNA/random primer mixture as follows: 2 µL of DNA (e.g., PCR product, 25–50 ng), 1 µL of random primer (50 ng), and 3 µL of sterile water to a final volume of 6 µL.
2. Incubate the DNA mixture at 95°C for 5 min, then chill on ice. A dry block heater is a safe and convenient method of heating the sample compared with a boiling water bath, which can result in contamination of the work area.
3. Add the following reaction mixture to the denatured DNA/primer solution: 1 µL of nucleotide mix, (3.3 m*M* each of dATP, dGTP, dTTP), 3 µL [α-^{32}P] dCTP (3000 Ci/mmol, 10 µCi/µL), 1.5 µL 10X Klenow enzyme buffer, 3 µL of sterile water, and 0.5–1 µL of DNA polymerase I Klenow fragment (5 U/µL).
4. Incubate the final reaction solution (15 µL) at 37°C for 15–30 min.
5. The labeled DNA fragment can be separated from unincorporated radioactive nucleotides using Sephadex G-25 (*see* **Subheading 3.6.**).

3.2.2. Radioactive Labeling of Double-Stranded Oligonucleotide Probes

1. Prepare the oligonucleotide mixture as follows: 1 µL of forward 30'mer oligonucleotide (25 ng), 1 µL of reverse 30'mer oligonucleotide (25 ng), and 4 µL of sterile water to a final volume of 6 µL.
2. Incubate the oligonucleotide mixture at 80°C for 5 min, then allow to slowly cool to room temperature.
3. Add the following reaction mixture to the oligonucleotide solution: 1 µL of nucleotide mix, (3.3 m*M* each of dATP, dGTP, and dTTP), 3 µL [α-^{32}P] dCTP (3000 Ci/mmol, 10 µCi/µL), 1.5 µL 10X Klenow fragment buffer, 3 µL of sterile water, and 0.5–1 µL of DNA polymerase I Klenow fragment (5 U/µL).

4. Incubate the final reaction solution (15 µL) at 37°C for 15–30 min.
5. The labeled DNA fragment can be separated from unincorporated radioactive nucleotides using Sephadex G-25 (*see* **Subheading 3.6.**).

3.2.3. Removal of Unincorporated Radioactive Nucleotides

1. Add 35 µL of 1X TE buffer to the reaction mixture and load onto a small Sephadex G-25 Medium column (70 × 10 mm) equilibrated in 1X TE buffer.
2. Collect approx 0.3-mL fractions in sterile microcentrifuge tubes.
3. Identify fractions containing the labeled DNA (first peak) using a hand-held radioactive monitor.

3.3. Nonradioactive Labeling of DNA

3.3.1. Random Primed DIG Labeling of a DNA Fragment

1. Prepare the DNA/random primer mixture as follows: 2 µL of DNA (e.g., PCR product, 25–50 ng), 1 µL of random primer (50 ng), and 9 µL of sterile water to final volume of 12 µL.
2. Incubate the DNA mixture at 95°C for 5 min, then chill on ice (*see* **Note 5**).
3. Add the following reaction mixture to the denatured DNA/primer solution: 2 µL 10X DIG labeling mix (*see* **Subheading 2.3.**), 2 µL 10X Klenow enzyme buffer, 3 µL of sterile water, and 1 µL of DNA polymerase I Klenow fragment (5 U/µL).
4. Incubate the final reaction solution (20 µL) at 37°C for 1 h or 20 h. Time course studies by Roche Applied Science indicate that higher yields of DIG-labeled DNA are obtained with longer incubation times (approximately fivefold increase in yield).
5. The labeled DNA fragment can then be used in hybridization techniques. It may be necessary to optimize the concentration of DIG-labeled DNA in the hybridization solution. If the concentration of probe is too high, then background problems may appear, whereas low concentrations may result in weak signals.

3.3.2. Detection of DIG-Labeled DNA

DIG-labeled DNA is detected by a DIG antibody that is conjugated to alkaline phosphatase. The enzyme will then catalyze a color or chemiluminescent reaction, depending on the type of substrate added. Chemiluminescent substrates are 10^4 to 10^5 more sensitive than colorimetric substrates. However, the colorimetric systems are generally more appropriate for *in situ* hybridization methods, whereas the chemiluminescent methods are more appropriate for blots.

Two chemiluminscent substrates are available: CSPD and CDP-Star (both available from Applied Biosystems and Roche Applied Science). CDP-Star produces a more intense and rapid signal compared to CSPD. DIG-labeled DNA also can be detected by DIG antibodies conjugated to different fluoro-

chromes, which can be visualized directly. This method is more suitable for *in situ* hybridization applications, however, sensitivity will be lower compared with chemiluminescence.

4. Notes

1. It is recommended that the PCR fragment for DNA labeling is generated from a well-characterized source to ensure that the valid gene sequence is used as a probe. This is the reason that a characterized recombinant plasmid containing the gene sequence of interest is suggested as the template for generating the PCR product. It is possible to label a PCR fragment generated from cellular mRNA or genomic DNA, however, they should be used with caution until their identity is validated. Uncharacterized PCR products should be subcloned and subjected to partial DNA sequencing. The subcloned PCR fragment can then be used as the source for generating the labeling template.
2. Overlapping oligonucleotide probes are particularly useful if a cloned DNA fragment is not available. A labeled DNA probe of 50–55 bases can easily be produced that can be used with traditional hybridization conditions. These probes are ideal for examining alternatively spliced mRNA transcripts. The fill-in reaction will add approx 20 nucleotides to each strand of the template, thus, ensuring a high level of radionucleotide incorporation. The specific activity of the probe will be far greater compared to 5' end labeling of oligonucleotides with [γ-^{32}P] dATP.
3. Commercial random primed labeling kits are available from many suppliers; however, they can be expensive. The technique described here is simple and only a few components are required, therefore, it is quite easy to label DNA using individually purchased enzyme, random primers, and nucleotide stocks.
4. The silica membrane kits are rapid, convenient, and can produce high-quality DNA. This DNA is suitable for all standard protocols, for instance, PCR template, restriction enzyme digestions, DNA sequencing, and template for riboprobe synthesis. The anion-exchange kits produce excellent quality DNA for the more demanding applications, for instance, mammalian cell transfections. These kits should be considered for riboprobe templates because the DNA will be of the highest quality.
5. A dry block heater is a safe and convenient method of heating the sample, compared with a boiling water bath, which can result in contamination of the work area.

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3

In Situ Hybridization Using cRNA Probes

Isotopic and Nonisotopic Detection Methods

Ian A. Darby, Teresa Bisucci, Alexis Desmoulière, and Tim D. Hewitson

Summary

In this chapter we describe the use of cRNA (riboprobes) in the detection of gene expression in tissue sections. Riboprobes offer good sensitivity and allow the detection of low-level mRNA expression. In some cases, the use of radiolabeling is justified because this method is still sensitive. However, recent advances in nonisotopic detection methods mean that in some cases digoxigenin (DIG) or biotin labeling also may be sufficiently sensitive to detect mRNA expression in tissues of interest. The use of alkaline phosphatase conjugated anti-DIG antibodies improves the sensitivity of DIG detection over peroxidase systems, and the use of amplification systems based on biotinyl tyramide has improved the sensitivity of biotin labelled probe detection. Finally, it can be shown that low-level mRNA expression is easier to detect in frozen sections than in paraffin-embedded material, with a consequent loss in quality of morphology.

Key Words: *In situ* hybridization; digoxigenin; biotin; tyramide; autoradiography.

1. Introduction

Since the first description of the technique of *in situ* hybridization to detect specific mRNA species on tissue sections, a variety of methods have been used. Treatment of tissue and cells ranges from the use of frozen sections, which show good preservation of mRNA but poor morphology, to standard histological methods for tissue preservation, such as paraformaldehyde (PFA) or formaldehyde fixation followed by paraffin embedding. This latter method may show reduced preservation of tissue mRNA levels but gives better structural information as the tissue morphology is better preserved (*see* **Note 1**). Similarly, a variety of probe types have been used, beginning with double-stranded cDNA probes, peptide nucleic acid probes, and moving on to single-stranded

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RNA probes (riboprobes). In addition, short single-stranded oligonucleotide probes also have been used, allowing the user to synthesize specific probes from published sequences. Finally, various methods of probe labeling and detection have been used, including different radioactive isotopes and, more recently, nonradioactive methods such as biotin, fluorescein, and digoxigenin (DIG). For examples of the use of these methods in various tissues, *see* **refs. 1–11**.

In this chapter, we will describe the standard methods used in our laboratory for detecting target mRNA in tissue sections and in cultured cells. In general, single-stranded RNA probes are used on formalin or PFA-fixed paraffin sections, frozen sections, or cell preparations. A number of advantages of RNA probes over cDNA or oligonucleotide probes exist, which makes them preferable for *in situ* hybridization. These include the formation of tighter (RNA:RNA) hybrids, the possibility of higher stringency posthybridization washing (using RNase to remove unbound probe), and the lack of a competing reaction that occurs in the case of double-stranded DNA probes, which re-anneal in addition to binding to target mRNA.

The choice of label and detection system depends to some extent on the abundance of the target mRNA; in the case of low-abundance mRNA species, we have continued to use radioactively labeled probes, which give better sensitivity in our hands, with ^{33}P now being the isotope of choice because of its relative safety in the laboratory and its compromise between reasonably short exposure times and good resolution. However, when mRNA species of higher abundance are being detected, probe labeling with nonradioactive methods becomes more feasible. Signal amplification methods, such as those discussed in this chapter and described in Chapter 4 of this volume by Speel et al., may further improve the sensitivity of the technique.

2. Materials

2.1. Tissue Preparation and Embedding

1. PFA (Merck, Darmstadt, Germany).
2. Phosphate-buffered saline (PBS), pH 7.4.
3. Ethanol, laboratory grade.
4. Chloroform (BDH, Poole, UK).
5. Paraffin wax, for instance, Paraplast (melting point 56°C).
6. Stainless-steel embedding molds (Tissue Tek).
7. Glass vials for tissue processing.
8. Freeze embedding compound, such as OCT (Tissue Tek; for frozen sectioning only).

2.2. Slide Preparation

1. Glass slides.
2. Plastic or metal slide racks.
3. Decon or similar detergent.

4. 3-Aminopropyltriethoxysilane (Sigma, St. Louis, MO).
5. Acetone.

2.3. Pretreatment of Tissue Sections or Cell Culture Preparations

All buffers are treated with 0.05% diethylpyrocarbonate (DEPC; Sigma), with the exception of Tris-based buffers.

1. Histolene (Histolabs/Fronine, NSW, Australia) or xylene.
2. Ethanol.
3. Phosphate-buffered saline (PBS): 0.14 *M* NaCl, 0.003 *M* KCl, 0.008 *M* Na_2HPO_4, 0.0015 *M* KH_2PO_4.
4. Pronase buffer (P buffer): 50 m*M* Tris-HCl, pH 7.5, 5 m*M* ethylene diamine tetraacetic acid (EDTA), pH 8.0.
5. Pronase E (Sigma).
6. Antigen retrieval solution, e.g., Citra (BioGenex, San Ramon, CA) or 0.01 *M* citrate buffer.
7. 0.1 *M* Sodium phosphate buffer, pH 7.2.
8. 4% PFA in PBS.
9. Double distilled water, DEPC treated.
10. 70% Ethanol.

2.4. Labeling of the Probe

2.4.1. Isotopic Labeling

1. cDNA in the appropriate in vitro transcription vector providing polymerase sites for cRNA production (T7, T3, SP6).
2. 5X transcription buffer: 200 m*M* Tris-HCl, pH 7.5, 30 m*M* $MgCl_2$, 10 m*M* spermidine (Sigma), 50 m*M* NaCl.
3. 100 m*M* Dithiothreitol (DTT; Roche Diagnostics, Mannheim, Germany).
4. RNasin, ribonuclease inhibitor (Promega Corporation, Madison, WI).
5. 10 m*M* Adenosine 5'-triphosphate (ATP), 10 m*M* cytidine 5'-triphosphate (CTP), 10 m*M* Guanosine 5'-triphosphate (GTP), 12 μ*M* uridine 5'-triphosphate (UTP; Promega Corporation).
6. RNA polymerases T7, T3, and SP6 (Promega Corporation).
7. Radionucleotide; 5' [α-^{33}P]UTP (Amersham, Chalfont, UK).
8. DNase I (Promega Corporation).
9. Transfer RNA (tRNA) 20 mg/mL stock (Roche).
10. 7.5 *M* Ammonium acetate.
11. 3 *M* Sodium acetate, pH 5.2.
12. Ethanol.
13. Hydrolysis buffer: (80 m*M* $NaHCO_3$, 120 m*M* Na_2CO_3, 20 m*M* β-mercaptoethanol).
14. Stop buffer (200 m*M* sodium acetate pH 6.0, 1% glacial acetic acid, 10 m*M* DTT).
15. DEPC-treated double distilled H_2O.
16. Dry heat block or water bath accurately set at 37°C.
17. Microcentrifuge.

2.4.2. Nonradioactive Probe Labeling Using DIG

All tissue is prepared in the same manner as for radioactive probes, i.e., **Subheadings 2.1.–2.3.**

1. cDNA in appropriate in vitro transcription vector, with polymerase sites for cRNA production (T7, T3, SP6).
2. 5X transcription buffer (*see* **Subheading 2.4.1.**, **step 2**).
3. DIG 10X labeling mix (Roche).
4. RNasin (Promega).
5. RNA polymerase (T7, T3, SP6).
6. 0.2 *M* EDTA, pH 8.0.
7. 4 *M* LiCl.
8. 100 and 75% ethanol.
9. Hydrolysis buffer: 0.06 *M* Na_2CO_3, 0.04 *M* $NaHCO_3$.
10. Neutralization buffer: 0.2 *M* $NaC_2H_3O_2$, 1% acetic acid.
11. DEPC-treated double distilled H_2O.

2.4.3. Nonradioactive Probe Labeling With Biotin

Tissue and slides are prepared the same as for DIG labeling, except that biotin RNA labeling mix is substituted at **Subheading 2.4.2.**, **step 3**.

2.5. Dot Blot Analysis of Nonradioactive Probes

2.5.1. Dot Blot Analysis of DIG-Labeled Probe

To determine the concentration of the DIG-labeled riboprobe a dot blot is necessary.

1. Nylon membrane (Amersham).
2. DIG-labeled RNA control (Roche).
3. Buffer 1: 0.1 *M* (maleic acid) $C_4H_4O_4$, 0.15 *M* NaCl, pH 7.5.
4. Buffer 2: 1% skim milk powder in buffer 1.
5. Anti-DIG alkaline phosphatase antibody (Roche).
6. Buffer 3: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 0.05 *M* $MgCl_2$.
7. Alkaline phosphatase substrate (5-bromo,4-chloro, 3-indolyphosphate/nitroblue tetrazolium [BCIP/NBT]).

2.5.2. Dot Blot Analysis of Biotin-Labeled RNA Probes

1. Same procedure as for DIG-labeled probes except at **step 5**, substitute avidin-biotin-peroxidase complex (e.g., ABC from Vectastain kit, Vector, Burlingame, CA) and at **step 7**, color reaction is performed with diaminobenzidine-4HCl (DAB; Sigma).

2.6. Hybridization

1. 10X salts solution: 3 *M* NaCl, 100 m*M* Na_2HPO_4, 100 m*M* Tris-HCl, pH 7.5, 50 m*M* EDTA; 0.2% bovine serum albumin; 0.2% Ficoll; 0.2% polyvinylpyrrolidone.
2. Formamide (BDH, Poole, UK).
3. Dextran sulfate (Amersham).
4. tRNA, 20 mg/mL.
5. DEPC-treated distilled H_2O.

2.7. Posthybridization Washes

1. 20X standard saline citrate (SSC) solution: 3 *M* NaCl, 0.3 *M* sodium citrate.
2. Wash buffer: 2X SSC, 50% formamide.
3. RNase A (Sigma).
4. RNase buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.5 *M* NaCl.

2.8. Autoradiography and Emulsion for Radioactive Riboprobes

1. X-ray film cassette.
2. Film (XAR-5 or Hyperfilm).
3. Liquid nuclear research emulsion (gel form) (e.g., K5 nuclear emulsion, Ilford, Cheshire, UK or NTB-2, Kodak, Rochester, USA).
4. Developer, Phenisol (Ilford), diluted 1 in 4 with distilled H_2O.
5. Hypam fixer (Ilford), diluted 1 in 4 with distilled H_2O.
6. Harris' hematoxylin stain.
7. Eosin stain.
8. Scott's tap water: 82 m*M* $MgSO_4$, 42 m*M* $NaHCO_3$.
9. Mounting medium, nonaqueous.

2.9. DIG-Labeled Probe Detection

1. Tris-buffered saline: 0.9% NaCl, 50 m*M* Tris-HCl, pH 7.5.
2. Antibody diluent: 1X TBS, 0.1% gelatin, 0.2% BSA.
3. NT buffer: 0.1 *M* NaCl, 0.1 *M* Tris-HCl, pH 9.5.
4. BCIP/NBT (Roche).
5. 10% Polyvinyl alcohol.
6. Harris' hematoxylin stain.
7. Aqueous mountant.

2.10. Biotin-Labeled Probe Detection (With Signal Amplification)

1. Tris-buffered saline (1X TBS): 0.15 *M* NaCl, 5 m*M* KCl, 0.25 *M* Tris-HCl, pH 7.5.
2. TBS-Tween: TBS with 5% Tween-20 (BDH Chemicals, Poole, UK).
3. 0.3% H_2O_2 in 100% methanol.

4. Blocking solution: 1.5% horse serum in TBS, 0.1% gelatin, 0.2% BSA.
5. Amplification kit, for instance, DAKO GenPoint kit for catalyzed signal amplification containing primary streptavidin-horseradish peroxidase, biotinyl tyramide, secondary streptavidin-horseradish peroxidase (DakoCytomation, Carpinteria, CA).
6. Diaminobenzidine-4HCl (DAB).

3. Methods

3.1. Tissue Fixation, Processing, and Embedding

1. Place tissue biopsy in 4% PFA/PBS, overnight at room temperature.
2. Wash the tissue in 7% sucrose/0.1 *M* sodium phosphate buffer overnight at 4°C.
3. Dehydrate tissue through graded alcohols 50, 70, 90, 100%, and then two changes of 100% chloroform.
4. Place tissue in molten paraffin wax (approx 58°C) and leave tissue in wax for a minimum of 4 h.
5. Discard primary wax and replace with fresh wax and leave for 4 h.
6. Ensuring correct orientation of the tissue, embed the tissue in wax using the stainless steel molds. Place the molds at –20°C for 1 h and then remove the wax block from the mold.

3.2. Cell Preparation

The most convenient way to perform *in situ* hybridization on cultured cells, particularly on monolayer cultures, is to grow the cells directly onto glass or plastic slides or cover slips. Slides with sterile plastic culture chambers are available commercially (LabTek, Nunc, Roskilde, Denmark), and these are ideal for hybridization of monolayer cultures. For cells growing in suspension, cytospin preparations may be used.

1. Aspirate cell culture medium.
2. Wash cells twice with cell culture PBS.
3. If using slide chambers, remove the plastic chamber and silicone gasket at this point.
4. Fix the cells by immersion in 4% PFA at room temperature for 20 min.
5. Dehydrate through graded ethanols: 50, 70, 95, 100%.
6. Store in an airtight container at 4°C until use. Pretreat cells according to the labeling technique to be employed, *see* **Subheading 3.5.** or **3.5.1.**

3.3. Coating Slides With 3-Aminopropyltriethoxysilane

1. Place glass slides in racks and wash in an alkaline detergent overnight.
2. Rinse the slides thoroughly with running water and then allow them to dry.
3. Wrap the slides in aluminium foil and bake at 180°C for 3 h.
4. Place slides in racks and immerse in a 2% solution of 3-aminopropyltriethoxysilane in acetone for 20 s.
5. Rinse slides in acetone for 20 s and then in distilled H_2O, twice.
6. Dry the slides at 37°C overnight and store in an air tight container.

3.4. Tissue Sectioning

1. Fill a small container with distilled H_2O and prepare a water bath at 42°C.
2. Cut 4- to 5-µm sections of the paraffin embedded tissue on a microtome.
3. Place the sections into the H_2O and then with an uncoated glass slide transfer the section into the water bath. The section should flatten.
4. Mount the section with a coated slide and allow the section to dry overnight at 42°C.

3.5. Pretreatment of Tissue Sections and Cells

1. Dewax the sections in histolene or xylene and rehydrate through graded alcohols and, finally, DEPC-treated distilled H_2O.
2. Microwave sections in 1X Citra solution, to boiling point, add extra buffer if the level falls significantly and slides are not fully covered. Allow to cool to 37°C.
3. Rinse the sections in prewarmed (37°C) P buffer.
4. Digest tissue with Pronase E in P buffer (125 µg/mL) at 37°C for 10 min.
5. Rinse twice in 0.1 *M* sodium phosphate buffer.
6. Postfix the sections in 4% PFA/PBS at room temperature for 10 min.
7. Rinse twice in 0.1 *M* sodium phosphate buffer.
8. Wash the sections in distilled H_2O and dehydrate in 70% ethanol twice.
9. Air-dry sections and store at room temperature in a closed container until required.

3.5.1. Pretreatment of Tissue for DIG- or Biotin-Labeled Probes

1. The steps are identical to pretreatment of tissue previously described except at **step 3**, Pronase E is incubated for 20 min.

3.6. Labeling the Riboprobe

3.6.1. Radioactive Probe Preparation

Template concentration is important in the labeling procedure and for riboprobe synthesis 500–1000 ng of template is recommended.

1. For one transcription reaction the following final concentrations of reagents are required; 1X transcription buffer, 16 m*M* DTT, 20 U RNasin, 400 µ*M* ATP, 400 µ*M* CTP, 400 µ*M* GTP, 12 µ*M* UTP, template (500–1000 ng), 20 U appropriate RNA polymerase, 50 µCi 5' [α-^{33}P]UTP and distilled H_2O, to a final volume of 20 µL.
2. Incubate the reaction mixture at 37°C for 1 h in a dry heat block or water bath.
3. Digest the template DNA with 1 U of DNase I and incubate the reaction at 37°C for a further 15 min.
4. Add 40 µg of tRNA and adjust the reaction volume to 100 µL with DEPC-treated distilled H_2O.
5. Set aside 1 µL for scintillation counting.

6. Precipitate the riboprobe by adding 50 µL of 7.5 *M* ammonium acetate and 300 µL of 100% ethanol and place at –70°C for 20 min.
7. Pellet the riboprobe by centrifugation at 10,000*g* for 20 min at room temperature.
8. Remove the supernatant and wash the pellet with 70% ethanol. Allow to dry.
9. Resuspend the riboprobe in 100 µL of DEPC-treated distilled H_2O and remove 1 µL for scintillation counting.
10. In the case of long probes, access to the target mRNA in the tissue may be limited. To improve penetration of the probe hydrolysis may be necessary. We have chosen a probe length of approx 0.15 kb. For hydrolysis, add 100 µL of hydrolysis buffer to the riboprobe and incubate at 65°C for the appropriate length of time (*see* **Note 2**).
11. Terminate the hydrolysis reaction by adding stop buffer and then precipitate the hydrolyzed probe by adding: 40 µL of 3 *M* sodium acetate, 40 µg of tRNA and 800 µL of 100% ethanol.
12. Precipitate as described in **steps 7** and **8**.
13. Resuspend in 100 µL of DEPC-treated distilled H_2O, and take 1 µL for scintillation counting.

3.6.2. Labeling the Probe—DIG

1. To a microcentrifuge tube add the following final concentration of reagents: 2 µg of template cDNA, 1X transcription buffer, 1X DIG labeling mix (from labeling kit), 20 U of RNasin, 20 U of the appropriate RNA polymerase, and DEPC-treated double distilled H_2O to a final volume of 20 µL.
2. Incubate the reaction mixture at 37°C for 2 h in a dry heat block or water bath.
3. Remove template DNA by digestion with DNase 1, see **Subheading 3.6.1.**, **step 3**.
4. Precipitate DIG-labeled riboprobe by stopping the reaction with addition of 2 µL of 0.2 *M* EDTA, then add 2.5 µL of LiCl, 75 µL of 100% ethanol, and place at –70°C for 2 h.
5. Pellet DIG-riboprobe by centrifugation at 10,000*g* for 20 min at room temperature.
6. Remove supernatant and wash pellet with 70% ethanol.
7. Resuspend pellet in 50 µL of DEPC-treated double distilled H_2O.
8. The riboprobe may require hydrolysis to ensure the correct size (*see* **Subheading 3.6.1.**, **step 10**, also *see* **Note 2**). Add 100 µL of hydrolysis buffer and incubate at 60°C for the appropriate length of time; also *see* **Note 1** for calculation of hydrolysis times.
9. Stop hydrolysis by adding 150 µL of neutralization buffer and 900 µL of 100% ethanol.
10. Place at –70°C for 2 h and pellet as described earlier.

3.6.3. Labeling the Probe—Biotin

1. As for labeling with DIG except substitute 1X biotin labeling mix for 1X DIG labeling mix.

3.7. Dot Blot of Nonradioactive Probes

3.7.1. Dot Blot of DIG-Labeled Probe

Prepare serial dilutions of a DIG-labeled RNA control, available from Roche.

1. Spot 1 µL of the DIG-labeled controls and experimental probe onto a nylon filter. Make a number of dilutions of the experimental probe to gauge labeling efficiency. Probes to be spotted are diluted in a mixture of formaldehyde and SSC.
2. Fix the RNA onto the membrane by baking at 120°C for 30 min.
3. Rinse in buffer 1.
4. Incubate the membrane in buffer 2 for 30 min at room temperature.
5. Incubate the membrane in anti-DIG peroxidase or anti-DIG alkaline phosphatase diluted 1:1000 in buffer 2 for 45 min.
6. Wash membrane in buffer 1, twice.
7. Equilibrate membrane for 2 min in buffer 3.
8. Wash membrane in 1X PBS, three times.
9. Using DAB according to manufacturer's instructions perform the color reaction by immersing the membrane in DAB/H_2O_2. Watch for brown color to develop. Alternatively, if using alkaline phosphatase detection, dissolve one tablet of fast red in Tris buffer and immerse the membrane.
10. Membranes can be washed in PBS and then kept as a record by sealing them in polythene film.

3.7.2. Dot Blot of Biotin-Labeled Probe

1. As in **Subheading 3.7.1.**, i.e., from **steps 1–4**, and **steps 6–9**; only **step 5** differs. Instead of **step 5**, biotinylated probes can be easily detected on blots using avidin-biotin peroxidase complex followed by DAB detection.

3.8. Hybridization

3.8.1. Hybridization of Radioactive Probes

1. Make hybridization buffer consisting of: 1X salt solution, 50% formamide, 10% dextran sulfate, 360 µg/mL tRNA in a total volume of 500 µL. Five hundred microliters is sufficient for approx 10 sections.
2. Add labeled riboprobe to the hybridization buffer at a concentration of 20×10^6 *d*pm per 500 µL of hybridization buffer.
3. Heat the probe/hybridization buffer mix to 85°C for 5 min before placing on the sections.
4. Cover slip the sections and place in a humidified airtight chamber.
5. Hybridize overnight at 60°C.

3.8.2. DIG-Labeled Probes

Follow **Subheading 3.8.1.**, **steps 1–4**.

1. Hybridization with DIG-labeled probes is performed at 42°C overnight.

3.8.3. Biotin-Labeled Probes

Follow **Subheading 3.8.1.**, **steps 1–4**.

1. Hybridization with biotin-labeled probes generally is performed at 37°C overnight.

3.9. Posthybridization Washes

3.9.1. Radioactive Probes

1. Heat wash buffer to 55°C and soak slides to remove cover slips.
2. Wash slides at 55°C for 30 min. Replace wash buffer and wash slides for a further 30 min.
3. Wash slides in three changes of RNase buffer and then incubate the sections with 150 µg/mL RNase A in RNase buffer at 37°C for 1 h, with agitation (shaking water bath).
4. Wash the sections in 2X SSC for 45 min at 55°C and then dehydrate through 70, 80, 90, and 100% ethanol followed by air-drying.

3.9.2. Posthybridization Washes—DIG

1. Heat wash buffer to 42°C and soak slides to remove cover slips.
2. Wash slides for a total of 60 min in wash solution, replacing after 30 min.
3. Rinse slides in three changes of RNase buffer and then incubate slides with RNase A (150 µg/mL) at 37°C for 1 h.
4. Wash slides in 2X SSC, 45 min at 37°C.

3.9.3. Posthybridization Washes—Biotin

1. As previously described for DIG probes except that washes are performed at 37°C, instead of 42°C.

3.10. Autoradiography

1. Sections that have been hybridized with ^{33}P-labeled probes can be placed on X-ray film (e.g., XAR-5 Kodak, Hyperfilm) to provide an idea of the success or otherwise of the hybridization reaction. This preliminary autoradiography also can serve as a guide for exposure times required in the liquid emulsion autoradiography step. However, for small pieces of tissue or where only a few cells are labeled in the tissue section, this step may be omitted. Similarly, if a phosphorimager is available, this can be used to obtain a rapid autoradiographic result.
2. In a darkroom under safelight illumination (Ilford safelight filter number 904 or Kodak safelight filter number 2), weigh out 10 g of emulsion, add 6 mL of distilled H_2O, and incubate at 42°C for 2 h to allow the emulsion to melt.
3. Pour the liquefied emulsion into a glass dipping chamber (available from Amersham) and "dip" the experimental slides ensuring all slides are coated evenly and that there are no air bubbles.

4. Remove excess emulsion by allowing slides to drain vertically on absorbent paper in the dark.
5. Place slides into a plastic slide rack and store in a lightproof box containing dessicant.
6. Expose in the lightproof container for 10–20 d, depending on the strength of the hybridization signal.

3.11. Signal Development of Radioactive Probes

1. In a darkroom under safelight illumination, place slides in the diluted developer for 2 min with mild agitation.
2. Stop development by immersion in 0.5% acetic acid for 30 s.
3. Immerse the slides for 2 min in rapid fixer.
4. Rinse slides in running tap water for 5 min.
5. Stain slides with Harris' hematoxylin, rinse in tap water and place in Scott's tap water for 30 s or until hematoxylin appears blue, rinse in water, and then stain with eosin.
6. Dehydrate sections through graded alcohols, rinse in two changes of histolene and mount in nonaqueous mountant.

3.12. Detection of DIG-Labeled cRNA

1. Rinse slides in 1X TBS, three times.
2. Incubate the sections in 1.5% fetal calf serum made in antibody diluent for 30 min.
3. Incubate sections in anti-DIG-alkaline phosphatase, diluted 1:250, for 1 h at room temperature.
4. Wash the slides in 1X TBS, three times.
5. Add alkaline phosphatase substrate BCIP/NBT diluted in NT buffer containing 1% polyvinyl alcohol.
6. Color reaction should then be conducted in the dark. Sections may continue to develop color over several hours or overnight.
7. Wash sections in TBS, counterstain if desired and mount in aqueous mountant.

3.13. Detection of Biotin-Labeled Probe (Amplification Method)

1. Rinse slides in 1X TBS.
2. Quench slides in 0.3% H_2O_2 in methanol, 30 min at room temperature to remove endogenous peroxidase.
3. Wash slides three times in 1X TBS for 5 min each.
4. Block sections using 1.5% serum in 1X TBS for 30 min at room temperature.

Using reagents from a biotinyl tyramide amplification kit (e.g., GenPoint, DakoCytomation) perform the following steps:

5. Add primary streptavidin (1:100–1:1000) and incubate sections for 15 min at room temperature.

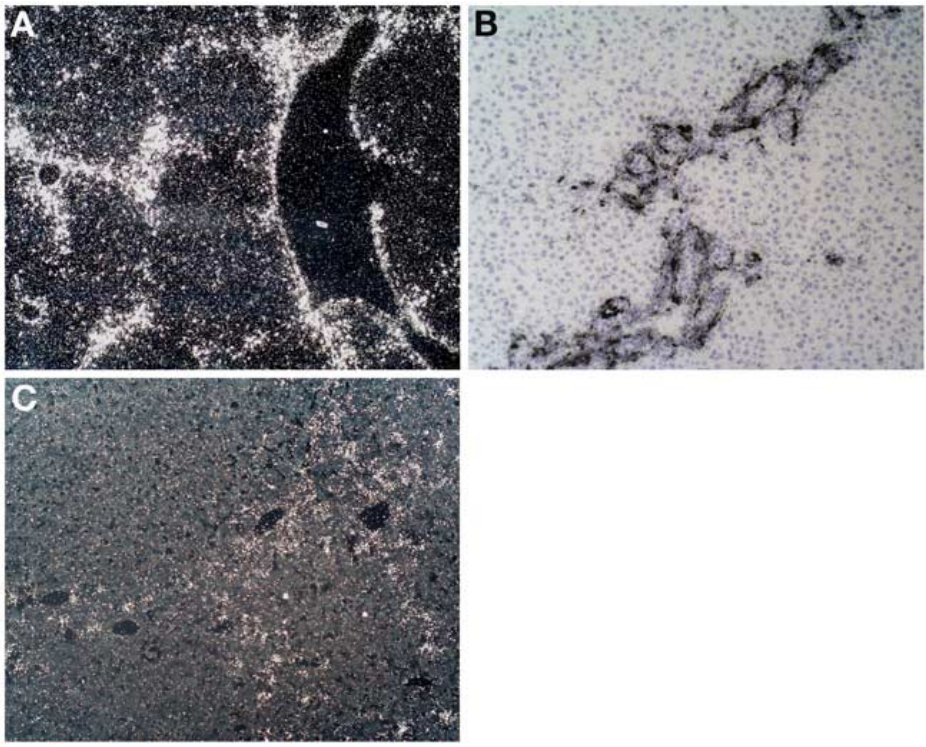

Fig. 1. Procollagen I labeling in frozen sections is considerably stronger than in paraffin sections of the same tissue. Sections are cut on a cryostat and fixed in 4% paraformaldehyde, then treated as for paraffin-embedded tissue. There is a considerable loss of morphology in frozen sections, however the intensity of labeling is much higher in frozen fibrotic liver sections **(A**,**B)** than in paraffin-embedded tissue from the same experiment **(C)**.

6. Wash slides three times in 1X TBS + 0.5% Tween-20, for 5 min.
7. Add biotinyl tyramide (undiluted) to sections and incubate 15 min at room temperature.
8. Repeat **step 6**.
9. Add secondary streptavidin (undiluted) and incubate for 15 min.
10. Repeat **step 6** (*see* **Note 3**).
11. Apply activated DAB (containing H_2O_2) and monitor color development.
12. Counterstain using hematoxylin, dehydrate, and mount using nonaqueous mountant.

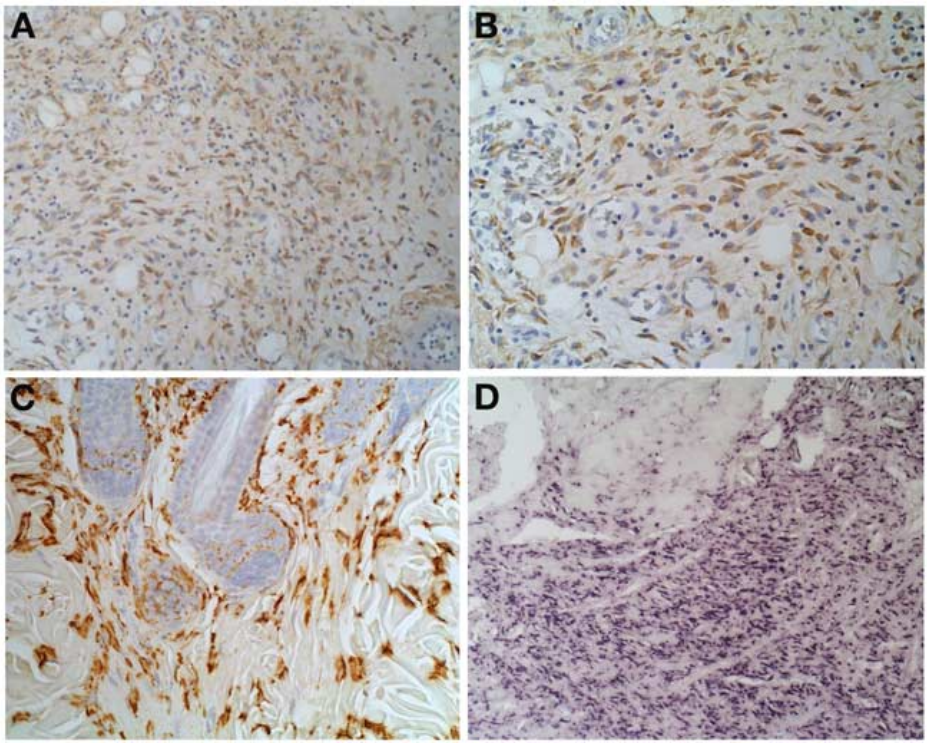

Fig. 2. Skin wound tissue embedded in paraffin labeled with a procollagen I riboprobe. **(A)** The probe is labeled with biotin and detected using ABC; **(B)** the probe is labeled with DIG and detected with anti-DIG horseradish peroxidase; **(C)** the probe is biotinylated but detected using the GenPoint amplification system; and **(D)** the probe is DIG labeled and detected using anti-DIG alkaline phosphatase. The alkaline phosphatase system is more sensitive than horseradish peroxidase using DIG labeling, but the biotin-GenPoint system is the most sensitive of all, detecting procollagen I expression strongly in fibroblasts throughout the dermis.

4. Notes

1. Paraffin embedding is preferable for preservation of morphology; however, it does result in loss of mRNA and consequently reduced sensitivity of mRNA detection by *in situ* hybridization. In some cases, frozen sectioning of unfixed tissue, post-fixation in 4% PFA, and then *in situ* hybridization conducted as described in this chapter can result in markedly increased sensitivity (*see* **Fig. 1** for examples) and in other cases, frozen sectioning may allow detection of expression of genes that prove very difficult to detect in paraffin embedded tissue. An example of this is IL-6 in muscle biopsies (*see* **ref. *11***).

2. Hydrolysis formula:

$$\text{Hydrolysis time T (min)} = \frac{\text{lenth of probe (kb)} - \text{length of desired end product (kb)}}{\left[0.11 \times \text{lenth of probe (kb)} \times \text{length of desired end product (kb)}\right]}$$

For example, starting with a probe that is 1.5 kb and requiring an end product of 0.15 kb, the hydrolysis time is

$$T = 1.5 - 0.15 / 0.11 \times 0.15 = 54.5 \text{ min}$$

3. The tyramide amplification system can be used to increase the sensitivity of *in situ* hybridization when the target mRNA is in low abundance. However, the conditions, including dilutions of the various components need to be optimized for each probe. Increasing the number of cycles of amplification may also lead to an increase in background. Even a single amplification step results in considerably stronger signal when compared to the same probe using biotin and a single streptavidin-peroxidase detection step (for examples, *see* **Fig. 2**).

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4

Tyramide Signal Amplification for DNA and mRNA *In Situ* Hybridization

Ernst J. M. Speel, Anton H. N. Hopman, and Paul Komminoth

Summary

In situ hybridization (ISH) has significantly advanced the study of gene structure and expression in cells and tissues, but its application is often limited by detection sensitivity. The introduction of signal amplification after ISH using tyramides has greatly advanced *in situ* detection methods that also are now applicable in routine diagnostics. In this chapter, we provide detailed step-by-step protocols for synthesis of biotinylated tyramides, multiple-target deoxyribonucleic acid–ISH on cell preparations, both DNA– and messenger ribonucleic acid (mRNA)–ISH on formalin-fixed, paraffin-embedded tissue sections, and tyramide signal amplification for signal detection.

Key Words: *In situ* hybridization; tyramide; signal amplification.

1. Introduction

In situ hybridization (ISH) permits the localization of specific unique or repeated DNA and RNA sequences at the level of individual cells *(1–5)*. It has significantly advanced the study of gene structure and expression and, in addition to morphological identification of cell types involved, ISH also allows some quantification of observations, for instance, with respect to tumor burden or viral load. Despite its high degree of detection specificity, the technique does still not allow the routine visualization of DNA sequences less than 5 kb in size by conventional detection methods and, in the case of tissue sections, the detection sensitivity is even more limited. The threshold levels for mRNA detection are more difficult to determine, with the reported sensitivity limits of 1–20 copies of mRNA per cell being approached only in the most sensitive protocols *(6,7)*.

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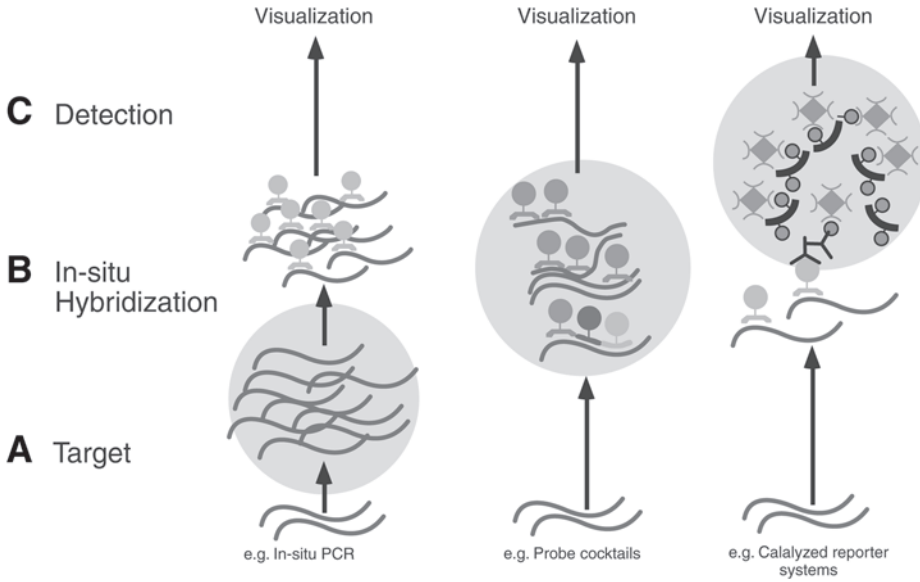

Fig. 1. Principles of amplification systems that have been used for the detection of nucleic acid sequences *in situ*.

In recent years, strategies to improve the threshold levels of nucleic acid detection in ISH studies have included (*see* **Fig. 1** and **Table 1** ***[8–39]***):

1. The use of increased absolute amounts of hybridized probes, for instance, cocktails of oligonucleotide probes or multiple ccRNA probes ***(40–42)***.
2. Protocols to amplify the target nucleic acid sequences before ISH, for example, by *in situ* polymerase chain reaction (PCR ***[8,9,24,43]***), *in situ* self-sustained sequence replication (3SR ***[28,29]***), and cycling or repeated primed *in situ* (PRINS) nucleic acid labeling ***(9,12–15)***, which is not meaningfully different from direct *in situ* PCR. And, in addition, protocols to amplify the probe, for example, by rolling circle amplification of hybridized padlock probes ***(21–23)***.
3. Protocols to amplify the signals produced by the hybridization procedures, for instance, by catalyzed reporter deposition (CARD), now better known as tyramide signal amplification (TSA ***[5,44–48]***), and branched DNA amplification ***(33,34)***.

Target amplification techniques, thus, combine PCR and hybridization *in situ* and have been used to visualize specific amplified DNA sequences (repeated, single-copy, viral) as well as RNA sequences within isolated cells and tissue sections and of DNA sequences in chromosomal preparations ***(8–15,24,28,29,43)***. Theoretically *in situ* PCR techniques are straightforward: fixation of cells or tissue samples; generating semipermeable cell membranes allowing the primers, nucleotides, and enzymes to enter the cell but avoiding

Table 1
Approaches to Amplify Nucleic Acid Target Sequence and (Immuno) Cytochemical Detection Signals *In Situ*[a]

Nucleic acid target amplification	Target	References
In situ polymerase chain reaction (*in-situ* PCR)	DNA	***8–11***
Primed *in situ* labeling (PRINS) and repeated/cycling PRINS	DNA	***10–20***
Rolling circle amplification	Hybridized oligonucleotide (Padlock probe)	***21–23***
In situ reverse transcriptase (RT) PCR	RNA	***9,24–27***
In situ self-sustained sequence replication (3SR)	RNA	***28,29***
In situ transcription/PRINS	RNA	***30–32***
Detection signal amplification		
Branched DNA amplification		***33,34***
Catalyzed reporter deposition/tyramide signal amplification (CARD/TSA)		This chapter
Mirror image complementary antibodies (MICA)		***35***
Enzyme antibody polymer system (EPOS/EnVision)		***36,38***
Enzyme-labeled antibody-avidin conjugates		***38***
End Product Amplification (anti-DAB antibody strategy)		***39***

[a]Adapted from **refs.** ***5*** and ***9***.

the loss of the generated amplificants; in cell PCR amplification; and direct or indirect (by ISH) detection of the amplificants *(9,25)*. The practical procedure, however, is associated with several obstacles, such as low amplification efficiency (restricted sensitivity), poor reproducibility (restricted specificity), and difficulties in quantification of the results *(9,24,49)*. These findings are caused by a number of artifacts as a consequence of PCR amplification *in situ*, such as diffusion of PCR products during and after denaturation from the site of synthesis inside and/or outside the cells, extracellular generation of amplificants and, in the case of direct incorporation of labeled nucleotides during direct *in situ* PCR, the generation of nonspecific PCR products resulting from mispriming, from fragmented DNA undergoing "repair" by DNA polymerase ("repair" artifacts), or from priming of nonspecific DNA or cDNA fragments ("endogenous priming" artifacts). Repair artifacts also may occur in apoptotic cells or samples that have been pretreated with DNase before *in situ* reverse transcriptase (RT)-PCR for mRNA detection *(9,24–27)*. Therefore, it has been recommended that one use a multitude of different controls to allow adequate interpretation of results *(9,50)*. In summary, it appears that the theoretical potential of *in situ* PCR is still much greater than its current practical impact, both in research and clinical studies. However, interesting alternative nucleic acid target amplification procedures are being explored, such as *in situ* strand-displacement amplification *(51)* and the rolling circle amplification approach in combination with so-called padlock probes (*see* **refs.** *21–23* and **Table 1**).

On the other hand, more and more literature is becoming available that describes approaches to amplify the signals after ISH, of which the most promising is the TSA technique. This method, which was introduced by Bobrow et al. *(52)* as CARD for use in immunoblotting and enzyme-linked immunosorbent assays, is based on the deposition of haptenized tyramide molecules by peroxidase activity (**Fig. 2**). The highly reactive intermediates of this reaction are bound to electron-rich moieties of proteins, such as tyrosine, at or near the site of the peroxidase binding site. Visualization of deposited tyramides *in situ* can be either directly after the TSA reaction with fluorescence microscopy, if fluorochrome-labeled tyramides are used, or indirectly with either fluorescence or brightfield microscopy, if biotin, digoxigenin, di- or trinitrophenyl are used as haptens, which can act as further binding sites for anti-hapten antibodies or (strept)avidin conjugates (in the case of biotinylated tyramides; *see* **Table 2**).

In addition, fluorescein and rhodamine can be used efficiently as haptens because specific antibodies against these fluorochromes are commercially available (from, e.g., Dako, Glostrup, Denmark, and Molecular Probes, Eugene, OR). TSA has been implemented easily in immunohistochemistry,

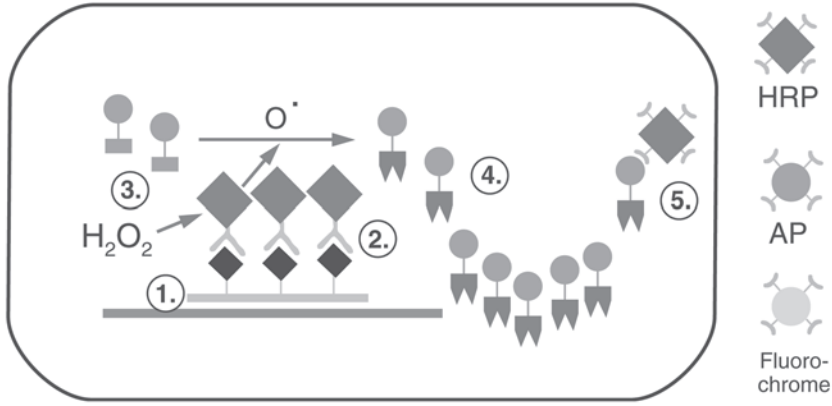

Fig. 2. Principles of ISH combined with TSA using biotinylated tyramides. (1) Hybridization with digoxigenin-labeled probe, (2) anti-digoxigenin HRP detection of the probe, (3) peroxidase–tyramide reaction (TSA) with H_2O_2 and biotinylated tyramides (tyramide and O radical formation), (4) deposition of activated tyramide molecules onto protein moieties, (5) detection of biotin groups with (strept)avidin conjugates using fluorescence of enzyme cytochemical visualization (HRP, horseradish peroxidase; AP, alkaline phosphatase).

allowing an enormous (up to 1000-fold) increase in sensitivity (based on primary antibody dilution) when compared with conventional avidin biotinylated enzyme complex procedures *(53–57)*. In most cases, however, the increase of sensitivity seems to be in the range of 5- to 50-fold or even less. Hence, the optimal dilution for each primary antibody needs to be determined. TSA also has been applied to visualize antigens or incorporated BrdU in fluorescence and electron microscopy with increased sensitivity, as well as in multiple-target approaches *(58–61)*.

Since 1995, TSA has further been introduced in detection procedures for both DNA and RNA ISH on cell preparations and tissue sections, with reported amplification factors in the range of 5- to 10-fold or even higher with preservation of distinct localization of ISH signals. These approaches enabled the detection of: (1) single-copy DNA sequences up to the level of 1 kb *(44,45,47,62–64)*, (2) the simultaneous localization of as many as three different DNA sequences (repetitive as well as single-copy; **Fig. 3A–D**; **Subheading 3.2.**; **Table 3**; *[47,59,65,66]*), and (3) ribosomal RNA and mRNA, ranging from high to low abundance in cell and tissue preparations (*see*, e.g., **Figs. 3E–K** and **4** for a comparison of insulin mRNA signal intensity after conventional detection as well as TSA; **Subheading 3.4.**; *[45,63,67–70]*).

Table 2
Cytochemical Detection Systems That Are Frequently Used for ISH[a]

Label	Detection		
	First layer	Second layer	Third layer
Biotin	Avidin*[b,c]		
Biotin	Avidin*	Biotin-labeled anti-avidin Ab	Avidin*
Hapten[d]	Anti-hapten Ab*		
Hapten	Mouse[e] anti-hapten Ab	Anti-mouse Ab*	
Hapten	Mouse anti-hapten Ab	Rabbit anti-mouse Ab*	Anti-rabbit Ab*
Hapten	Mouse anti-hapten Ab	Biotin-labeled anti-mouse Ab	ABC
Hapten	Mouse anti-hapten Ab	Digoxigenin-labeled anti-mouse Ab	Anti-digoxigenin Ab*

[a]Further amplification of ISH signals can be achieved by combining these detection systems with signal amplification using labeled tyramides (*see* **Subheading 3.5.**).

[b]Ab, antibody; ABC, Avidin Biotinylated enzyme (horseradish peroxidase or alkaline phosphatase) Complex; *, fluorochrome (e.g., coumarin, fluorescein, Cy2, rhodamine, Texas Red, Cy3, Cy5, Cy5.5, Cy7, Alexa dyes) or enzyme (horseradish peroxidase or alkaline phosphatase).

[c]Detection conjugate dilutions should be optimized for one's own experiments. Usually the optimal dilutions of the commercially available conjugates are indicated by the manufacturer.

[d]Hapten = biotin, digoxigenin, dinitrophenyl (DNP), trinitrophenyl (TNP), fluorescein, rhodamine or Alexa dyes.

[e]Anti-hapten Ab raised in another species (e.g., rabbit, goat, swine) also can be used as primary Ab in ISH detection schemes; then, adapted secondary antigens also should be used, if needed.

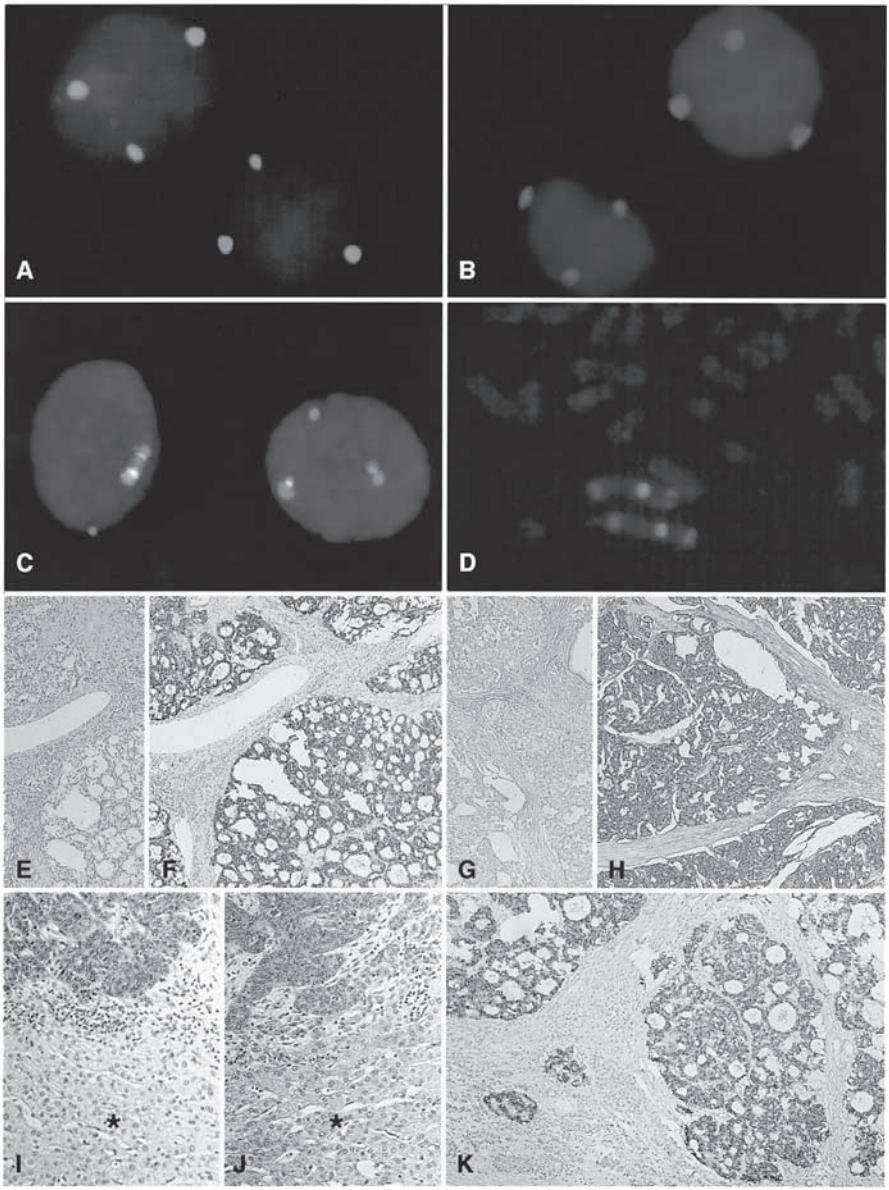

Fig. 3. Results after ISH and TSA on human cell preparations **(A–D)** and routinely fixed, paraffin-embedded tissue sections (**E–K**; *see* **Tables 2** and **3**). **(A**,**B)** Fluorescence detection of three centromere 1 copies in bladder tumor cell line T24 using a chromosome 1-specific centromere probe (biotin-labeled), AvPO/fluorescein-tyramide **(A)** or AvPO/rhodamine-tyramide **(B)** visualization, and PI **(A)** or DAPI **(B)** counter

(*continued on page 40*)

Moreover, TSA can be implemented in diagnostic ISH procedures, including, for instance, the visualization of low and single-copy human papillomaviruses in cell and tissue preparations (**Fig. 5**; **Subheading 3.3.** ***[71–74]***). This approach allows optimal localization of signals and discrimination between viral replication and integration within well-preserved cell nuclei, the latter of which has been associated with malignancy in uterine cervical lesions ***(73–75)***. Furthermore, we have used the TSA technology to increase the sensitivity of our diagnostic mRNA ISH procedure and to shorten the overall turnaround time of the assay (also *see* **Figs. 3E–K** and **4** ***[46,68]***). This approach allows, e.g., the detection of peptide hormone mRNA within one working day and makes the assay suitable for routine diagnostic purposes. Furthermore, it allows the use of diaminobenzidine (DAB) as a chromogen and as a consequence the application of conventional counterstains and the mounting of slides in xylene-based mounting solutions, making the procedure more acceptable to perform in a diagnostic setting.

To date, most of the protocols use biotinylated tyramides in the amplification step, which are commercially available (PE Life Science Products, Dako) or can be synthesized in the laboratory ***(44,45,52,53,65)***. However, the use of biotin can be disadvantageous in tissues with high amounts of endogenous biotin, such as liver or kidney, leading to low signal-to-noise ratios as the result

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Fig. 3. (*continued*) staining. **(C)** Fluorescence detection of three centromere 11 and two 11p15 copies in bladder tumor TCC 9 using a chromosome 11-specific centromere and 11p15 probe (digoxigenin- and biotin-labeled), ShADigPO/fluorescein-tyramide and MADig-GAMPO/rhodamine-tyramide visualization, and DAPI counterstaining. **(D)** Fluorescence detection of chromosome 1q42–43, 1p36, and centromere loci on a lymphocyte metaphase spread using specific biotin-, digoxigenin- and fluorescein-labeled probes, and AvPO-BioGAA-AvPO/coumarin-tyramide, MADig-GAMPO/rhodamine-tyramide and AFluPO/fluorescein-tyramide visualization without counterstaining. **(E,F)** Brightfield detection of insulin mRNA in an insulinoma with **(F)** or without **(E)** antisense oligonucleotide probe (digoxigenin-labeled) using ShADigPO/digoxigenin-tyramide/ShADigPO detection, PO-DAB visualization and hematoxylin counterstaining. **(G,H)** Brightfield detection of vasoactive intestinal polypeptide (VIP) mRNA in a VIPoma with **(H)** or without **(G)** signal amplification using the same visualization procedure as in **E–F**. **(I,J)** Brightfield detection of insulin mRNA in a liver metastasis of an insulinoma, showing digoxigenin-tyramides **(I)** providing less background than biotin-tyramides **(J)** after signal amplification due to the presence of endogenous biotin in liver tissue. Visualization in **(I)** as described in **(E–F)**, in **(J)** with ShADigPO/biotin-tyramide/StreptavidinPO (PE), followed by the PO-DAB reaction and hematoxylin counterstaining. **(K)** Brightfield detection of insulin mRNA in an insulinoma as in **E–F**, now using trinitrophenyl-tyramides and peroxidase-conjugated rabbit anti-trinitrophenyl (Dako) for detection.

Table 3
Protocol for the Fluorescence Detection of Three Nucleic Acid Sequences in Cell Preparations, Hybridized With a Biotin-, Digoxigenin-, and Fluorescein-Labeled DNA Probe, Using Multiple TSA Reactions[a]

Detection step	Time	Temperature
1. Detect biotin with AvPO[b,c] (diluted 1:50–200)	30 min	37°C
2. Visualize PO activity in blue (coumarin-tyramide) as described in **Subheading 3.5., step 2b**	5–15 min	37°C
3. Inactivate residual PO activity with 0.01 *N* HCl	10 min	RT
4. Detect digoxigenin with ShADigPO[c] (diluted 1:100–200)	30 min	37°C
5. Detect PO activity in red (rhodamine-tyramide) as described in **Subheading 3.5., step 2b**	5–15 min	37°C
6. Inactivate residual PO activity with 0.01 *N* HCl	10 min	37°C
7. Detect fluorescein with AFluPO[c] (diluted 1:2000)	30 min	37°C
8. Detect PO activity in green (fluorescein-tyramide) as described in **Subheading 3.5., step 2b**	5–15 min	37°C
9. Embed in Vectashield without DNA counterstain	10 min	RT

[a]For details of detection systems, *see* **Table 2** and **refs. *5,47,48***.

[b]AfluPO, PO-conjugated anti-fluorescein (PE Life Science Products); AvPO, PO-conjugated avidin (Dako); PO, horseradish peroxidase; RT, room temperature; ShADigPO, PO-conjugated sheep anti-digoxigenin Fab fragments (Roche).

[c]For smaller DNA targets, an amplification step with biotinylated goat anti-avidin (1:200; Vector) and AvPO (1:50–200) can be applied in **step 1**, with monoclonal mouse anti-digoxin (1:20,000; Sigma) and PO-conjugated goat anti-mouse IgG (1:200; Dako) in **step 4**, and with rabbit anti-fluorescein (1:2000; Dako) and PO-conjugated swine anti-rabbit IgG (1:200; Dako) in **step 7**.

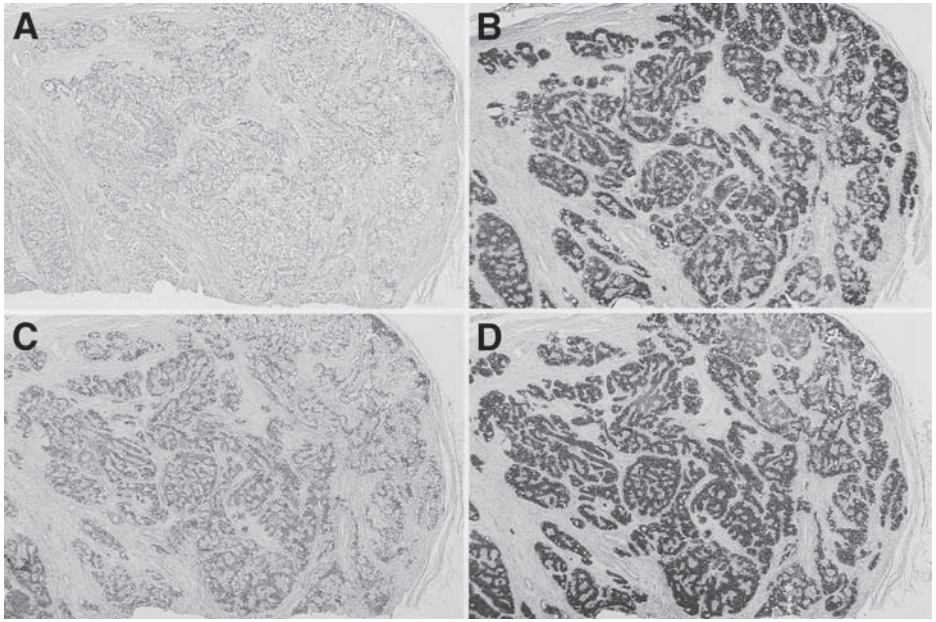

Fig. 4. Comparison of TSA **(B,D)** with conventional detection **(A,C)** of insulin mRNA in an insulinoma, showing higher signal-to-noise ratios with than without the use of TSA. The hybridized antisense digoxigenin-labeled oligonucleotide probe was visualized by the PO-DAB reaction and tissues were counterstained by hematoxylin (*see* **Tables 2** and **3**). Detection with **(A)** ShADigPO, **(B)** ShADigPO/digoxigenin-tyramide/ShAdigPO, **(C)** MADig-Biotinylated horse anti-mouse-Avidin Biotinylated peroxidase Complex (ABC), and **(D)** ABC (*see* **C**)/digoxigenin-tyramide/ShADigPO (*see* **Tables 2** and **3**).

of a high background staining. It is, therefore, desirable to be able to rely on differently labeled tyramides, for instance with digoxigenin, di- or trinitriphenyl, or fluorochromes, which is now possible (PE Life Science Products; *see* **Fig. 3I–K** ***[65,68]***). In addition, these different tyramide conjugates allow the signal amplification of multiple nucleic acid targets or protein targets *in situ* or of a combination of both, if applied consecutively ***(47,65,76)***.

Closer examination of the literature shows that a number of different combinations of cytochemical probe detection (one to three detection conjugate layers) and TSA (using different tyramides, TSA buffer, reaction time, and temperature) systems are applied in various studies, and that optimization of each detection system was necessary to obtain a high signal-to-noise ratio. Because with TSA both specific and nonspecific (background) ISH signals will be amplified, it is essential that nonspecific probe binding and detection

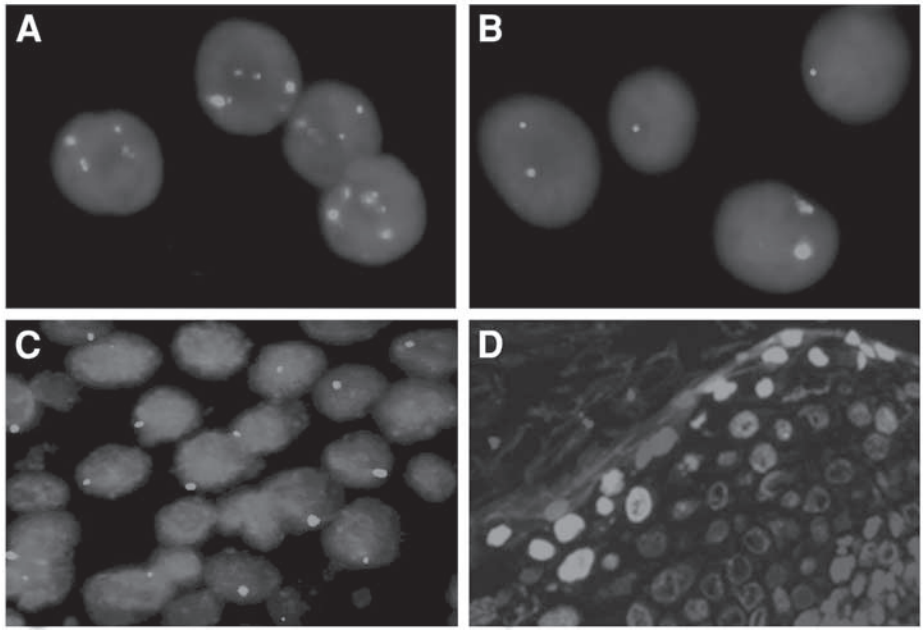

Fig. 5. Fluorescence ISH detection of **(A)** 25–50 HPV 18 genomes (approx 8 kb per genome) at 5 genomic integration sites (8q24) in the HeLa cell line, and **(B)** 1–2 integrated single-copy HPV 16 genomes (at 13q21) in the SiHa cell line, using digoxigenin-labeled HPV 16 genomic DNA and rhodamine-labeled tyramide signal amplification, and Vectashield embedding with DAPI counterstaining. **(C**,**D)** Fluorescence ISH detection of integrated (punctate signals) and episomal (diffuse nuclear staining) HPV 16 genomic DNA in formalin-fixed, paraffin-embedded tissue sections of, respectively, a tonsillar carcinoma **(C)** and a cervical intraepithelial neoplasia lesion **(D)**. Probe, detection, embedding, and nuclear counterstaining is as described in **A** and **B**.

has to be avoided or kept at a minimum to apply this procedure successfully. Therefore, probe hybridization, cytochemical probe detection, and TSA always need to be optimized. For this purpose, an appropriate detection system can be selected from **Table 2** and combined with either a commercially available TSA kit (available from PE Life Science Products as TSA kits, or from Dako as Genpoint kit), used according to the manufacturers instructions, or TSA using freshly synthesized hapten- or fluorochrome-labeled tyramides (*see* **Subheading 3.1.** ***[44,45,47,52,53,65]***) in a phosphate-buffered saline (PBS) buffer of pH 7.6 containing 0.1 *M* imidazole and 0.001% H_2O_2. In our hands, discretely localized ISH signals of high intensity can be obtained by adjusting the number of cytochemical detection layers, the dilution of detection conjugates (usually the first detection layer can be diluted 2- to 10-fold further than in conventional

detection systems), the tyramide concentration in the TSA buffer, and the reaction time (at 37°C *[47,48,68]*). In addition, probe concentrations may be diluted in combination with TSA, which seems to be especially advantageous in cases in which complex DNA probes are used (e.g., chromosome painting, YAC, P1, PAC, BAC, or cosmid probes containing repetitive elements that need to be blocked by competitor DNA, such as Cot-I DNA *[47,59]*). An even quicker but less sensitive way to detect nucleic acids *in situ* is the use of peroxidase-labeled oligonucleotide probes, which can be detected directly with signal amplification using fluorochrome-labeled tyramides *(59,63,69)*. However, these procedures proved to be clearly less sensitive than the indirect procedures previously described, and an additional TSA step may be required to compensate for this *(69)*.

In conclusion, TSA using labeled tyramides has already proven to substantially enhance the detection sensitivity and efficiency in current ISH approaches, and still holds enormous potential to significantly influence future directions in the development of ISH. It is anticipated that efforts for a signal amplification are the key strategy for the future to make ISH more easy to perform, fast, highly sensitive and efficient method than it is to date, and suitable for use in diagnostic laboratories.

2. Materials

2.1. Synthesis of Biotinylated Tyramides for Use in TSA

1. *N*-hydroxysuccinimide ester of biotin (sulfosuccinimidyl-6-[biotinimide]hexanoate; MW 557; Pierce, Rockford, IL).
2. Dimethylformamide (DMF; Pierce).
3. Tyramine-HCl (MW 173; Sigma, St. Louis, MO).
4. Triethylamine (TEA, 7.2 *M*; Pierce).
5. 100% Ethanol (Merck, Darmstadt, Germany).

2.2. Multiple-Target DNA ISH on Cell Preparations

1. Slides with fixed cells and/or chromosomes.
2. RNase A (Roche, Mannheim, Germany).
3. 20X standard saline citrate (SSC): 3 *M* NaCl, 300 m*M* trisodium citrate, pH 7.0.
4. 2X SSC (diluted from stock 20X SSC).
5. Pepsin from porcine stomach mucosa (2500–3500 U/mg; Sigma).
6. PBS: 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.6.
7. 1% formaldehyde (diluted from 37% Formaldehyde [Merck] in PBS with 50 m*M* $MgCl_2$ (for chromosome preparations), without magnesium in the case of cell preparations.
8. Dehydration series of 70, 96, and 100% ethanol.
9. DNA probes (*see* **ref. 47**).

10. Nick translation mix for biotin, digoxigenin, and/or fluorochrome labeling (Roche), Bionick labeling system (Invitrogen, Carlsbad, CA), or separate labeling components (*see* **ref. 77**).
11. Hybridization mixture, pH 7.0: 50–60% formamide, 10% dextran sulfate (Sigma), 2X SSC, 0.05–0.5 mg/mL of sonicated herring sperm DNA (Sigma) as well as yeast transfer (t)RNA (50X excess; Roche).
12. Human Cot-I DNA (Invitrogen).
13. Genomic human placenta DNA (Sigma).
14. 70% Formamide/2X SSC at 70°C.
15. Metal box or heating plate.
16. Wash buffer I: 50–60% formamide, 2X SSC (diluted from 20X SSC), 0.05% Tween-20.
17. Wash buffer II: 2X SSC, 0.05% Tween-20 (repetitive probes) or 0.1X SSC (single copy probes).
18. Rubber cement.
19. Water bath at 37°C.
20. Water bath at 70–75°C.
21. Water bath at 42°C.
22. Humid chamber.
23. Incubator at 37°C.

2.3. DNA ISH on Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Tissue blocks of formalin fixed, paraffin-embedded samples.
2. Super Frost Plus object slides (Merck).
3. Xylene.
4. Dehydration series of 70, 96, and 100% ethanol.
5. 85% formic acid (diluted form 100% [Merck]) and 0.3% H_2O_2 (diluted from 30% [Merck]) in Milli-Q.
6. 0.01 and 0.02 *M* HCl (diluted from 37% HCl [Merck]).
7. Dehydration series of 70% (in 0.01 *N* HCl), 96, and 100% ethanol.
8. 1 *M* NaSCN (Merck).
9. Pepsin (*see* **Subheading 2.2., item 5**).
10. 20X SSC (*see* **Subheading 2.2., item 3**).
11. DNA probes, labeling and hybridization components, and wash buffers as described in **Subheading 2.2., items 9–23**.

2.4. mRNA ISH on Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Tissue blocks of formalin fixed, paraffin-embedded samples.
2. Super Frost Plus object slides (Merck).
3. Xylene.
4. Dehydration series of 70, 96, and 100% ethanol.
5. 0.1% diethylpyrocarbonate (DEPC; Sigma)-treated Milli Q H_2O.
6. PBS (*see* **Subheading 2.2., item 6**).

7. PBS/0.5% SDS: PBS containing 0.5% sodium dodecyl sulfate (SDS).
8. Pepsin (*see* **Subheading 2.2., item 5**).
9. 4% Paraformaldehyde (Merck) in PBS at 4°C.
10. 0.1 *M* Glycine in PBS.
11. 0.3% H_2O_2 in methanol (diluted from 30% H_2O_2 [Merck]).
12. 4X SSC (diluted from stock 20X SSC, *see* **Subheading 2.2., item 3**).
13. Oligonucleotides synthesized without or with internally amino-dTTP-labeled nucleotides. The amino groups can be coupled to different haptens, such as biotin, digoxigenin, or fluorochromes.
14. DIG oligonucleotide tailing kit (Roche).
15. EasyHyb (Roche): 4X SSC, 10% dextrane sulfate, 0.5% SDS, 0.5 mg/mL of salmon testes ssDNA (Sigma), 0.25 mg/mL yeast tRNA (Roche), 100 mg/mL of polyadenylic acid (Sigma), 0.125 U/mL of polydeoxyadenylic acid (Sigma), and 50 pmoles/mL Randomer (PE Life Science Products, Boston, MA).
16. 2X SSC, 1X SSC, and 0.5X SSC (diluted from stock 20X SSC, *see* **Subheading 2.2., item 3**).
17. Plastic hydrophobic cover slips (Gelbond Film; FMC Bioproducts, Vallensbaek Strand, Denmark).
18. Humid chamber.
19. Water bath at 37°C.

2.5. Cytochemical Probe Detection

1. Nonfat dry milk powder or blocking reagent (Roche).
2. Normal goat serum (NGS).
3. 20X SSC (*see* **Subheading 2.2., item 3**).
4. PBS (*see* **Subheading 2.2., item 6**).
5. Tween-20 (Merck).
6. 0.1 *M* Phosphate buffer, pH 7.3.
7. Detection conjugates (*see* **Tables 2** and **3**).
8. Dehydration series of 70, 96, and 100% ethanol.
9. 30% H_2O_2 (Merck).
10. DAB (Walter, Kiel, Germany).
11. 1% Cobalt (II) chloride ($CoCl_2.6H_2O$) (Merck).
12. 1% Nickel (II) sulfamatetetrahydrate ($H_4N_2NiO_6S_2.4H_2O$; Merck).
13. PO-DAB buffer: 50 m*M* Tris-HCl, pH 7.6.
14. Vectashield fluorescence embedding medium (Vector, Burlingame, CA).
15. DAPI: 4',6-diamidino-2-phenyl indole (Sigma).
16. Propidium iodide (PI; Sigma).
17. YOYO (Invitrogen).
18. Hematoxylin: Hematoxylin (Solution Gill no. 3; Sigma):distilled water (1:2–4).
19. Nuclear fast red.
20. Embedding media for brightfield microscopy: we usually use 0.2 *M* Tris-HCl, pH 7.6:glycerol (1:9, v/v) as aqueous-based and TissueTek, Entellan (Merck) or immersion oil (Zeiss) as organic-based embedding medium.

21. DNA blocking buffer: 4X SSC (diluted from stock 20X SSC), 5% nonfat dry milk.
22. RNA blocking buffer: 0.5% blocking reagent in RNA detection buffer.
23. RNA detection buffer: 50 m*M* Tris-HCl, pH 7.4, 300 m*M* NaCl.
24. DNA washing buffers: 4X SSC (diluted from stock 20X SSC), 0.05% Tween-20, and PBS, 0.05% Tween-20.
25. RNA washing buffer: RNA detection buffer containing 0.1% Tween-20.
26. Incubator at 37°C.
27. Fluorescence and brightfield microscope (Leica DM-RE and Zeiss Axiophot).
28. Photographic film (64-100 ASA film for brightfield images and 400–640 ASA for fluorescence images).
29. Charge-coupled device (CCD) camera and image processing software, for instance, Vysis Quips Genetic Workstation (Vysis, Downers Grove, IL) or Metasystems Image Pro System (Metasystems, Sandhausen, Germany).

2.6. CARD Signal Amplification

1. Detection conjugates labeled with peroxidase (*see* **Subheading 2.5.** and **Tables 2** and **3**).
2. PBS (*see* **Subheading 2.2.**, **item 6**).
3. RNA detection buffer (*see* **Subheading 2.4.**, **item 23**).
4. Imidazole (Merck).
5. 30% H_2O_2 (Merck).
6. TSA reaction buffer: 1X Amplification Diluent (PE Life Science Products) or PBS containing 0.1 *M* imidazole, pH 7.6, and 0.001% H_2O_2.
7. Tyramide stock solutions freshly synthesized in DMF (*see* **Subheading 3.1.** and **ref.** ***65***) and diluted to stock solutions of 1 mg/mL with ethanol, or DMF: biotin-, digoxigenin-, trinitrophenyl-, coumarin-, fluorescein-, rhodamine-, and Cy3-labeled tyramides (biotin-labeled tyramides also are available commercially from PE and Dako, and dinitrophenyl- and fluorochrome-labeled tyramides from PE).
8. Washing buffer: PBS containing 0.05% Tween-20.
9. RNA washing buffer: RNA detection buffer containing 0.1% Tween-20.
10. Fluorescence and brightfield microscope (Leica DM-RE and Zeiss Axiophot).
11. Photographic film (64–100 ASA film for brightfield images and 400–640 ASA for fluorescence images).
12. CCD camera and image processing software, for instance, Vysis Quips Genetic Workstation (Vysis) or Metasystems Image Pro System (Metasystems).

3. Methods

3.1. Synthesis of Biotinylated Tyramides for Use in TSA

1. Dissolve 1 mg of the *N*-hydroxysuccinimide ester of biotin in 100 µL of DMF (=1.8 µmol) (*see* **Notes 1** and **2**).
2. Dissolve 10 mg of tyramine-HCl in 1 mL of DMF (=58 µmol) and add an 1.25X equivalent amount of TEA (=10 µL; *see* **Note 3**).
3. 28.4 µL of tyramine/TEA stock solution (=1.76 µmol, *see* **step 2**) is added to 100 µL of biotin ester stock solution (=1.8 µmol, *see* **step 1**), mixed, and left at room temperature in the dark for 2 h (*see* **Notes 3** and **4**).

4. The synthesized biotinylated tyramides are then further diluted with 872 µL of ethanol to obtain a stock solution of 1 mg/mL, which can be stored for several years at 4°C and used without further purification (*see* **Note 5**).

3.2. Multiple-Target DNA ISH on Cell Preparations

1a. Metaphase chromosomes are freshly prepared from peripheral blood lymphocytes by standard methods (*see*, e.g., **refs.** *78–81*), fixed in methanol:acetic acid (3:1), and spread on acid/alcohol cleaned slides (*see* **Note 6**). Alternatively, slides with chromosome spreads may be obtained from Vysis.

1b. Prepare preparations from routinely cultured normal diploid cells and tumor cell lines by: (1) trypsinization (if necessary), harvesting, washing in PBS, and fixation in 70% ethanol (–20°C) (=ethanol suspension; *see* **Note 7**); (2) growing cells on glass cover slips and fixation in cold methanol (–20°C) for 5 s and acetone (4°C) for 3 × 5 s. Air-dry and store at –20°C. (Optionally other fixatives may be utilized, such as 2–4% [para]formaldehyde); (3) cytospinning floating cells onto glass slides (1000 rpm for 5 min), air-drying for 1 h at room temperature, and fixation and storage as described for cells on cover slips.

2. Incubate cell preparations optionally with 100 µL of 100 µg/mL RNase A in 2X SSC under a cover slip for 1 h at 37°C to digest RNA in the cells; wash 3 × 5 min with 2X SSC.

3. Incubate with 50–100 µg/mL pepsin (Sigma) in 0.01 *M* HCl for 10–20 min at 37°C to improve probe accessibility. A stock of pepsin can be made in Milli-Q H_2O (10 mg/mL) and stored at –20°C. This stock can then be diluted with 0.01 *M* HCl to the desired final concentration. Wash slides 1 × 2 min with 0.01 *M* HCl, and 2 × 5 min with PBS (*see* **Note 8**).

4. Post-fix slides for 20 min at 4°C (or 10 min at room temperature) in 1% formaldehyde in PBS with (chromosomes) or without (cells) 50 m*M* $MgCl_2$, wash 2 × 5 min with PBS, and dehydrate through a series of ethanol (70, 96, 100%).

5. Label the DNA probes of interest (repetitive, unique, or repetitive element-containing probes) with hapten (biotin, digoxigenin, dinitrophenyl or fluorochrome)-labeled dUTP according to the nick translation procedure (*see* **Notes 9–11** *[77,82,83]*).

6. Place 10 µL of hybridization mixture containing 1–2 ng/µL of labeled repetitive probe DNA under a 20 × 20-mm cover slip on the slide, and seal optionally with rubber cement. In case of unique and repetitive element-containing DNA probes usually 2–10 ng/µL probe DNA is used, in the latter case together with an excess of Cot-I or total human DNA (up to 100- to 500-fold).

7. Denature probe and cellular DNA simultaneously at 70–75°C for 3–5 min on the bottom of a metal box or on a metal heating plate. Optionally, in the case of repetitive element-containing probes, denature probe DNA and repetitive components in hybridization mixture for 5 min at 75°C, preanneal for 0.5–2 h at 37°C, and apply to cell preparations that have been denatured separately in 70% formamide/2X SSC at 70°C, followed by dehydration in chilled ethanol and air-drying.

8. Perform ISH for 2–16 h at 37°C in a humid chamber.
9. Perform stringent posthybridization washes at 42°C in wash buffer I for 2 × 5 min, and wash buffer II (2X SSC, 0.05% Tween-20) for 2 × 5 min. In case of single-copy DNA probes washing with wash buffer II (0.1X SSC) should be at 60°C (*see* **Subheading 2.2.**, **step 17**).

3.3. DNA ISH on Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Cut serial sections (4–5 µm) from tissue blocks that were formalin-fixed and paraffin-embedded according to standard procedures and mount them on Superfrost Plus object slides. Air-dry and heat the slides overnight at 56°C. Store slides at room temperature until use.
2. Deparaffinize tissue sections in three changes of fresh xylene, and rehydrate in graded alcohols (100, 96, and 70% ethanol, 5 min each) and distilled water (Milli-Q, 5 min).
3. Immerse slides in freshly made 85% formic acid/0.3% H_2O_2 for 20 min at room temperature in a Coplin jar (*see* **Note 12**).
4. Acid dehydrate slides by incubating 3 min each in 70% (in 0.01 *M* HCl), 96, and 100% ethanol and air-dry (*see* **Note 8**).
5. Soak slides in prewarmed 1 *M* NaSCN at 80°C for 10 min in a plastic Coplin jar.
6. Rinse slides for 5 to 10 s in H_2O in the Coplin jar and acid dehydrate and air-dry as in **step 4**.
7. Incubate slides 5–20 min at 37°C in 4 mg/mL pepsin in 0.02 *M* HCl in a prewarmed Coplin jar (*see* **Note 13**).
8. Rinse slides three times shortly in 0.01 *M* HCl in a Coplin jar.
9. Acid dehydrate slides and air-dry as in **step 4**.
10. Label the DNA probes of interest as in **Subheading 3.2.**, **step 5**.
11. Place 10 µL of hybridization mixture containing labeled probe DNA under a 20 × 20-mm cover slip on the slide as in **Subheading 3.2.**, **step 6**.
12. Denature probe and cellular DNA as in **Subheading 3.2.**, **step 7**.
13. Perform ISH for 2–16 h at 37°C in a humid chamber.
14. Perform stringent posthybridization washes as in **Subheading 3.2.**, **step 9**.

3.4. mRNA ISH on Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Cut serial sections (4–5 µm) from tissue blocks that were formalin-fixed and paraffin-embedded according to standard procedures and mount them on Superfrost Plus object slides.
2. Deparaffinize tissue sections in three changes of fresh xylene, and rehydrate in graded alcohols (100, 96, and 70% ethanol, 5 min each) and DEPC-treated distilled water (Milli-Q, 2 × 5 min; *see* **Note 14**).
3. Permeabilize for 15 min in PBS/0.5% SDS at room temperature and for 5–30 min in 0.1% pepsin in 0.2 *N* HCl at 37°C with short Milli-Q washes in between (*see* **Note 15**).
4. Wash two times shortly with PBS at 4°C, post-fix the sections in 4% paraformaldehyde in PBS for 5 min at 4°C, and incubate in 0.1 *M* glycine in PBS twice for 3 min at room temperature to stop the reaction.

5. Incubate sections for 15 min in PBS/0.5% SDS at room temperature and rinse twice for 3 min with Milli Q.
6. Treat sections for 20 min at room temperature with 0.3% H_2O_2 in methanol to inactivate endogenous peroxidase, and rinse again twice for 3 min in Milli Q, and twice for 3 min in 4X SSC at room temperature.
7. Label the oligonucleotide probes of interest with digoxigenin either at their 3' end using a DIG-oligonucleotide-labeling kit or chemically by incorporating amino-dT nucleotides in the oligonucleotide during synthesis which can be coupled to digoxigenin afterwards (*see* **Note 15**).
8. Prehybridize the sections with 30 µL of EasyHyb under a plastic hydrophobic cover slip for 1 h at room temperature, and dip the slides shortly in 4X SSC to remove the cover slips.
9. Hybridize with 30 µL of EasyHyb containing 2–10 ng of Dig-labeled oligonucleotide probe for 2–16 h at room temperature.
10. Perform a quick wash of the slides in 4X SSC to remove ISH coverslips and excess EasyHyb, and follow with stringent washes in 2X SSC, 1X SSC, and 0.5X SSC for twice for 15 min each at 37°C.

3.5. Cytochemical Probe Detection

1. Place 50–100 µL of DNA or RNA blocking buffer on the slide and leave for 5–15 min at room temperature to reduce background staining in the detection procedures. For DNA detection in cell preparations and tissue sections 5% nonfat dry milk powder in 4X SSC is a sufficient blocking buffer, whereas for RNA detection in tissue sections 4% nonfat dry milk powder in RNA detection buffer is needed for sufficient blocking (*see* **Note 12**). Instead of nonfat dry milk powder also 0.5% blocking reagent from Roche in RNA detection buffer has proven to be an appropriate blocking buffer, which we prefer for standardization (*see* **Note 16**).
2. Dilute detection conjugates for DNA detection as follows: dilute avidin conjugates in DNA blocking buffer, and antibody conjugates in PBS, 0.05% Tween-20, 2% NGS. Dilute all conjugates for RNA detection in RNA blocking buffer.
3. For single-target probe detection, incubate the slides for 30–60 min at 37°C with the first detection layer (**Table 2**) and wash twice for 5–10 min in the appropriate DNA washing buffers (4X SSC, 0.05% Tween-20 for avidin conjugates; PBS, 0.05% Tween-20 for antibody conjugates) or RNA washing buffer. Repeat this step with the next detection layer(s) until all incubations are complete.
4. After the last detection layer, wash samples with the appropriate washing buffer twice for 5–10 min, followed by PBS or RNA detection buffer for 5–10 min at room temperature, in the case of DNA or RNA detection, respectively. For fluorescence probe detection, dehydrate samples in an ethanol series (70, 96, and 100% ethanol) and embed as described in **step 6**. For enzyme cytochemical probe detection, visualize the nucleic acid targets by an appropriate enzyme reaction (*see*, e.g., the peroxidase-diaminobenzidine [PO-DAB] reaction with or without nickel-cobalt intensification [**step 5**], or other peroxidase or alkaline phosphatase reactions as described elsewhere ***[5]*** and embed as described in **step 6**).

5. Visualize the nucleic acid targets with the PO-DAB reaction: mix 120 mg of DAB in 200 mL of PO-DAB buffer and add 80 µL of 30% H_2O_2 just before use. Incubate the slides for 5–10 min at room temperature and rinse with tap water. For nickel-cobalt intensification of ISH signals: mix 100 mg of DAB in 200 mL of 0.1 *M* phosphate buffer, pH 7.3, and slowly add 5 mL of 1% cobalt chloride and 4 mL of 1% nickel sulfamate during light vortex mixing. Incubate sections for 5 min at room temperature in this mixture, and then add 66 µL of 30% H_2O_2. Incubate sections for another 5–10 min at room temperature and rinse with tap water (*see* **Note 17**).
6. After all detection layers have been applied to the slides, dehydrate the slides in the case of fluorescence detection in an ethanol series (70, 96, and 100% ethanol) and embed them in Vectashield with or without the addition of 0.2–0.5 µg/mL DAPI (blue fluorescent), PI (red fluorescent), or YOYO (green fluorescent) as DNA counterstain. Chromosomes, nuclei, or tissue sections with nucleic acid targets visualized by enzyme precipitates are counterstained by hematoxylin (e.g., in the case of PO-DAB visualization) or other appropriate DNA counterstains (e.g., 0.1% nuclear fast red in the case of PO-DAB visualization with nickel-cobalt intensification, or (m)ethylgreen. Dehydrate slides with PO-DAB precipitates in an ethanol series (70, 96, and 100% ethanol) and embed in Tissue Tek, or any other aqueous- or organic-based mounting medium. Sections stained with other enzyme precipitates should be counterstained with the appropriate DNA counterstain and embedded in an aqueous- or organic-based mounting medium dependent on the solubility of the enzyme precipitate used ***(5)***.
7. To detect multiple nucleic acid targets labeled with different haptens, choose a combination of cytochemical detection systems from **Table 2**. Multiple DNA targets can be visualized simultaneously by suitable combinations of detection systems using conjugated fluorochromes, or enzymes in combination with different enzyme precipitation reactions (**Table 3** ***[5]***).
8. Examine slides under a fluorescence (fluorochromes) or brightfield (enzyme precipitates) microscope with appropriate filter sets. Microscope images can be recorded directly on photographic film, or with a CCD camera.

3.6. TSA

1. To use TSA, ISH probes should be detected with peroxidase conjugates (*see* **Tables 2** and **3**, and **Subheading 3.5.**), applied subsequently to the slides. Instead of performing an enzyme precipitation reaction, the peroxidase activity is now used to deposit labeled tyramide molecules in the vicinity of the enzyme molecules. These deposited tyramide molecules then can be detected directly, if labeled with fluorochromes, or indirectly with subsequent detection layers (*see* **Fig. 2** and **Table 2**), if labeled with haptens.

2a. For single-target ISH, wash slides with PBS or RNA detection buffer for 5–10 min at room temperature, after application of the last peroxidase detection layer (*see* **Subheading 3.5.**, **step 4**, and **Note 18**). Detect peroxidase activity on the slides by application of 50–100 µL of reaction mixture containing the desired

labeled tyramides for 5–15 min at room temperature or 37°C. For DNA detection on cell preparations and tissue sections, optimal reaction mixtures contain 1:250–1:1000 dilutions of 1 mg/mL stock solutions of coumarin-, fluorescein-, rhodamine-, Cy3-, biotin-, digoxigenin-, and trinitrophenyl-tyramides in TSA reaction buffer (**Note 19**). For RNA detection on tissue sections, optimal reaction mixtures contain 1:50–100 dilutions of the stock solutions in TSA reaction buffer. Thereafter, wash slides 2 × 5 min with washing buffer or RNA washing buffer. For direct fluorescence detection, wash slides 1 × 5 min with PBS or RNA detection buffer, dehydrate in an ethanol series, and embed in Vectashield (*see* **step 3**). For indirect detection (fluorescence or brightfield), apply the next anti-hapten detection layer and follow the protocol in **Subheading 3.4.**, from **step 3** on.

2b. To detect multiple DNA targets *in situ* with different labeled tyramides, hybridize with differently labeled probes and visualize them with a combination of cytochemical detection systems from **Table 2**, all using peroxidase-conjugates. Visualize the probes consecutively with the appropriate detection system and TSA reaction. As an example, **Table 3** shows the detection of three DNA targets with TSA using three different fluorochrome-labeled tyramides. A similar protocol may be utilized for multiple-target mRNA ISH.

3. Embed slides with fluorochrome-labeled tyramides in Vectashield with or without the addition of 0.2–0.5 µg/mL DAPI, PI, or YOYO as DNA counterstain. Chromosomes or nuclei with DNA targets visualized by enzyme precipitates are counterstained by and embedded as described in **Subheading 3.5., step 6**.
4. Examine slides under a fluorescence (fluorochromes) or brightfield (enzyme precipitates) microscope with appropriate filter sets. Microscope images can be recorded directly on photographic film, or with a CCD camera.

4. Notes

1. Synthesis of other tyramide conjugates (e.g., with digoxigenin, trinitrophenyl, coumarin, fluorescein, rhodamine, Cy3, and other haptens) that have been used for *in situ* nucleic acid detection are described elsewhere ***(63,65)***. All synthesized tyramides could be applied at the same concentrations as commercially available tyramides (2–10 m*M*).
2. Because *N*-hydroxysuccinimide esters are prone to hydrolysis and are light sensitive, they need to be freshly dissolved only shortly before tyramide synthesis.
3. Hydrolysis of the biotin ester can be circumvented by performing the tyramide synthesis reaction in water-free medium (e.g., DMF) and by adding TEA in an 1.25X equivalent amount to deprotonate the amino group of tyramine-HCl (final pH should be between 7.0 and 8.0).
4. For an efficient biotin coupling, the biotin ester was added in a 1.1X molar excess compared to the tyramine/TEA.
5. Synthesized tyramides can also be further diluted in DMF or dimethylsulfoxide.
6. After dehydration and air-drying, slides should be used directly, stored for up to 4 wk at room temperature, or stored in a dry box (sealed with a plastic bag) at –70°C for up to several years. Cell suspensions can be stored for up to 3 mo at –20°C.

7. Ethanol suspensions can be stored in this way for several years.
8. After the 0.01 *M* HCl wash, preparations can be dehydrated optionally in 70% (ethanol/0.01 *M* HCL pH 2.0), 96 and 100% ethanol followed by air-drying. This procedure increases cell adhesion (and avoid losing a part of the tissue section during the hybridization step) and preserves cellular and chromosomal morphology.
9. Apart from nick translation other specialized labeling reactions are available to label DNA probes with modified nucleotides, including random primed labeling, PCR, and chemical labeling using, for instance, the ULS system (Kreatech, Amsterdam, The Netherlands; *see also* **refs. *5*,*84***).
10. For good ISH with a high signal-to-noise ratio, labeled probe molecules should be in the range of 100–500 nucleotides long.
11. Selected, labeled probes and probe mixtures for more and more DNA and RNA targets, particularly those that can be used for molecular diagnostics in pathology and genetics, become commercially available from different companies, including Vysis, Dako, Zymed, and PanPath.
12. Pretreatment of sections with formic acid/H_2O_2 and NaSCN strongly improves the efficacy of the subsequent pepsin digestion step, resulting in reproducible and efficient ISH results (*see* **ref. *66***). H_2O_2 may introduce nicks in the DNA, leading to DNA fragmentation. This may in turn cause reduced thermal stability of the formed hybrids. If this is the case, the formic acid/H_2O_2 incubation time should be reduced.
13. Ten minutes is a standard digestion time. Optionally, the degree of digestion may be evaluated under the phase contrast microscope before moving on to the hybridization step. If there is a loss of cell borders, the sample is overdigested. If no individual nuclei are observed, the sample is underdigested and the pepsin incubation may be prolonged. When the nuclear image becomes gray/opaque, the incubations should be stopped.
14. All solutions containing water are prepared with 0.1% DEPC-treated water.
15. A number of steps in our mRNA ISH protocol proved to be essential to obtain high signal-to-noise ratios, including treatments with SDS and pepsin for optimal tissue pretreatment, the use of blocking reagents during detection, and the use of chemically labeled oligonucleotide probes instead of 3' end tailed oligonucleotides ***(68)***.
16. Blocking with 5% nonfat dry milk in 4X SSC proved to be less efficient than with 4% in RNA detection buffer to obtain high signal-to-noise ratios for mRNA ISH.
17. It is recommended to follow every enzyme reaction under the microscope to ensure discrete localization of the *in situ* signals.
18. In comparison with conventional ISH procedures, the same standard probe concentrations were generally used for signal amplification, whereas the detection conjugates, in particular applied in the first layer, were 2- to 10-fold further diluted to obtain high signal-to-noise ratios ***(47*,*68)***. Dependent on the size and amount of nucleic acid target to be detected, one to three detection layers were applied to the preparations before TSA. Generally, one or two layers were sufficient for repetitive DNA sequences (e.g., chromosome centromeres) and high

abundant mRNA, whereas two or three layers were needed for single-copy DNA sequences and intermediate/low abundant mRNA. With respect to repetitive element-containing probes, such as BAC and cosmid probes containing Alu sequences, a high excess of competitor DNA (total genomic or Cot-I DNA) need to be hybridized simultaneously with the probe to prevent reduction of the signal-to-noise ratio. In the light of reducing experimental costs (e.g., if expensive probes are used), optimal amplification conditions can also be found by reducing the probe concentrations in combination with more detection layers and/or higher tyramide concentrations (*see also* **Note 19**).

19. Besides establishing probe concentration and detection system for efficient signal amplification (*see* **Note 18**), the tyramide and H_2O_2 concentration as well as the TSA reaction time are further factors influencing the final result. We recommend to optimize the tyramide concentration in a PBS/0.1 *M* imidazole, pH 7.6, buffer containing 0.001% H_2O_2 during a TSA reaction time of 5–15 min at room temperature or 37°C as starting point.

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5

Expression Analysis of Murine Genes Using *In Situ* Hybridization With Radioactive and Nonradioactively Labeled RNA Probes

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Summary

The term *in situ* hybridization (ISH) refers to all methods allowing the detection of specific DNA (gene loci) or RNA (gene expression products) sequences, using molecular hybridization (base pairing) of labeled nucleic acid probes to target molecules within "intact" cell populations in tissue sections or whole organisms, cultured cells, or chromosomal spreads. For more than two decades, ISH has been one of the main approaches used to characterize gene expression patterns in all laboratory animal models, especially in the context of embryonic development, as well as in human tissue or cell samples for both research and diagnostic purposes. Here, we describe several ISH protocols applied to the analysis of mouse embryos and tissues; this organism has become a reference for mammalian experimental genetics. These protocols use in vitro transcribed RNAs as probes for detection. Radiolabeled probes (using ^{35}S as a radioisotope) allow sensitive ISH on sections of paraffin-embedded material, whereas nonradioactively (digoxigenin) labeled probes can be used both for hybridization of whole embryos (whole-mount ISH) and frozen tissue sections.

Key Words: *In situ* hybridization; gene expression; mouse; embryos; riboprobes; digoxigenin; paraffin sections; cryosections; whole-mount.

1. Introduction

Analyses of the biological function of gene products require detailed characterization of their expression patterns throughout development and in adult organs. *In situ* hybridization (ISH) was developed approx 25 yr ago as a method to detect specific mRNA at the cellular level within tissue sections. It has since been widely used in various experimental animal models, as well as human tissue samples for both diagnostic and research purposes.

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The laboratory mouse has become an indispensable mammalian model for studies of gene function in development, physiology, and/or disease. This is largely a result of the advent of powerful methods allowing transgenesis or generation of site-specific (germinal or somatic) gene mutations. The mouse also has become the chosen mammalian model for large-scale phenotypic screens after random chemical mutagenesis or gene trapping methods (e.g., **ref.** *1*). Mouse functional genomic studies will be further powered by the completion of genome sequencing and the development of tools (DNA microarrays) for transcriptome analysis. For all these reasons, there should be increasing needs for studying murine gene expression patterns at the cellular level, including for large scale projects.

Here, we describe several procedures for ISH analysis of developing mouse embryos or postnatal tissues. All these protocols use in vitro transcribed antisense RNA (riboprobes) as detection probes. The first procedure describes ISH of ^{35}S-labeled probes on tissue sections of paraffin-embedded material (*see* **Note 1**). This method has been extensively used over the last decades and is both sensitive and resolving as a result of the use of a "weak" β-emitter and emulsion autoradiography revelation.

Despite the clear success of radioactive probes for ISH, some associated disadvantages (e.g., the safety measures required for their handling, their limited half-life, or the extensive time required for autoradiography) may refrain their general application. Nonradioactive labeling methods have therefore been developed and have actually opened new opportunities, such as the possibility to detect target mRNAs within whole, unsectioned specimens (whole-mount ISH) or to combine different labels for simultaneous detection of distinct mRNAs in the same tissue sample. Usually, these methods use an indirect detection procedure, in which the RNA probe contains an antigenic (hapten) moiety that can be detected immunocytochemically. In 1987, one of the most widely used system was developed and uses digoxigenin (DIG) as a label for indirect detection.

DIG is a steroid that is only produced by the digitalis plants, which means that an anti-DIG antibody does not bind to any other biological material. When linked to uridine nucleotides (DIG-11-uridine-5'-triphosphate), it can be incorporated into antisense RNA probes generated by standard in vitro transcription reactions. The hybridized probe is then detected using a high affinity anti-DIG antibody that is conjugated to an enzyme, such as alkaline phosphatase or peroxidase (for details, *see* **ref.** *2*). Alternatively, the antibody can be conjugated to fluorescein, rhodamine, or colloidal gold. The detection sensitivity will then depend upon the method used to visualize the antibody conjugate. In the case of alkaline phosphatase, as described herein, the conjugate is visualized with the colorimetric substrates 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and

nitroblue tetrazolium chloride (NBT), which produce a blue precipitate. Two procedures involving DIG-labeled probes are described: (1) one for whole-mount ISH, which is commonly used for post-implantation mouse embryos from 7.5 to 10.5 d post-coitum (dpc) and also can be used for isolated organs at later stages; and (2) one for ISH on frozen sections that can be used for embryos at any stage of development, as well as for adult tissues (*see* **Note 2**).

2. Materials

As a general rule, precautions should be taken to avoid the degradation of RNA probes during synthesis and of cellular mRNA at all steps up to the hybridization reaction. Solutions should be prepared in RNase-free conditions, and manipulators should wear gloves during the procedure. Instruments (e.g., spatulae, dissection tools, magnetic stirrers) and glassware should be cleaned and oven-baked for 4 h at 200°C. After hybridization, the probe-mRNA duplexes are nuclease-resistant, and there is no need to work in RNase–free conditions. However, because some stock solutions are common to prehybridization and posthybridization steps, care should be taken to manipulate solutions during the posthybridization as well. For general instructions about work in RNase-free conditions, *see* **refs. *3*** and ***4***.

2.1. RNA Probe Synthesis

2.1.1. Linearization of Template DNA

1. Purified plasmid DNA containing part or totality of the cDNA (or genomic DNA with exon sequence) of interest, cloned into a vector having SP6, T7, or T3 RNA polymerase promoters.
2. Appropriate restriction enzyme and buffer for plasmid linearization.
3. Water (*see* **Note 3**).
4. Agarose gel 1% in Tris-acetate–ethylene diamine tetra-acetic acid EDTA (TAE) buffer (0.04 *M* Tris-acetate, 0.001 *M* EDTA) and electrophoresis device.

2.1.2. In Vitro Transcription

2.1.2.1. For ^{35}S-Labeled Probes

1. Linear DNA at 1 µg/µL.
2. SP6, T7, or T3 RNA polymerase and manufacturer's transcription buffer (5X concentrated).
3. Dithiothreitol (DTT): 1 *M* stock solution, stored in aliquots at –20°C. Do not autoclave (*see* **Note 4**).
4. 3 *M* Sodium acetate.
5. Absolute ethanol and aqueous 70% dilution.
6. Riboprobe system buffers: ATP, GTP, UTP, CTP (10 m*M* each), 100 m*M* DTT, 5X Transcription Buffer, nuclease-free water (Promega, Madison, WI).

7. RNasin Ribonuclease Inhibitor (Promega).
8. RQ1 RNase-free DNase and manufacturer's buffer (Promega).
9. *Escherichia coli* transfer (t)RNA 5 mg/mL (Roche Diagnostics, Penzberg, Germany).
10. [α^{35}S]-CTP (800 Ci/m*M*, approx 30 TBq/m*M*; Amersham Biosciences, Buckinghamshire, UK; *see* **Note 5**).
11. TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1 [pH 4.5]).
12. Quick Spin columns for radiolabeled RNA purification (Sephadex G-50) (Roche).
13. Scintillation counter.

2.1.2.2. For DIG-Labeled Probes

1. Linear DNA at 1 µg/µL.
2. SP6, T7, or T3 RNA polymerase and manufacturer's transcription buffer (5X concentrated).
3. Dithiothreitol (DTT): 1 *M* stock solution, stored in aliquots at –20°C. Do not autoclave (*see* **Note 4**).
4. 3 *M* Sodium acetate.
5. Absolute ethanol and aqueous 70% dilution.
6. DIG RNA Labeling Mix (Roche).
7. RNase Inhibitor 40 U/µL (Roche).
8. Agarose gel 1% in TAE buffer (*see* **Subheading 2.1.1.**).

2.2. ISH of Paraffin Sections

2.2.1. Collection, Fixation, and Embedding of Specimens

1. Dissection equipment (e.g, forceps, scissors) suitable for the size of the specimens to be collected.
2. Stereomicroscope (e.g., Leica MZFLIII) for dissection of small embryos or tissue samples.
3. Rocking tray allowing gentle agitation during fixation (e.g., Speci-mix, Bioblock Scientific, Illkirch, France).
4. Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO): prepare a 10X stock solution by diluting the powder with 1 L of water, autoclave. The stock solution should be diluted to 1X in water before use (*see* **Note 6**).
5. Paraformaldehyde (PFA): prepare a stock solution at 20% (w/v) in PBS, stored at –20°C (*see* **Note 7**). Warm at 65°C and dilute with PBS to 4% the day of use. PFA at 4% is kept on ice and should be used within 24 h.
6. Paraplast Plus (Fluka, Sigma), melted in a 56°C oven.
7. Absolute ethanol and 25, 50, 75, 100% dilutions in PBS.
8. Xylene (*see* **Note 8**).
9. Embedding moulds or cassettes (e.g., Electron Microscopy Sciences, Hatfield, PA).

2.2.2. Preparation of Paraffin Sections

1. Microtome (e.g., Leica RM2155).
2. Superfrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany) (*see* **Note 9**).

3. Heating plate.
4. Plastic boxes for storage of slides.

2.2.3. Prehybridization Treatments

1. Glass staining jars and slide-holding racks (*see* **Note 10**).
2. Proteinase K: stock solution at 17.7 mg/mL (Roche).
3. Proteinase K digestion buffer: 100 m*M* Tris-HCl, pH 8.0; 50 m*M* EDTA, pH 8.0.
4. Glycine solution: 0.1 *M* glycine, 0.32 *M* Tris-HCl, pH 7.4.
5. 0.1 *M* Triethanolamine, pH 8.0.
6. Acetic anhydride.
7. Xylene (*see* **Note 8**).
8. Absolute ethanol and aqueous 30, 70, and 95% dilutions.

2.2.4. Hybridization

1. Large water bath or hybridization oven set at 60°C.
2. Denhardt's 100X stock solution: 2 g of Ficoll, 2 g of polyvinylpyrrolidone, 2 g of bovine serumalbumine (BSA) for 100 mL of water. Use BSA Pentax Fraction 5. Do not autoclave. Filter through a 0.22-µm filter and store in aliquots at –20°C.
3. Dextran sulfate: prepare a stock solution at 50% (w/v). Warm and vortex thoroughly to obtain complete dissolution. Do not autoclave. Aliquot and store at –20°C.
4. *E. coli* tRNA: 5 mg/mL stock solution, stored at –20°C.
5. DTT: 1 *M* stock solution (*see* **Subheading 2.1.2.**).
6. Hybridization mix: 300 m*M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA, pH 8.0, 1X Denhardt's, 10% dextran sulfate, 0.5 mg/mL tRNA, and 100 m*M* DTT. Can be stored for a few weeks at –70°C.
7. Labeled riboprobe(s) (*see* **Subheading 3.1.2.**).
8. Formamide (*see* **Note 11**).
9. Slide cover slips (GelBond Film, TEBU or Parafilm, Greenwich; *see* **Note 12**).
10. Humidified incubation chamber (*see* **Note 13**).

2.2.5. Posthybridization Washes

1. Two water baths with gentle agitation.
2. Washing solution: 150 m*M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA, pH 8.0, 50% formamide, 10 m*M* DTT.
3. RNase A: 1 mg/mL stock solution, stored in aliquots at –20°C.
4. RNase buffer: 400 m*M* NaCl, 10 m*M* Tris-HCl pH 7.5, 50 m*M* EDTA, pH 8.0.
5. SSC 20X stock solution: 175.3 g of NaCl, 88.2 g of sodium citrate for 1 L of water. Adjust to pH 5.0 and autoclave.
6. Absolute ethanol and 30, 70, and 95% aqueous dilutions.

2.2.6. Autoradiography

1. Darkroom equiped with safe light, water bath, and light-tight entry/exit.
2. HyperFilm^{MP} (Amersham Biosciences).
3. Autoradiography cassettes.

4. Autoradiography emulsion (Kodak NTB-2, Eastman-Kodak, Rochester, NY).
5. 0.6 *M* ammonium acetate.
6. Dipping chamber (e.g., Electron Microscopy Sciences).
7. Silica gel.
8. Dark storage boxes.

2.2.7. Developing, Staining, and Examination

1. Developing solution (Kodak D19).
2. Fixative solution (Kodak AL4).
3. Hoechst 33258 solution in PBS (1/250 w/v).
4. Mounting medium: Glycergel (Dako Cytomation, Glostrup, Denmark).
5. Microscope equipped with dark field illumination and fluorescent lighting, and photography or digital acquisition system (e.g., CoolSnap, Photometrics, Tucson, AZ).

2.3. Whole-Mount ISH

2.3.1. Collection and Fixation of Specimens

1. Dissection equipment (*see* **Subheading 2.2.1.**).
2. PBS 1X (*see* **Subheading 2.2.1.**).
3. PBT: PBS 1X with 0.1% Tween-20.
4. PFA 4% in PBS (*see* **Subheading 2.2.1.**).
5. Ethanol: 25, 50, and 75% dilutions in PBT.

2.3.2. Pretreatments and Hybridization

1. Hybridization oven with a rocking platform (e.g., Hybridization oven/shaker, Amersham Life Science) set at 70°C.
2. 24-well tissue culture plates (e.g., Falcon).
3. PBT (*see* **Subheading 2.3.1.**).
4. Ethanol: 25, 50, and 75% dilutions in PBT.
5. Proteinase K: stock solution at 17.7 mg/mL (Roche), diluted before use with PBT to 10, 5, or 2.5 µg/mL (*see* **Note 14**).
6. Glycine: stock solution at 100 mg/mL, stored at –20°C. Dilute to 2 mg/mL in PBT before use.
7. Postfixation mix: 4% PFA, 0.2% glutaraldehyde in PBS. PFA is prepared as in **Subheading 2.2.1.** Glutaraldehyde 25% (Sigma) is stored in aliquots at –20°C and diluted to 0.2% in PFA before use.
8. Hydrogen peroxide (H_2O_2): stock solution at 30%, kept at 4°C in dark. Dilute to 6% in PBT before use.
9. Formamide (*see* **Subheading 2.2.4.**).
10. SSC 20X (*see* **Subheading 2.2.5.**).
11. SDS 20%: 100 g electophoresis-grade sodium dodecyl sulfate for 500 mL of water. Adjust pH to 7.2. Do not autoclave.
12. tRNA from baker's yeast, at 10 mg/mL (Sigma), store at –20°C.
13. Heparin, sodium salt (Grade I-A, from Porcine intestinal mucosa, Sigma), stock solution at 100 mg/mL, store at –20°C.

14. Prehybridization mix: 50% formamide, 5X SSC, 50 µg/mL tRNA, 1% SDS, 50 µg/mL heparin. Prepare the day of use.
15. DIG-labeled probe stock solution (*see* **Subheading 3.1.3.**).

2.3.3. Posthybridization Washes and Immunocytochemistry

1. Solution 1: 50% formamide, 5X SSC, 1% SDS, prepared the day of use.
2. Solution 2: 50% formamide, 2X SSC, prepared the day of use.
3. TBST: 0.15 *M* NaCl; 0.1 *M* Tris-HCl, pH 7.5; 0.1% Tween-20. A 10X concentrated solution can be prepared and stored at room temperature after autoclaving (*see* **Note 15**).
4. Blocking solution: blocking reagent for nucleic acid hybridization (Roche) at 1.5% (w/v) in TBST.
5. Antibody: anti-DIG alkaline phosphatase conjugated, Fab fragment (Roche). Because this is a polyclonal antibody, a step of pre-adsorption with embryo powder may be performed in order to prevent the nonspecific binding to embryonic tissue and to reduce the background (*see* **Note 16**). Alternatively, the antibody may be used without any purification step and diluted at 1/1000 in the blocking solution.

2.3.4. Staining Reaction and Bleaching

1. TBST (*see* **Subheading 2.3.3.**).
2. NTMT: 0.1 *M* NaCl, 0.1 *M* Tris-HCl, pH 9.5, 50 m*M* $MgCl_2$, 0.1% Tween-20. This solution is not stable and should be prepared the day of use.
3. BCIP: 5-bromo-4-chloro-3-indolyl-phosphate (Roche). Stock solution at 50 mg/mL in 100% dimethylformamide, kept in dark at –20°C.
4. NBT: Nitroblue tetrazolium chloride (Roche). Stock solution at 75 mg/mL in 70% dimethylformamide, kept in dark at –20°C.
5. Staining solution: NTMT with 3.5 µL/mL BCIP and 3.5 µL/mL NBT.
6. PBT (*see* **Subheading 2.3.1.**).
7. Ethanol: 25, 50, and 75% dilutions in PBT.
8. Glycerol 50% in PBT.

2.4. ISH on Frozen Sections

2.4.1. Collection, Fixation, and Embedding of Specimens

1. Dissection equipment (*see* **Subheading 2.2.1.**).
2. PBS 1X (*see* **Subheading 2.2.1.**).
3. PFA 4% in PBS (*see* **Subheading 2.2.1.**).
4. Sucrose, solution at 20% (w/v) in PBS, autoclaved at 0.5 atmosphere and stored at 4°C.
5. Dry ice.
6. Frozen specimen embedding medium (e.g., Shandon Cryomatrix, Thermo Electron Corporation, Pittsburgh, PA).
7. Embedding moulds (e.g., Histomold, Leica microsystems, Wetzlar, Germany).

2.4.2. Preparation of Frozen Sections

1. Cryostat (e.g., Leica CM3050 S).
2. Heating plate.
3. Superfrost Plus glass slides (*see* **Subheading 2.2.2.**).
4. Plastic boxes for storage of slides.

2.4.3. Hybridization

1. Glass staining jars and slide-holding racks.
2. Slide cover slips (*see* **Subheading 2.2.4.**).
3. Humidified incubation chamber (*see* **Note 13**).
4. One large water bath set at 70°C.
5. PBS (*see* **Subheading 2.2.1.**).
6. PFA 4% in PBS (*see* **Subheading 2.2.1.**).
7. Formamide (*see* **Subheading 2.2.4.**).
8. Denhardt's 100X stock solution (*see* **Subheading 2.2.4.**).
9. Salts 10X stock solution: 114 g of NaCl, 14.04 g of Tris-HCl, 1.34 g of Tris-Base, 7.8 g of $NaH_2PO_4.2H_2O$, 7.1 g of Na_2HPO_4, 100 mL of EDTA 0.5 *M* for 1 L of water. Autoclave and store at 4°C.
10. Dextran sulfate (*see* **Subheading 2.2.4.**).
11. tRNA from baker's yeast, at 10 mg/mL (Sigma), store at –20°C.
12. Hybridization buffer: 50% formamide, 1X salts, 10% dextran sulfate, tRNA 1 mg/mL, 1X Denhardt's. This buffer can be prepared in advance and stored at –20°C for several weeks.
13. DIG-labeled probe stock solution (*see* **Subheading 3.1.3.**).

2.4.4. Posthybridization Washes and Immunocytochemistry

1. Glass staining jars, slide racks, cover slips, and humidified chamber (*see* **Subheading 2.2.**).
2. Wax pen (e.g., DAKO).
3. Washing solution: 50% formamide, 1X SSC, 0.1% Tween-20, prepared the day of use and prewarmed at 65°C.
4. TBST (*see* **Subheading 2.3.3.**).
5. Blocking solution: TBST with 2% (w/v) blocking reagent (*see* **Subheading 2.3.3.**) and 20% (v/v) heat-inactivated goat serum (Sigma). The serum is inactivated at 56°C during 30 min. Aliquot and store at –20°C.
6. Antibody: anti-DIG alkaline phosphatase conjugated, Fab fragment (Roche). For immunocytochemistry on sections the antibody is used without pre-absorption, diluted at 1/2000 in the blocking solution.

2.4.5. Staining Reaction and Section Mounting

1. TBST (*see* **Subheading 2.3.3.**).
2. NTMT (*see* **Subheading 2.3.4.**).
3. BCIP and NBT (*see* **Subheading 2.3.4.**).

4. Staining solution: (*see* **Subheading 2.3.4.**).
5. PBT (*see* **Subheading 2.3.1.**) with EDTA 1 m*M*.
6. Ethanol 30, 70, 96, and 100% in water.
7. Clearing solvent (e.g., Histosol Plus, Shandon).
8. Glass cover slips and permanent mounting medium (e.g., Pertex, Labonord, France).

3. Methods

3.1. Probe Synthesis

Both radioactive- and digoxigenin-labeled riboprobes are generated by standard in vitro transcription reactions from a DNA template (approx 300–3000 bp) inserted into a transcription vector. Before transcription, the recombinant plasmid has to be linearized using a restriction enzyme that cuts at the end of the insert (or at any convenient internal site) opposite the appropriate promoter used to generate the transcript. Two linearizations are generally performed, one at each end of the insert to generate either sense or antisense probe. To avoid multiple purification steps, we linearize plasmids in a small amount of buffer in order to obtain a concentrated solution of linear DNA, and use the digestion reaction directly for transcription (*see* **Note 17**). The length of the synthesized RNA will thus vary according to the size of the template DNA insert. We found that probes ranging from 200 bp to 1 kb give satisfactory results, both with radioactive and non-radioactive methods, in whole-mounts and sections. For best results, probes of greater length may be subject to partial alkaline hydrolysis (*see* **Note 18**).

3.1.1. Preparation of Template DNA

1. Set up a 20 µL digestion reaction using 20 µg of plasmid (e.g., 10 µL of a 2 µg/µL plasmid solution), 1–2 U of restriction enzyme per microgram of plasmid and 2 µL of the appropriate reaction buffer.
2. Incubate at 37°C for 2 h to overnight.
3. Verify complete linearization of the DNA by running an aliquot (0.5–1 µL) of the digest in a 1% agarose gel.
4. This digestion reaction yields 1 µg/µL of linear DNA that can be stored at –20°C and used as such for transcription, without further purification (*see* **Note 19**).

3.1.2. In Vitro Transcription of ^{35}S-Labeled Riboprobes

1. Prepare the following AUG mix (sufficient for 10 reactions, can be stored at –20°C): 40 µL of 5X transcription buffer, 20 µL of 100 m*M* DTT, 10 µL of 10 m*M* ATP, 10 µL of 10 m*M* GTP, and 10 µL of 10 m*M* UTP.
2. Prepare the transcription mix (20 µL) by adding 1 µL of linear DNA (from a stock at 1 µg/µL), 4 µL of transcription buffer (5X), 9 µL of AUG mix, 1 µL of RNasin (40 µ/µL), 1 µL of tRNA (5 mg/mL), 3 µL of [α^{35}S]-CTP (800 Ci/m*M*) and 1 µL of RNA polymerase (20 µ/µL).

3. Incubate 2 h at 37°C.
4. Add 10 µL of RQ1 DNase buffer (10X), 65 µL of water and 5 µL of RQ1 RNase-free DNase (1 µ/µL).
5. Incubate 15 min at 37°C.
6. Add 200 µL of water and 300 µL of phenol/chloroform. Vortex, centrifuge for 2 min at 12,000*g*, and transfer the aqueous (upper) phase to a clean tube.
7. Add 0.1 vol of 3 *M* sodium acetate and 2.5 vol of absolute ethanol to the aqueous phase.
8. Vortex and place at –20°C for at least 30 min.
9. Centrifuge for 30 min at 12,000*g* at 4°C (*see* **Note 18**).
10. Discard the supernatant and suspend the pellet in 20 µL of tRNA (5 mg/mL), 10 µL DTT 100 m*M*, and 70 µL of water.
11. Purify on Quick Spin Column, according to manufacturer's recommendations (*see* **Note 20**).
12. Precipitate the purified probe as in **steps 7–9**.
13. Discard the supernatant and wash pellet with cold 70% ethanol.
14. Centrifuge 10 min at 4°C. Discard the supernatant and air-dry the pellet.
15. Dissolve the pellet in 100 µL of 10 m*M* DTT.
16. Proceed to scintillation counting of a 2 µL aliquot.
17. Dilute the probe(s) with 100 m*M* DTT to obtain an activity of 1000–20,000 cpm/µL (*see* **Note 21**).

3.1.3. In Vitro Transcription of Digoxigenin-Labeled Riboprobes

1. Prepare a transcription mix (20 µL) containing 1 µL of linear DNA (from a stock at 1 µg/µL), 4 µL of transcription buffer (5X), 2 µL of DTT (100 m*M*), 2 µL of DIG RNA labeling mix, 2 µL of RNasin (40 U/µL), 1 µL of RNA polymerase (50 U/µL), and 8 µL of water.
2. Incubate for 2 h at 37°C.
3. Check the amount and integrity of transcripts by running a 1 µL aliquot of the transcription reaction on a 1% agarose gel (*see* **Note 22**). During the electrophoresis, the transcription reaction can either be stored on ice, or immediately processed to **step 4**.
4. Add 80 µL of water to the transcription reaction to improve the efficiency of the purification step (*see* **Note 23**).
5. Add 15 µL of 3 *M* sodium acetate and 250 µL of absolute ethanol (*see* **Note 24**). Vortex and place at –20°C for at least 30 min.
6. Centrifuge for 30 min at 12,000*g* at 4°C (*see* **Note 18**).
7. Discard the supernatant, suspend the pellet in 100 µL of water and repeat the purification step (from **step 5**).
8. Wash the pellet with 50 µL of ice-cold 70% ethanol.
9. Centrifuge for 15 min at 4°C.
10. Discard the supernatant and air-dry the pellet.

11. Resuspend the pellet in water, to an estimated 1 µg/µL probe concentration (usually in 100 µL: *see* **Note 22**). The RNA probe can be stored at –20°C at least for months, preferably in aliquots if it has to be used repeatedly.

3.2. ISH of Paraffin Sections

3.2.1. Collection, Fixation, and Embedding of Specimens

1. Dissect the embryos or tissues in PBS under a stereomicroscope, as quickly as possible to avoid RNA degradation and dip them in the 4% PFA solution. Specimens can be pooled for fixation, but the volume of fixative should be at least 10 times that of the sample(s). Accordingly, the containers should be adapted to the size of the specimens (e.g., 2 mL Eppendorf or 12 mL Falcon tubes).
2. Fix overnight, gently shaking, at 4°C (*see* **Note 25**).
3. The following day, wash the specimens in PBS for 2× 10 min.
4. Dehydrate in graded series of ethanol in PBS: 25, 50, 75, 100% for 30 min each and 100% ethanol for 2× 30 min.
5. Transfer in xylene for 2× 1 h (*see* **Note 8**).
6. Place in Paraplast premelted in a 56°C oven and incubate for at least 3 h, with three changes of Paraplast and a final overnight incubation for specimens greater than 5 to 6 mm thick (*see* **Note 26**).
7. Place specimens in disposable moulds, orientate with warm forceps, and let the Paraplast solidify at room temperature.
8. Store at 4°C until use.

3.2.2. Preparation of Paraffin Sections

1. Histological sections are performed using a standard rotary microtome at 5- to 7-µm thickness to generate Paraplast ribbons.
2. SuperFrost slides are overlaid with clean water and placed on a 37°C heating plate.
3. Sections are floated on the slides until they spread out, then water is gently excluded by sloping on clean absorbent paper (*see* **Note 27**).
4. Place the slides in adapted boxes. Leaving the boxes open, air-dry the slides in a 37°C oven for overnight to 2 d.
5. Seal the boxes with tape and store at 4°C until use (*see* **Note 28**).

3.2.3. Prehybridization Treatments

All steps except **steps 5** and **6** are performed at room temperature.

1. Place the slides on slide-holding racks and allow them to reach room temperature (15–30 min).
2. Place the racks in xylene twice for 10 min (deparaffinization).
3. Rehydrate in a graded series of ethanol in PBS: 95, 70, and 30% twice for 5 min and finally in water twice for 5 min.

4. Incubate in glycine solution for 10 min.
5. Equilibrate in prewarmed (37°C) proteinase K buffer for 10 min.
6. Transfer in proteinase K diluted to 1 µg/mL in prewarmed proteinase K buffer and incubate for 30 min at 37°C (*see* **Note 29**).
7. Wash briefly in distilled water and then in freshly prepared 0.1 *M* triethanolamine buffer for 10 min.
8. Dilute acetic anhydride just before use (acetic anhydride is highly instable) to 0.245% (v/v) in 0.1 *M* triethanolamine buffer (agitate thoroughly) and immerse the slides for 10 min.
9. Wash briefly in water and dehydrate in 30, 70, 95, and 100% ethanol for 5 min each (*see* **Note 30**).
10. Allow the slides to air-dry vertically in a rack on a paper towel (*see* **Note 31**).

3.2.4. Hybridization

1. Estimate the total slide area in square centimeters to be covered by each probe.
2. Cut cover slips as small as possible to cover the section(s) to be hybridized. Use 5–10 µL of hybridization mix per cm^2 to be overlaid (as an indication, use 50 µL for a 22 × 40-mm cover slip; *see* **Note 32**).
3. Prepare the amount of hybridization solution required for each probe by adding 4/10 of hybridization mix, 5/10 of formamide and 1/10 of diluted probe.
4. Vortex and heat at 80°C for 2 min.
5. Lay the hybridization solution on slides, at the center of each surface to be covered (*see* **Note 33**).
6. Cover with pre-cut cover slips, handled with forceps. Apply gently to avoid trapping air bubbles.
7. Place the slides horizontally in a moist chamber containing paper towels soaked in 50% formamide, 2X SSC. Seal the box and incubate overnight in a 60°C water bath (*see* **Note 34**).

3.2.5. Posthybridization Washes

All washing steps are performed with gentle agitation.

1. Prewarm all solutions at the appropriate temperatures (37°C and 60°C).
2. Place the slides vertically in temperature-resistant racks.
3. Incubate at room temperature in 4X SSC, 10 m*M* DTT for 30 min. Replace solution and further incubate for 30 min to 1 h. Cover slips should float off from the slides and/or can be gently detached with forceps.
4. Place the racks in washing solution for 30 min at 60°C.
5. Place in RNase digestion buffer twice for 10 min at 37°C.
6. Incubate in RNase A diluted to 20 µg/mL in RNase digestion buffer for 1 h at 37°C.
7. Wash in RNase digestion buffer for 5 min at 37°C.
8. Incubate successively at 60°C, twice for 15 min in a large volume (approx 1 L; use plastic containers) of 2X SSC and twice for 15 min 0.1X SSC.
9. Dehydrate in 30, 70, 95, and 100% ethanol, each for 5 min and air-dry the slides.

3.2.6. Autoradiography

A low-resolution estimation of the signal can be obtained by direct exposure of the slides against a X-ray film. Although the resolution is too low for analysis, this step is useful to evaluate the signal intensity and thus adapt the duration of subsequent emulsion autoradiography. All steps must be performed in a darkroom equipped with a safe light.

3.2.6.1. Film Autoradiography

1. Place the hybridized slides in an autoradiography cassette with a high resolution X-ray film.
2. Expose for 1–4 d at room temperature (*see* **Note 35**).

3.2.6.2. Emulsion Autoradiography

1. Dilute the emulsion (1:1 v/v) with 0.6 *M* acetate ammonium after warming both at 45°C for 30 min. The diluted emulsion can be stored in aliquots at 4°C (*see* **Note 36**).
2. The day of use, place an aliquot of diluted emulsion at 45°C for 30 min. Transfer slowly into the dipping chamber and let stand for 30 min to allow elimination of air bubbles.
3. Slowly dip each slide for approx 4–5 s in the emulsion.
4. Carefully wipe the emulsion from the back of the slides with paper towels.
5. Drain excess emulsion by gently sloping on absorbent paper to obtain a thin, homogenous layer.
6. Place the slides vertically and allow them to air-dry for at least 1 h to overnight.
7. Transfer the slides to a dark box containing dessicant (silica gel), seal with tape adhesive, and wrap in dark plastic bags.
8. Store at 4°C away from any source of radiation, usually for 2–3 wk (*see* **Note 37**).

3.2.7. Developing and Staining

Steps 1 and **2** are performed in the dark room.

1. Allow the sealed boxes to warm up at room temperature for approx 30 min.
2. Place the slides in vertical slide-containing racks.
3. Incubate successively for 2 min in developing solution, 2 min in tap water, 5 min in fixative solution, and then 5 min in water (*see* **Note 38**).
4. Place the racks in Hoechst 33258 solution for 2 min (*see* **Note 39**).
5. Rinse in PBS twice for 5 min.
6. Without allowing the slides to dry, mount them with glass cover slips in Glycergel mounting medium prewarmed at 50°C. Apply slowly from one side to avoid air bubbles trapping.
7. Air-dry the mounted slides before examination.

3.2.8. Microscopy and Imaging

1. Use a microscope equipped with dark-field or epi-luminescent illumination, which allow the silver grains to be visible in white.
2. Hoechst staining requires a microscope equipped with fluorescence light, to visualize tissue structure (*see* **Note 39**).
3. Photograph or digital acquisition is realized with double exposure of bright-field and fluorescence (*see,* for illustration, **Fig. 1**).

3.3. Whole-Mount ISH

3.3.1. Collection and Fixation of Specimens

1. Dissect and fix the specimens as described in **Subheading 3.2.1.** (**steps 1** and **2**).
2. The following day, wash the specimens in PBT three times for 10 min at 4°C (on ice).
3. Dehydrate in graded series of ethanol in PBT (25, 50, 75, 100%) for 10 min each. Transfer to 100% ethanol and store at –20°C until use (*see* **Note 40**).

3.3.2. Pretreatments and Hybridization

The embryos are first rehydrated and transferred (at **step 2**) into 24-well plates. At the end of the rehydration, embryos are checked under the stereomicroscope to control their morphology and to open all cavities where reagents could be trapped and generate background (*see* **Note 41**). To preserve mRNA integrity, all steps preceding the post-fixation are performed on ice or at 4°C. The accessibility of the probe to the target mRNA is increased by moderate digestion with proteinase K. Alternatively, a milder permeabilization treatment with detergents is provided in **Note 42**. This treatment replaces **steps 4** and **5** below. Although the detection enzyme is alkaline phosphatase and not peroxidase, a pretreatment with H_2O_2 has been shown to reduce background, probably because it also inactivates endogenous phosphatases. An incubation at 70°C also is included because high temperatures are known to inhibit endogenous alkaline phosphatase.

1. Rehydrate samples in graded series of ethanol in PBT (75, 50, 25%) for 5–10 min each, on ice (*see* **Note 40**).
2. Wash in PBT three times for 5 min, on ice.
3. Open cavities under stereomicroscope if necessary.
4. Permeabilize samples by treatment with proteinase K (2.5–10 µg/mL), 5–10 min on ice (concentration and time of incubation have to be adapted to the size of the samples, *see* **Note 14**).
5. Stop digestion by incubating in glycine (2 mg/mL) for 5 min on ice.
6. Wash in PBT three times for 5 min on ice.
7. Postfix the samples in 4% PFA–0.2% glutaraldehyde for 20 min on ice (*see* **Note 43**).
8. Wash in PBT twice for 5 min at room temperature.
9. Incubate in prewarmed PBT for 30 min at 70°C (*see* **Note 44**).

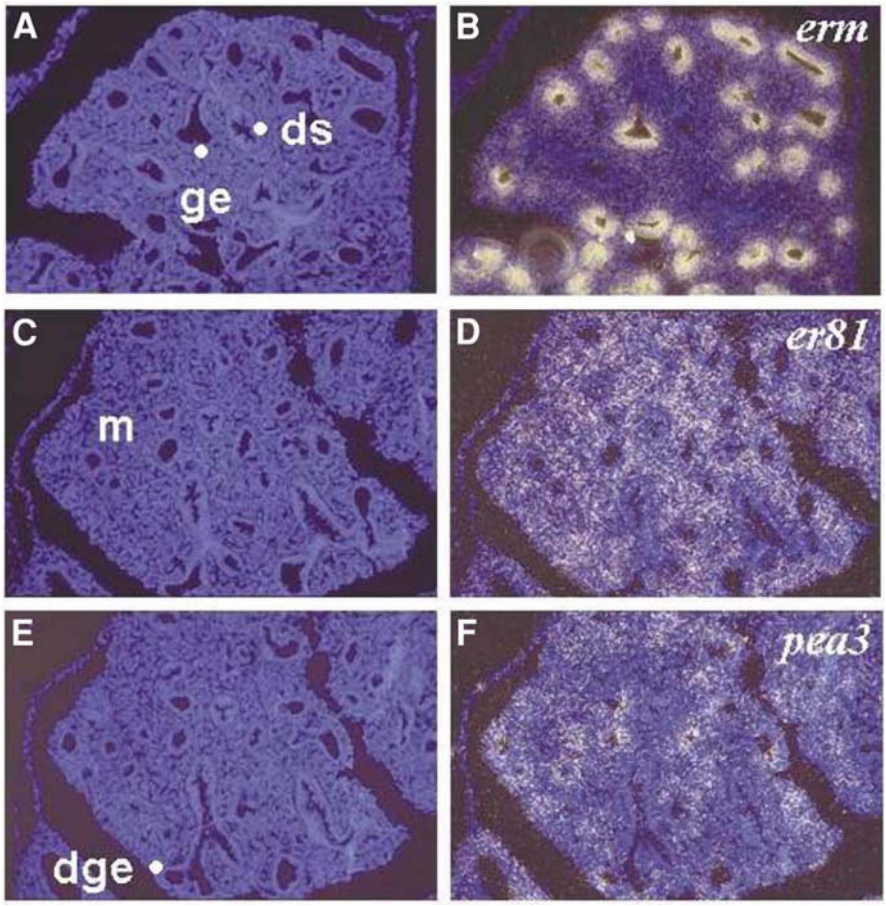

Fig. 1. Comparative ISH expression analysis of three related transcription factors (*erm*, *er81*, and *pea3*) belonging to the Ets family, on serial paraffin sections of lung from an E15.5 mouse fetus. Hybridization has been performed with ^{35}S-labeled *erm* **(B)**, *er81* **(D)**, and *pea3* **(F)** riboprobes and sections were counterstained with Hoechst fluorescent nuclear marker. Observation was performed with darkfield and fluorescent illuminations. **A, C,** and **E** correspond to fluorescent Hoechst staining, which depicts visualization of lung morphology. *Erm* is expressed in the distal growing epithelium (ge) but not in differentiated stalks (ds). *Er81* expression is observed in the mesenchymal compartment (m). *Pea3* is selectively expressed in the distal growing part of the ducts (dge). (Reproduced from **ref. *11*** with the permission of Elsevier Science.

10. Bleach with 6% H_2O_2 in PBT for 1 h at room temperature.
11. Wash in PBT three times for 5 min at room temperature.
12. Incubate in prehybridization buffer at 70°C for 1 h or more, gently shaking.

Fig. 2. Whole-mount ISH analysis of *pea3* **(A)** and *erm* **(B**,**C)** expression in postimplantation mouse embryos using DIG-labeled riboprobes. Expression sites are depicted by blue-purple coloration. **(A**,**B)** Differential expression of *pea3* and *erm* at late headfold stage (E8.5). **C,** *erm* expression pattern at E9.5. br, branchial arches; cd, caudal region; fl, forelimb bud; mb, midbrain; ot, otic placode/vesicle (otocyst); sl, sclerotomes; so, somites. (Reproduced from **ref.** ***12*** with the permission of Elsevier Science.)

13. Replace the prehybridization buffer by the same solution containing the RNA probe, and incubate at 70°C overnight, with gentle agitation (*see* **Note 45**). According to the probe, the concentration used varied from 500 to 1000 ng/mL.

3.3.3. Posthybridization Washes and Immunocytochemistry

Extensive posthybridization washes at different stringencies are performed to wash excess of nonhybridized probe and to dissociate nonspecific hybrids.

1. Wash the samples in prewarmed solution 1 three times for 30 min at 70°C, gently shaking.
2. Wash in a mix of solution three times for 30 min at 70°C (*see* **Note 46**).
3. Wash in solution 2 three times for 30 min at 65°C, gently shaking.
4. Wash in TBST three times for 5 min at room temperature.
5. Incubate in blocking solution, for a minimum of 1 h at room temperature.
6. Incubate overnight at 4°C in anti-DIG-AP-conjugated antibody prepared as detailed in **Subheading 2.3.3.**

3.3.4. Development and Bleaching

All steps are conducted at room temperature. The procedure can be stopped at **step 6**, as embryos can be observed and photographed in PBT, as illustrated in **Fig. 2** (and can be stored for a few weeks in PBT at 4°C). An additional step

of bleaching in ethanol also can be performed. This results in bleaching of nonlabeled tissue (from pinkish to white) and a change in color (purple to blue) for the signal. Altogether, this increases the signal/background ratio. Embryos are then transferred into a mix of glycerol/PBT that allows conservation almost indefinitely at 4°C. Tissues are more translucent in glycerol, and this can improve the observation of the signal and quality of the pictures.

1. Wash in TBST three times for 5 min.
2. Wash in TBST five times for 60 min.
3. Wash in NTMT three times for 10 min (*see* **Note 47**).
4. Incubate in the staining solution, protected from light, with gentle agitation. Check the color reaction regularly (*see* **Note 48**).
5. Wash in NTMT three times for 10 min.
6. Wash in PBT for 10 min.
7. Dehydrate in graded series of ethanol in PBT (25, 50, 75%) for 5–10 min each.
8. Bleach in absolute ethanol for 1 h.
9. Rehydrate in graded series of ethanol in PBT (25, 50, 75%) for 5–10 min each.
10. Wash in PBT for 10 min.
11. Wash in 25% glycerol in PBT for 5–10 min.
12. Store at 4°C in 50% glycerol in PBT.

3.4. ISH on Frozen Sections

This protocol is inspired both from radioactive ISH on sections and whole-mount ISH with DIG-labeled probes. The prehybridization steps are minimal. Indeed, because of the characteristics of frozen sections, no permeabilization treatment is required. A postfixation step is performed, which is critical especially for tissues that have not been fixed before freezing (*see* **Note 49**). The posthybridization washes and immunohistochemical procedure are directly inspired from whole-mount ISH, although the length and the number of washes are usually reduced.

3.4.1. Collection, Fixation, and Embedding of Specimens

1. Dissect and fix embryos or tissue samples as described in **Subheading 3.2.1.** (**steps 1** and **2**).
2. The following day, wash samples in PBS three times for 10 min at 4°C (on ice).
3. Soak in 20% sucrose for 2 h to overnight according to the size of the specimens (the samples must sink at the bottom of the tube).
4. Place samples in molds containing a thin layer of embedding medium. After proper orientation of the specimen, medium is added to fill the mold, which is then frozen on the surface of dry ice. Excess of sucrose solution, and possible air bubbles, must be drained before filling the mould, to avoid heterogeneity that would interfere with sectioning.
5. Once frozen, store at –80°C.

3.4.2. Frozen Sections

1. Tissue sections are performed on a standard cryostat. The tissue block is first allowed to reach –20°C by placing it in the cryostat chamber for at least 30 min.
2. Ten micrometer-thick sections are made at –20°C, and serially collected on slides (*see* **Note 27**). While collecting the sections, keep the slides on a 37°C heating plate. Allow further drying on the heating plate for at least 30 min after completion of a slide.
3. Place slides in sealed boxes and store at –80°C until use (*see* **Note 28**).

3.4.3. Hybridization

1. Defrost sections at room temperature for at least 30 min before use.
2. Transfer slides to a slide rack and post-fix in 4% PFA for 10 min at room temperature.
3. Rinse with PBS twice for 10 min. Slide are ready for hybridization and can be processed to **step 6**.
4. Prepare the probe as follows. Prewarm the hybridization buffer for 30 min at 70°C, in order to reduce the viscosity of the solution. Dilute the probe in the hybridization buffer (usually 1/200). Denature the probe solution for 10 min at 70°C. Vortex thoroughly and quick-spin. The probe is ready for use.
5. Prepare the humidified chamber with Whatman paper (Whatman, Brentford, Middlesex) moistened with a solution of 50% formamide in 1X salts.
6. Take the slides one after another from the PBS solution. Drain most of the liquid but do not let the sections dry. Add 100 µL of probe solution to the slide. Gently cover the slide with a cover slip, to avoid trapping of air bubbles.
7. Place the slides in the humidified chamber, and hybridize overnight at 70°C (*see* **Note 45**).

3.4.4. Posthybridization Washes and Immunocytochemistry

1. Transfer the slides into a slide rack that will allow the cover slips to fall freely in the middle.
2. Incubate in washing solution for at least 15 min at 70°C, until the cover slips have fallen at the bottom of the tank.
3. Incubate in washing solution twice for 30 min at 70°C.
4. Wash in TBST twice for 20 min at room temperature.
5. Prepare the humidified chamber with Whatman paper wetted with water.
6. Take the slides one-by-one from the TBST buffer. Drain the slides and dry carefully around the sections. Circle with a wax pen.
7. Add 200 µL of blocking solution per slide and incubate for at least 1 h in the humidified chamber at room temperature, without cover slips.
8. Drain the blocking solution and immediately add 100 µL of antibody per slide (diluted at 1/2000 in the blocking solution). Cover carefully with a cover slip. Incubate in the humidified chamber overnight, at 4°C.

3.4.5. Staining Reaction and Mounting

1. Transfer the slides into a slide rack that allows the cover slips to fall in the middle.
2. Wash in TBST until the cover slips have fallen at the bottom of the tank (approx 15 min).
3. Wash in TBST five times for 20 min at room temperature.
4. Rinse the slides in NTMT twice for 10 min at room temperature.
5. Prepare the humidified chamber with Whatman paper moistened with PBS or water.
6. Take the slides one-by-one from the NTMT buffer. Drain them and immediately add 100 µL of the staining solution (*see* **Note 47**). Cover with a cover slip. Incubate in the dark (e.g., in a cupboard). The slides can be taken at regular intervals and checked for staining under a dissecting microscope (avoid long exposure and high light intensity). The incubation may vary from a few hours to 3 d. For incubations longer than 24 h, remove the cover slips by washing in NTMT and add fresh developing solution every morning (*see* **Note 50**).
7. Stop the staining reaction by two washes in PBT/EDTA (*see* **Note 51**).
8. Dehydrate the sections in a graded series of ethanol in water (30, 70 96, 100, 100%) for 1 min each, then in clearing solvent twice for 5 min.
9. Mount the sections in permanent mounting medium.

3.5. Controls

When setting an experiment, adequate controls must always be included to ensure both for the sensitivity of detection and specificity of the signals. These should include positive controls to test for quality of the tissue samples (mRNA preservation) and hybridization procedure, and negative controls to assess for nonspecific (background) and/or artefactual labeling. Positive controls should include sample(s) known to contain the mRNA of interest and/or, if the expression pattern of the gene studied is unknown, the use of additional probe(s) whose expression pattern has already been characterized in the tissue(s) studied. As a negative control, a sample known to be devoid of the mRNA of interest should be included in the procedure (although one should be aware that background levels may vary according to the tissue). Sense probes are also commonly used to evaluate nonspecific background labeling. It should be stressed that some sense probes do not yield the same background levels that their corresponding antisense probes. Other valuable technical controls to evaluate specificity of the signal are the omission of either the probe or the antibody (for DIG methods), or the pre-treatment of sections with RNaseA (for both ^{35}S and DIG protocols).

4. Notes

1. Paraffin infusion allows best preservation of the morphological properties of the tissues, and offers the best histological resolution. ISH also can be performed

with radiolabeled probes on cryosections of freshly frozen tissues, which may lead to a more sensitive transcript detection (for a protocol, *see* **ref. *5***). However, histological resolution is much poorer resulting from the lack of tissue prefixation. Paraffin sections are thus recommended, especially for the analysis of early-stage embryos or organs with complex histological structure.

2. Although the protocol presented hereafter can be used for wide applications, some adult tissues may require particular care because of their inherent characteristics. For example, the intestine is rich in endogenous alkaline phosphatase, thus, its analysis requires the use of an alternate detection method. Some tissues, such as the brain, give better results if they are not fixed but fresh frozen. It is thus worth testing the protocol with adequate positive controls (e.g., with the help of gene probes known to be expressed in the tissue of interest), and adapt it to the characteristic of the tissue if necessary. With respect to sensitivity, we feel it is difficult to compare radioactive and nonradioactive methods because they involve very different detection procedures and may be prone to distinct artifacts. Novel users are therefore advised to consider both methods, especially if the gene(s) studied may be expressed at low levels.
3. Precautions should be taken to avoid the degradation of RNA during preparation of the probe. Notably, water used for DNA linearization and transcription of RNA probes needs to be RNase-free. We usually use distilled milliQ water taken directly from the source in oven-baked bottle and autoclaved. This water is also used during all steps of the hybridization protocol. Some users may be more confident using diethyl-pyrocarbonate (DEPC)-treated water, that can be home-prepared (**refs. *3*** and ***4***; or RNA Analysis Notebook, Promega, available on-line) or commercially purchased. Nevertheless, DEPC-treated water is not recommended for the probe synthesis to prevent a potential interference with enzymatic activity.
4. Unless explicitly mentioned, buffers, stock, and working solutions are prepared with water. Stock solutions are usually autoclaved at one atmosphere, unless mentioned otherwise.
5. Manipulation of ^{35}S-containing solutions (especially those involving the ^{35}S source) should be conducted under a chemical hood as a result of the potential risk of aerosol formation. Use of a protective screen is dispensable because ^{35}S will not irradiate through usual containers (including plastic pipet tips). General precautions for radioisotope handling (such as the use of designated radioactive waste) should of course be carefully followed.
6. We favor the use of commercial ready-made PBS because it is easy to prepare and reduces the risk of contamination by RNases. Alternatively, PBS can be prepared according to standard protocol. For PBS 10X: weight 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4.7H_2O$, and 2 g KH_2PO_4 for 1 L water.
7. PFA is difficult to dissolve. We recommend preheating PBS at 65°C and using a heating plate and magnetic stirrer for complete dissolution. PFA solution and vapor are toxic, thus, all steps involving this chemical should be handled in a fume hood, especially the weighting of the powder and heating of the solution during dissolution.

8. Xylene is suitable for most applications of this protocol but can be replaced by toluene, which is less damaging for prolonged immersion of tissues. Xylene and toluene are flammable and should be suitably contained. Manipulations of these agents must be done in resistant containers and under a chemical hood.
9. These slides can be used without any pretreatment. Alternatively, standard, precleaned glass slides can be used after coating with gelatin-alum chrome (for a protocol, *see* **ref. *5***).
10. These should be resistant to high temperature (autoclave, baking) and organic solvents. The equipment used for prehybridization steps (which must be RNase-free) and posthybridization washes (which include a RNase treatment) should imperatively be kept as separate batches. Although ISH with digoxigenin probes (**Subheading 3.4.**) does not include a post-hybridization RNase treatment, it is advised to do the same.
11. The formamide used for prehybridization and hybridization steps needs to be of "molecular grade," whereas "proanalysis p.a." formamide is sufficient for posthybridization washes. An alternative is to deionize p.a. formamide for the (pre-) hybridization steps. This is done by gently stirring Dowex XG8 resin beads (5 g/100 mL formamide) for 1 h (room temperature), followed by filtration through Whatman no. 1 paper. Deionized formamide can be stored in aliquots at –20°C. Note that formamide is toxic and teratogenic. It should thus be handled in a chemical fume hood. As it is not always possible to have the hybridization oven or water bath under the hood, this equipment should be located in an isolated room.
12. 25 × 60-mm cover slips are used if the whole surface of the slide has to be covered. Alternatively, pieces of Parafilm or GelBond Film cut at the appropriate dimension can be used. Glass cover slips usually are used without any pretreatment, but these may be silanized if loss of tissue is experienced during their removal (*see* **ref. *5***).
13. Any hermetic plastic box can be used. We use square (245 × 245 × 20 mm) Petri boxes with four thin stripes of 2-mm Plexiglas glued to support slides arranged in two rows. Humidified paper towels or Whatman paper can be placed between the rows of slides.
14. The permeabilization step with proteinase K is critical. The exact concentration and length of the incubation should be optimized for (a) each type of tissue and (b) each new batch of proteinase K. We found that the proteinase K solution at 17 mg/mL from Roche is stable at 4°C and gives rise to reproducible results. We use this enzyme at 2.5 µg/mL during 5 min for 8.5 dpc embryos, at 2.5 µg/mL during 10 min for 9.5 dpc embryos, at 5 µg/mL during 15 min for 10.5 dpc embryos, and at 10 µg/mL during 15 min for older embryos.
15. TBST can be replaced by MABT (100 m*M* maleic acid, 150 m*M* NaCl, 0.1% Tween-20) in all steps of immunocytochemistry without notable difference in the result. An advantage of MABT seems to be an easier dissolution of the blocking reagent. However, we found that prewarming TBST at 70°C with agitation allows dissolution of blocking reagent within an hour.

16. Preabsorption may be helpful for large (9.5 dpc and older) embryos. To prepare embryo powder (adapted from **ref. 7**), collect 12.5- to 14.5-dpc mouse embryos and homogenize them in a minimal volume of cold PBS, using a douncer. Add 4 vol of acetone, mix and incubate on ice for 30 min. Centrifuge at 10,000*g* for 10 min, discard the supernatant and wash the pellet with cold acetone. Centrifuge at 10,000*g* for 10 min, discard the supernatant. Spread the pellet and grind it to fine powder using a blade on a filter paper to absorb acetone. Store the powder at 4°C. To pre-adsorb the antibody, add 18 mg of embryo powder to 2 mL of TBST and heat at 70°C for 30 min. Cool on ice and add 30 µL of blocking solution and 9 µL of anti-DIG antibody. Gently shake at 4°C for 60 min. Centrifuge for 10 min at 4°C to pellet the embryo powder. Recover the supernatant and dilute it in 13 mL of blocking solution. The antibody is then ready to use.
17. This procedure requires that the template DNA is pure. We usually purify plasmid DNA using small anion-exchange resin column (kits from Qiagen or Roche), followed by extraction with phenol-chloroform and precipitation in ethanol.
18. The length of the probe influences the signal strength: the greater the sequence representation, the greater the signal, although very long probes give lower signals because they penetrate the tissue less efficiently. In addition, the inherent quality of some tissues may require smaller size probes. For example, the condensing mesenchymal cells in the developing limbs during cartilage formation are very compact and target mRNAs are not easily accessible during whole-mount ISH. Increasing the proteinase K treatment is not suitable as it may damage structures such as the ectodermal ridge. In such cases, alkaline hydrolysis of the probe to an average size of 200 bp may be required, according to the following procedure. After ethanol precipitation, discard the supernatant and resuspend the pellet in 200 µL of NaOH 0.1 *N*. Incubate on ice for "t" minutes as determined from the formula:

$$t = \frac{\text{initial length in kb} - \text{final Length in kb}}{0.11 \times \text{initial length} \times \text{final length}}$$

Add 1 µL of glacial acetic acid to stop the hydrolysis and precipitate with 20 µL of 3 *M* sodium acetate and 500 µL of absolute ethanol. Place at –20°C for 30 min and centrifuge at 12,000*g* for 30 min. These steps should be performed between **steps 9** and **10** (**Subheading 3.1.2.**) for ^{35}S-labeled probes, and should replace **step 7** (**Subheading 3.1.3.**) for digoxigenin probes.
19. The DNA also may be extracted (after adjusting the total volume to 100 µL) with an equal volume of phenol/chloroform, followed by an extraction with chloroform and a precipitation with 0.1 vol of 3 *M* sodium acetate and 2.5 vol ethanol. After precipitation and washing with 70% ethanol, resuspend the DNA in water.
20. Use the following procedure for best recovery of the probe. Remove the top cap from the column, then remove the bottom tip. This sequence is necessary to avoid creating vacuum and uneven flow of the buffer. Allow the buffer to drain by gravity and discard it. Place the column in a collection tube and centrifuge at 1100*g* for 2 min. Discard the collection tube and the eluted buffer. Keeping the

column in an upright position, carefully apply the RNA sample (as much as 100 µL) to the center of the column bed. Avoid applying to the sides of the column; if this occurs, nucleotides flow around the medium and are not retained. Overloading the column also results in nucleotides flowing through. Carefully keeping the column in an upright position, place it in a second collection tube. Maintaining the column in an upright position is very important, especially after centrifugation. Tipping the column causes back-flow of the RNA sample, resulting in reduced recovery. Centrifuge for 4 min at 1100*g*. The elution from the second collection tube contains the purified RNA sample.

21. We recommend testing several concentrations for each new probe, in order to find the one giving optimal ISH results. Activities greater than 20,000 cpm/µL are likely to generate elevated background. The specific activity of a given probe can be calculated according to its length and GC content (for details *see* **refs. *3*** and ***4***; or Promega manufacturer's protocol, also available on-line).
22. The transcription reaction usually yields approx 10 µg RNA for 1 µg template DNA. The amount of transcripts can be estimated on the electrophoresis gel: the RNA band should be approx 10-fold more intense than the plasmid DNA band. If the amount of RNA is lower than expected, the probe may be resuspended in 50 or 75 µL of water (**step 11**).
23. Because the amount of labeled RNA transcript is in large excess with respect to template DNA, it is usually unnecessary to remove the template by DNase treatment before an ISH experiment. Such treatment can, however, be performed at this step by incubating the transcription reaction with 2 µL of DNase I (20 U/µL) for 15 min at 37°C.
24. Some users recommend the use of lithium chloride 4 *M* instead of sodium acetate when precipitating RNA. We routinely use sodium acetate and never encountered problems.
25. Shorter fixation times are sufficient for small samples (e.g., 4 h for small embryos), but overnight fixation is often more convenient and will not interfere with subsequent ISH results.
26. Paraplast contains DMSO, which allows better penetration of tissues. Regular paraffin for histology also can be used. Glass containers are used for the intermediate incubation steps.
27. Adjacent sections may be serially placed on several sets of slides in order to perform comparative analyses with various probes, and/or for control experiments (for details, *see* **ref. *8***).
28. Sectioned material (both for paraffin-embedded and frozen tissues) can be stored up to at least 1–2 yr without affecting hybridization quality.
29. Although we routinely use this condition, the proteinase K concentration or incubation time may be adapted for specific tissues, to avoid overdigestion that may lead to loss of tissue integrity and/or of cellular RNA. After the proteinase K treatment, a post-fixation of the sections may be performed with 4% PFA (*see* **Subheading 2.2.1.**) for 15 min, followed by two rinses in PBS. In our experience, this step can be omitted without consequences.

30. The same ethanol series from **step 3** can be used, except the absolute ethanol, which is contaminated with xylene.
31. Slides usually are hybridized the same day, although they can be stored at room temperature, protected from dust, for a few days.
32. According to the experimental design, all sections of a given slide may be hybridized with the same probe under a single, large cover slip. Alternatively, two or three probes (e.g., sense and antisense or different probes) may be hybridized on adjacent sections placed on the same slide, which will then be covered by separate smaller cover slips. Some protocols recommend separating the areas, for instance, with adhesive tape. We find this to be unnecessary if the cover slips are kept well separate.
33. Prepare 10–20% extra hybridization solution to compensate for losses.
34. We routinely use a hybridization temperature of 60°C for many probes. Temperature may however be optimized in a 50–65°C range according to signal/noise ratio, especially in the case of specific probe characteristics (extremely short or long probes, high or low GC content).
35. As a guideline, we find that an absence of visible signal after one night of film exposure means that at least 2 wk of emulsion exposure will be required. Slides can be stored at room temperature if emulsion autoradiography is not performed the same day.
36. The diluted emulsion is stored in small aliquots in dark plastic containers in an area free of any source of radiation.
37. Probes can give highly variable signal/noise ratios. The time of exposure must therefore be adapted for each probe. Exposures longer than 3–4 wk are unlikely to give satisfactory signals.
38. It has been recommended (*see* **ref. *6***) that one equilibrate all solutions at 16°C to prevent emulsion peeling. We currently do this step at room temperature (19–20°C) without any problem. Tap water should however be equilibrated to the dark room temperature.
39. Hoechst is a nuclear fluorescent colorant and requires a fluorescence microscope. It can be replaced by a 0.02% toluidine blue solution, which allows visualization of morphological structures in bright-field illumination. In that case, a conventional microscope equipped with dark-field illumination is sufficient.
40. Ethanol and methanol can be used indifferently for the dehydration and conservation of embryos. In both cases, samples can thus be safely stored at least for months; they can even be placed at room temperature (e.g., for shipment) for a few days. Because of a higher toxicity of methanol, we favor the use of ethanol. Embryos may shrink more in ethanol than in methanol, so it is important to avoid osmotic shock during dehydration and especially rehydration. This is done by using graded series of ethanol in PBT (25, 50, and 75%). Alternatively, embryos can be transferred directly from PBT to absolute methanol (with two changes of 10 min before storage). For rehydration (**Subheading 3.3.2.**), use a graded series of methanol (75, 50, and 25%) in PBT.
41. To avoid such artifacts, enclosed cavities (such as the amniotic and yolk cavities for early post-implantation embryos, the brain vesicles) should be opened or

pierced. This may be done either during the dissection or at the beginning of the ISH procedure. We recommend performing it at this step, especially for people not used to manipulate embryos, as the specimens are fixed and less prone to degradation of cellular RNA. In addition, it is easier to pierce cavities without damaging surrounding tissues after fixation. This step will also allow one to check the integrity of the specimens to be hybridized before starting the procedure. Smaller cavities (e.g., optic and otic vesicles, heart) may be pierced after the ISH during final examination, if trapped precipitate is a problem.

42. Milder permeabilization treatments have been reported in the literature. We found that the mix of non-ionic and ionic detergents (RIPA: NaCl 150 m*M*, Nonidet P-40 1%, Na Deoxycholate 0.5%, SDS 0.1%, EDTA 1 m*M*, Tris-HCl, pH 8.0, 50 m*M*) described in (*see* **ref. 9**) gives good result for delicate structures such as the limb (preservation of the apical ectodermal ridge). To use this treatment, replace **steps 4** and **5** by the following: wash embryos three times in the detergent mix for 30 min at room temperature. Samples are then processed to **step 6**.
43. This post-fixation step is necessary after the permeabilization treatment, as the conditions for RNA probe hybridization are particularly harsh (high temperature and stringency), and embryos may disintegrate in the following steps.
44. This step can be extended to 1 h for large specimens (>10 dpc embryos).
45. Hybridization with DIG-labeled probes is carried out at a higher temperature than for radiolabeled probes. A temperature of 70°C is set up, to achieve a temperature of at least 65°C at the level of the samples.
46. An RNase treatment step may be included in the posthybridization washes. We found that this treatment does not effectively reduce the background. In our hands it results in an increase of the revelation time, thus finally increasing background during revelation. If necessary, the following procedure should be used instead of **step 2**: (1) wash with RNase buffer (0.5 *M* NaCl, 0.1 *M* Tris-HCl, pH 7.5, 0.1% Tween-20), 10 min at room temperature; (2) incubate in RNase buffer containing 20 µg/mL RNase A, 1 h at 37°C; and (3) wash with RNase buffer for 10 min at room temperature.
47. Levamisole at the concentration of 2 m*M* can be added to the solutions used for phosphatase reaction (NTMT and staining solution). Levamisole is included because it inhibits many alkaline phosphatases but not the calf intestinal enzyme that is coupled to the antibody. Because we include a high-temperature treatment in the prehybridization steps and because the hybridization is carried out at 70°C, most of the endogenous phosphatases have already been inactivated, and we usually do not include levamisole at this step.
48. According to the probe, the time required for staining can be highly variable (from 1 to 24 h). If the staining does not appear within the day (3–4 h), samples should be washed in NTMT and stored overnight at 4°C. On the following day the staining reaction can be started at **step 4** with freshly made solutions. In our experience, staining can also efficiently be resumed after several days of storage in PBT.
49. ISH also can be performed on samples that have been frozen immediately, without fixation (e.g., brain). This can be of interest, for instance if sections of the same sample are to be used for immunohistochemistry.

50. An option to increase the sensitivity of the reaction for probes that give a weak signal is to add 5% (w/v) of high molecular weight polyvinyl alcohol in the staining solution. The addition of this polymer enhances the alkaline phosphatase reaction and prevents diffusion of reaction intermediates that can occur during long incubation, resulting in a significant increase in sensitivity without increasing the background (*see* **ref.** ***10***). Polyvinyl alcohol (70–100 kDa, Sigma) is difficult to dissolve and requires heating of the solution at 90°C on a heated stirrer. It is thus better to first prepare a solution of Tris-HCl and NaCl to dissolve the PVA and cool it down before adding $MgCl_2$ and Tween-20. Once ready, add BCIP and NBT as usual. Amplification of the signal can also be performed using the TSA (Tyramide Signal Amplification) developed by Nen Life Science Products (PerkinElmer, Boston, MA). This system requires that the anti-DIG antibody is conjugated to horseradish peroxidase (HRP) instead of alkaline phosphatase. It then uses HRP to catalyze the deposition of the biotin-labeled tyramide (amplification reagent) onto tissue sections. Biotin can then be indirectly detected by chromogenic visualization techniques, via a streptavidin-enzyme conjugate, followed by the appropriate chromogen. For more details on this system, *see* manufacturer's instructions.
51. Slides can be counterstained after the staining reaction with methyl green 10% for 1 min or with eosin 0.1% for 90 s. We tend to avoid counterstaining as it may mask some areas of weak labeling. Sections can be observed under a phase-contrast microscope to identify histological structures if necessary.

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6

Nonradioactive *In Situ* Hybridization on Frozen Sections and Whole Mounts

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Summary

Nonradioactive *in situ* hybridization offers a unique opportunity to study gene expression on samples with preserved histological information. This method makes it possible to locate not only where in a tissue a particular gene is expressed, but in many cases also in which specific cell type it is active. Here, we describe our current protocols for *in situ* hybridization on frozen sections or whole mounts of mouse embryos. The protocols included describe synthesis of a digoxigenin-labeled probe, tissue handling, hybridization of the probe to the mRNA expressed in the sample and signal detection.

Key Words: *In situ* hybridization; gene expression; DIG, nonradioactive; frozen sections; whole mounts; mouse embryo.

1. Introduction

In situ hybridization (ISH) offers a unique opportunity to study gene expression. This method makes it possible to locate not only where in a tissue a particular gene is expressed, but in many cases also in which specific type of cells it is active. Moreover, it is an excellent tool to study gene expression in a limited time window, for instance, during a particular developmental stage. In the current "genomics" era, it can be used for further detailed analysis of genes showing up in transcription profiling experiments ***(1)*** and for comparison of expression patterns between the gene of interest and previously known genes. The drawback of ISH is that although the methodological design is fairly simple (reviewed in **ref.** ***2***), it is nevertheless technically quite challenging. A major source of frustration is the sensitive nature of RNA, which places high demands on reagents and tissue handling and makes low-abundance transcripts hard to detect. Numerous protocols have been developed using different starting mate-

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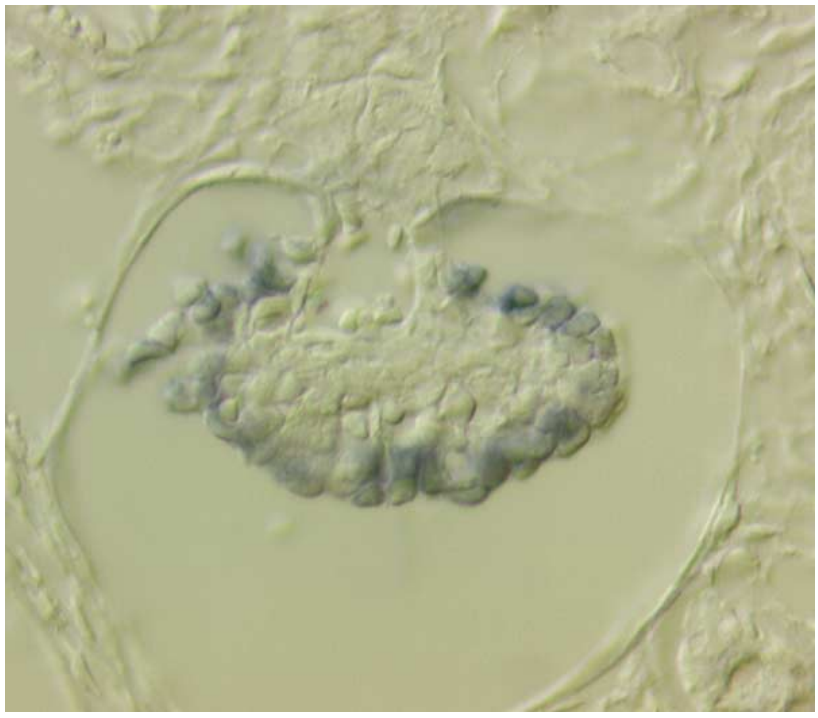

Fig. 1. Expression of podocin is observed in the podocytes, a glomerulus-specific cell type, in a 14-µm frozen section of a mouse embryo from embryonic d 18.5.

rial and signal detection system in hope to overcome such difficulties. The use of nonradioactive probes has not only eliminated the risks associated with radioactive hazards but also has increased the resolution in the results. The preserved histological information of the sample in a nonradioactive system is an immense advantage (**Fig. 1**). This information makes it possible to combine the mRNA expression data with a subsequent immunohistochemical staining on the same sample. Such analysis can provide unique information about expression pattern. By staining with an antibody which specifically labels a certain kind of cells, the mRNA expression revealed by ISH can be exactly localized ***(1,3)***.

In this chapter, we describe our current protocols for ISH on frozen sections and whole-mount *in situ* hybridization (WISH) of mouse embryos. The protocols included describe the synthesis of a digoxigenin (DIG)-labeled probe, tissue handling, hybridization of the probe to the mRNA expressed by cells in the sample and signal detection.

2. Materials

2.1. Common Reagents

1. Cloning vector containing RNA polymerase promoter sequences.
2. Restriction enzymes for linearization of vector.
3. Agarose and gel electrophoresis equipment.
4. Phenol:chloroform:isoamyl alcohol (25:24:1, Sigma, St. Louis, MO).
5. 4 *M* LiCl.
6. 99.5 and 70% ethanol.
7. RNase-free H_2O. We only use double-distilled water for this purpose.
8. DIG RNA-Labeling Mix, transcription buffer, and RNA polymerase (T7, T3, and SP6; Roche, Mannheim, Germany).
9. RNasin RNase inhibitor (Promega, Madison, WI).
10. 0.2 *M* Ethylene diamine tetraacetic acid (EDTA), pH 8.0.
11. DNA-free (Ambion, Austin, TX).
12. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4. Check pH. If necessary, adjust to pH 7.2 by saturated Na_2HPO_4.
13. 4% paraformaldehyde (PFA): dissolve PFA in PBS at 70°C. Chill on ice and set pH to 7.5 by adding NaOH. This solution must be prepared fresh the day of use or aliquoted and stored at –20°C. Do not use re-thawed PFA.
14. PBT: PBS with 0.1% Tween-20.
15. 20X standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* sodium citrate. Adjust pH to 7.0 with HCl. This stock solution is used for dilution as indicated in methods section.
16. Anti-Digoxigenin-AP Fab fragments (Roche).
17. Nitroblue tetrazolium chloride (NBT) 100 mg/mL and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) 50 mg/mL (Roche). These reagents are light sensitive and should be added to the indicated buffer shortly before use.
18. Levamisole (Sigma). A stock solution of 1 *M* is prepared by dissolving levamisole in H_2O. This solution is aliquoted and stored at –20°C.

2.2. Additional Reagents Required for ISH on Frozen Sections

1. Sucrose solution: 30% sucrose (Sigma) in PBS. Can be stored at 4°C if adding 0.02% NaAz.
2. Tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, The Netherlands).
3. Superfrost plus slides (Menzel-Gläser, Germany).
4. Equipment for cryosectioning.
5. Glass cuvets for slides.
6. 0.1% diethylpyrocarbonate (DEPC) in PBS. The DEPC (Sigma) is dissolved in PBS just prior to use. After use the solution is autoclaved to inactivate the DEPC before waste.
7. Liquid blocker (Daido Sangyo Co., Tokyo, Japan).
8. Hybridization solution: 50% deionized formamide (Sigma), 5X SSC, 40 µg/mL salmon sperm DNA (Ambion).

9. Humified box saturated with 50% formamide and 5X SSC for hybridization or with H_2O for antibody incubation and color development. This formamide does not need to be ionized.
10. Parafilm (Pechiney Plastic Packaging, Chicago, IL).
11. RNase A (Ambion), RPA grade.
12. Buffer 1: 100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
13. Blocking solution: 0.5% blocking reagent (Roche) in Buffer 1. The blocking reagent is dissolved according to manufacturer's instruction, aliquoted and stored at –20°C.
14. Buffer 2: 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 50 m*M* $MgCl_2$, 5 m*M* levamisole. This buffer is prepared fresh.
15. Stop solution: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0.
16. 95 and 99.9% ethanol, xylene, cover slips.

2.3. Additional Reagents Required for WISH

1. 2-mL Eppendorf tubes with snap lid and round bottom.
2. Methanol.
3. H_2O_2 (30%).
4. Proteinase K (Invitrogen, Carlsbad, CA): 10 mg/mL dissolved in 50% glycerol, aliquoted, and stored at –20°C.
5. Glycine (Sigma).
6. 25% glutaraldehyde.
7. Hybridization solution: 50% deionized formamide, 1.3X SSC, 50 µg/mL yeast RNA (Ambion), 0.2% Tween-20, 0.8% sodium dodecyl sulphate, 100 µg/mL heparin (Sigma), 5 m*M* EDTA, pH 8.0. This solution can be aliquoted and stored at –20°C. (One embryo needs approx 11 mL.)
8. 10X TBST: 1.37 *M* NaCl, 27 m*M* KCl, 0.25 *M* Tris-HCl, pH 7.5, 1% Tween-20. On day of use, dilute to 1X TBST with H_2O. Add levamisole to a final concentration of 2 m*M*.
9. MABT: 0.1 *M* maleic acid, 0.15 *M* NaCl, 1% Tween-20. Make fresh each time. Prepare a stock solution of 1 *M* maleic acid and adjust to pH 7.5 with NaOH. This stock solution can be stored for several months.
11. Sheep serum (Sigma). Before use, the serum is heat-treated at 55–60°C for 30 min and then stored frozen in aliquots at –20°C.
12. NTMT: 100 m*M* NaCl, 100 m*M* Tris-HCl, pH 9.5, 50 m*M* $MgCl_2$, 0.1% Tween-20, 2 m*M* levamisole.
13. BABB (benzyl alcohol:benzyl benzoate; 1:2).
14. Glycerol 87%.

3. Methods

The methods described in this chapter outline: (1) the synthesis of nonradioactive probes labeled with DIG, sample preparation and step-by-step instructions for ISH on frozen sections, (2) as well as on whole mounts (3) of mouse

embryos. Although these protocols differ in procedures and solutions, the same probes can be used. Consequently, reagents for signal visualization, including an alkaline phosphatase-coupled antidigoxigenin antibody and the BCIP and NBT substrates, are the same. It is important to note that all steps are performed RNase free from the start of purification of the vector in **Subheading 3.1.1.** until the end of the hybridization (*see* **Note 1**). Furthermore, one should be aware that the results from an ISH can sometimes be difficult to interpret correctly. For example, certain tissues contain a higher level of endogenous alkaline phosphatase, which may generate false-positive signals when using the DIG system. A probe that binds too much, which results in nonspecific signal, or a probe that binds too little, which appears as a lack of detectable signal, are other problems that can occur owing to unsuitable hybridization conditions for a particular probe. Therefore, it is important to include proper controls when setting up a new experiment. In addition to the antisense probe, which is used for the experiment, it is common to synthesize the corresponding sense probe as well. The sense probe can be used as a negative control because it has the same GC content as the antisense probe and therefore should hybridize under the same conditions. An experiment without addition of any probe can be useful to check for background signaling. A probe that detects a gene with known expression pattern can be useful as a positive control, to make sure that the sample material and solutions are working properly (**Fig. 2**). Finally, it is particular important to repeat the experiment.

3.1. Probe Synthesis

The synthesis of nonradioactive probes labeled with DIG is described in **Subheadings 3.1.1.–3.1.3.** We usually use probes between 300 and 1500 bp in length, which give good signal to background ratio.

3.1.1. Linearization and Purification of Vector

The first step in the ISH procedure is to synthesize the probe. We use riboprobes, which are made by in vitro transcription in the presence of DIG-labeled uracils. It is feasible (but not necessary, *see* **Note 2**) to use cDNA fragments cloned into a vector containing two of the RNA polymerase promoter sequences T7, T3, and SP6, which are located in opposite orientation on each side of the insert. The use of these fragments makes it possible to selectively synthesize either strand of the template sequence. Before in vitro transcription, the template plasmid is linearized by restriction enzyme cleavage to enable productions of "run off" transcripts (**Fig. 3**). We usually cleave up to 20 µg vector in a 100-µL reaction overnight under appropriate conditions for the restriction enzyme used, as described by the manufacturer. An enzyme that creates 5'-overhang should be chosen for the cleavage. Because it is important

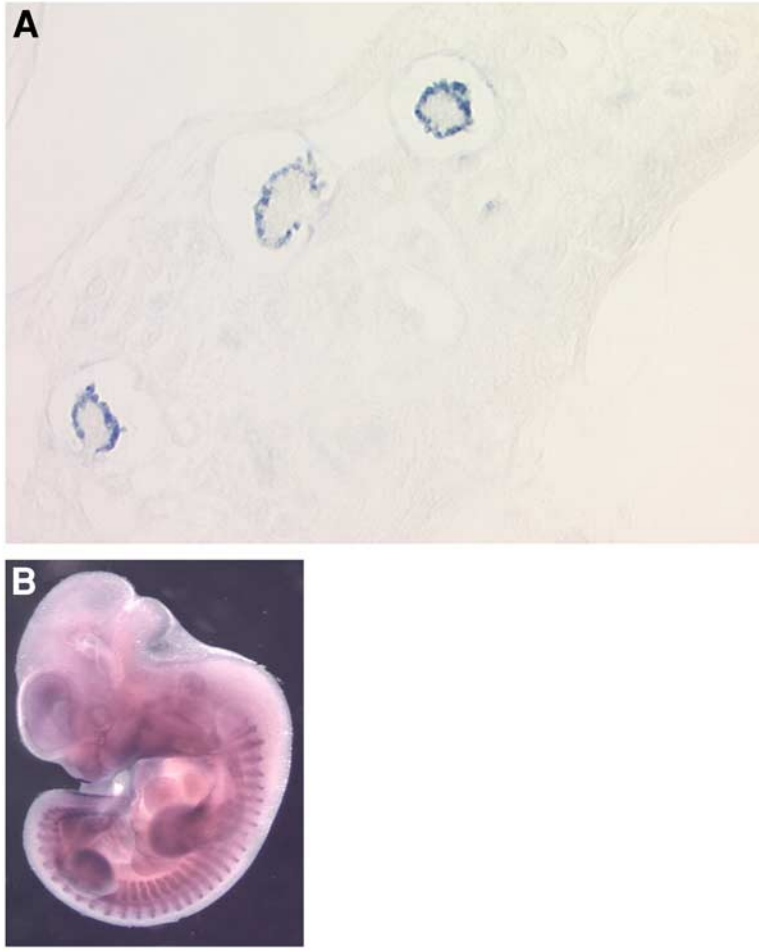

Fig. 2. Illustrations of the results from *in situ* hybridization (ISH) on frozen sections and whole-mount (W)ISH of mouse embryos. **(A)** ISH of the podocin gene, which is expressed in podocytes in the kidney glomerulus, on a frozen section of a mouse embryo from embryonic d 18.5. **(B)** WISH of PDGFC in a mouse embryo from embryonic d 11.

that the cleavage is complete, 2 µL of each reaction is checked on a 1% agarose gel before purification of the linearized plasmid. To remove proteins, the DNA in the restriction mix is purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation as described below. From here it is important to work RNase free.

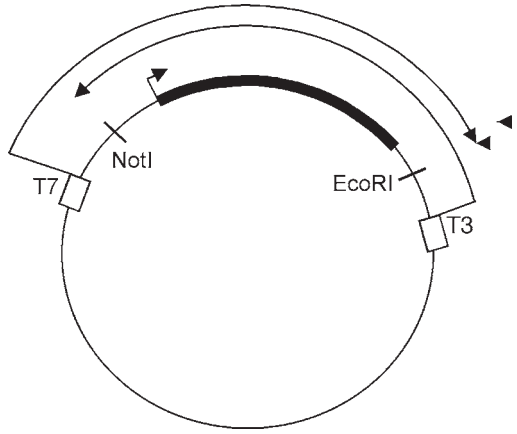

Fig. 3. Schematic drawing of a vector used for in vitro transcription. The insert used as a template for probe synthesis is shown as a bold line with an arrow indicating the direction of transcription. In this example, the vector is first cleaved with *Not*I and then in vitro transcribed with T3 RNA polymerase to generate the antisense probe used for *in situ* hybridization (ISH). Opposite, *Eco*RI and T7 RNA polymerase is used to synthesize a sense probe, which can be used as a negative control.

1. Add 1 vol of phenol/chloroform/isoamyl alcohol to the linearized vector. Vortex for 20 s and centrifuge at 10,000*g* for 10 min at room temperature (RT).
2. Transfer the upper phase containing the linearized vector to a new tube. Precipitate DNA with 208 µL of 4 *M* LiCl and 728 µL of ice-cold 99.5% ethanol at –80°C for 20 min.
3. Centrifuge as above and wash with ice-cold 70% ethanol.
4. Dissolve the pellet in a small volume RNase-free H_2O. Measure DNA concentration.

3.1.2. In Vitro Transcription of the Probe

The choice of RNA polymerase for the following in vitro transcription depends on which strand of DNA that is to be synthesized.

1. DIG-labeling reaction: 2 µL of 10X transcription buffer (included with the RNA polymerase); 2 µL of DIG RNA Labeling Mix; 2 µL of RNA polymerase (20 U/µL); 0.5 µL of RNasin (40 U/µL); and 1–2 µg of linearized vector. Add up to 20 µL with RNase-free H_2O. Mix and spin down. Incubate for 2 h at 37°C to perform the in vitro transcription. It is favorable to do this in an incubator to avoid condensation in the lid.
2. Stop the reaction by adding 2 µL of 0.2 *M* EDTA, pH 8.0.
3. Precipitate the transcribed RNA with 2.5 µL of 4 *M* LiCl and 75 µL of 99.5% ice-cold ethanol at –80°C for at least 30 min or –20°C for several hours.

4. Centrifuge down probe precipitate at 10,000*g* for 20 min at 4°C and wash pellet with ice-cold 70% ethanol.
5. Dissolve pellet in 50 µL of RNase-free H_2O. Add 1 µL of RNase inhibitor and incubate at 37°C for 30 min.
6. According to the manufacturer, about 10 µg DIG-labeled RNA is synthesized from 1 µg of linearized vector in the standard case. The efficiency of both the in vitro transcription and the labeling reaction can be checked rather easily. To make sure the in vitro transcription and the following precipitation have been successful before proceeding to the next step, 2 µL of the probe can be run on a 1% agarose gel. To check the labeling efficiency (*see* **Note 3**).

3.1.3. DNase Treatment of the Probe

When synthesis of the DIG-labeled riboprobe is finished, the probe is treated with DNase to destroy the DNA template. Although this step is not always necessary, it is recommended since the DNA may interfere with the probe in the hybridization step. We routinely use DNA-free (Ambion) according to the manufacturer's instruction. Because the kit contains a DNase Inactivation Reagent, no further precipitation step is required. Before the probe is used in the hybridization protocol, 2 µL of probe is checked on a 1% agarose gel. The probes are stored at –80°C and can be kept there for several months. However, repeated freezing and thawing should be avoided.

3.2. ISH on Frozen Sections

Subheading 3.2.1. describes the preparation of frozen sections. The hybridization protocol we currently use for frozen sections is outlined in **Subheading 3.2.2.–3.2.6.** This hybridization protocol is a modified version of a protocol previously described by Braissant and Wahli ***(4)***.

3.2.1. Preparation of Frozen Sections

According to our experience, the quality of the sections is a key to successful results. It is important that both the embryo preparation and the sectioning are performed RNase free.

1. Dissect whole-mouse embryos in cold PBS.
2. Fix overnight in 4% PFA at 4°C.
3. Wash twice in PBT.
4. Place the embryos in sucrose solution at 4°C. The embryos should be left in this solution until the tissue has sunk to the bottom of the tube.
5. Embed the embryo in Tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, Netherlands). Embedded tissue can be stored at –80°C.
6. Cut 14-µm thin sections on a cryostat before use. Collect the sections on Superfrost Plus slides. After sectioning, the slides are stored at –80°C or, preferably, used directly for hybridization (*see* **Note 4**).

3.2.2. Pretreatment of Tissue Sections

All steps in this protocol (up until **Subheading 3.2.6.**) are performed in glass cuvets without agitation unless otherwise stated. It is important to take care that the sections do not dry out at any point.

1. Postfix the sections in 4% PFA 10 min at RT.
2. Incubate twice for 15 min in PBS-DEPC solution at RT.
3. Equilibrate in 5X SSC for 15 min at RT. Proceed immediately to the prehybridization.

3.2.3. Prehybridization and Hybridization

During the hybridization reaction, several parameters including temperature, duration of time and amount of probe added can be optimized for different probes (*see* **Note 5**). Next, our standard protocol applied to new probes is outlined.

1. Wipe off the slides with a Kleenex without letting the sections dry. Draw around the sections with a liquid blocker and place the slides in a humidified box.
2. Add 150–500 µL of hybridization solution to each section, depending on size.
3. Prehybridize for 2 h at 58°C.
4. Toward the end of the pre-hybridization, prepare a tube with 20 µL of hybridization solution for each section. Add 1 µL of probe to each tube and place the tubes in a heating block at 80°C for 5 min to denature the probes. Put on ice until use.
5. Pour off the solution used for prehybridization from the sections. Add the contents of one tube to each section and cover with a square of parafilm or a cover slip.
6. Hybridize at 58°C for approx 40 h.

3.2.4. Posthybridization Washes

After hybridization, the slides are placed back in glass cuvettes for washings. Inspect the sections to make sure that they have not dried out during the hybridization. From this step, there is no longer a need to work RNase free.

1. Wash 30 min in 2X SSC at RT.
2. Wash 30 min in prewarmed RNase A solution at 37°C (*see* **Note 6**).
3. Wash 1 h in prewarmed 2X SSC at 65°C.
4. Wash 1 h in prewarmed 0.1X SSC at 65°C. Proceed immediately to the antibody staining.

3.2.5. Antibody Staining

1. Equilibrate slides 5 min in Buffer 1 at RT.
2. Wipe off the slides with a Kleenex without letting the sections dry. Fill in around sections with liquid blocker and place the slides in a humidified box saturated with H_2O.

3. Dilute the DIG-conjugated antibody 1:5000 in the blocking solution. Add approx 200 µL (depending on size of the section) of antibody/blocking solution to each section and incubate at 2 h at RT.
4. Wash twice for 15 min in Buffer 1 at RT. Proceed immediately to the color development.

3.2.6. Color Development

1. Equilibrate the slides 5 min in Buffer 2 (*see* **Note 7**) at RT.
2. Prepare the developing solution. To 10 mL of Buffer 2, add 50 µL of NBT and 37.5 µL of BCIP. We usually add around 250 µL of this solution to each section and cover with a square of parafilm.
3. Incubate in the dark at RT in a humified box over night (*see* **Note 8**).
4. When the color development reaction is finished, wash the slides in the stop-solution with Tris/EDTA for 10 min.
5. The slides are then washed in 95% ethanol by gentle agitation for a few hours (up to overnight). This step removes nonspecific background staining and makes the blue signal easier to detect.
6. Wash in deionized water for 15 min to remove any precipitates that may be on the slides.
7. Dehydrate sections by washing slides in 70, 95, 99.9% ethanol for 2 min each, followed by a 2 min wash in xylene. Mount. Before this step, it is possible to use the ISH sections for further applications, such as immunohistochemistry (*see* **Note 9**).

3.3. WISH

The preparation of mouse embryo is outlined in **Subheading 3.3.1.**, followed by the hybridization protocol in **Subheadings 3.3.2.–3.3.6.** Our hybridization protocol used for whole mounts is modified from the protocols of Dr. C. Mailhos, ICRF, London and Dr. D. G. Wilkinson, NIMR, London. All procedures are performed in 2-mL Eppendorf tubes with a round bottom.

3.3.1. Embryo Preparation

1. Dissect embryos in ice-cold PBS and remove as much of the extra-embryonic membranes as possible.
2. Rinse once in ice-cold PBS.
3. Fix in 4% PFA (approx 10 times the volume of the embryo) overnight at 4°C.
4. Wash twice in PBT at 4°C.
5. Wash 5 min each in PBT containing 30, 50, and 80% methanol and then twice in 100% methanol at RT. Embryos will float momentarily but allow embryos to settle during each wash. The embryos can be stored in methanol in 2-mL snap tubes at –20°C up to several weeks.

3.3.2. Pretreatments: Day 1

Steps 1–9 are conducted on a roller. Rinses are immediate and washes are 10 min if not otherwise stated. Let the embryos settle in the bottom of the tube after each wash.

1. Rehydrate embryos through washings in PBT containing 75, 50, and 25% methanol and wash twice in PBT. Embryos will float momentarily but allow embryos to settle during each wash.
2. Bleach embryos in 6% H_2O_2 in PBT for 60 min. Wash three times with 1.5 mL of PBT.
3. Treat with 10 µg/mL proteinase K in PBT. Incubate E9.5 embryos for 15 min, E10.5 for 25 min, E11.5 for 30 min, and E12.5 for 40 min (*see* **Note 10**).
4. Wash with 2 mg/mL glycine in PBT for 10 min, and then wash twice with PBT for 5 min each. Treat embryos very carefully because they are fragile at this stage.
5. Postfix for 20 min in 4% PFA including 0.1% glutaraldehyde.
6. Rinse and wash once with PBT.
7. Rinse once with 1:1 mixture of PBT/hybridization solution. Let embryos settle.
8. Rinse with 1 mL of prewarmed hybridization solution. Let embryos settle. Replace with 1 mL of prewarmed hybridization solution and incubate horizontally for more than 1 h at 70°C.
9. Add 1 mL of prewarmed hybridization mix containing 0.2 µg/mL DIG-labeled RNA probe (*see* **Note 11**).
10. Incubate gently rolling/rocking at 70°C overnight.

3.3.3. Posthybridization Washes: Day 2

During **steps 1–3**, is it important to keep tubes at 70°C using heating block or water bath. Let embryos settle by incubating tubes vertically at 70°C after each wash. Change supernatant in one tube at a time to avoid cooling of samples. Keep wash solutions at 70°C in water bath.

1. Remove hybridization solution while keeping tubes heated in a heating block or water bath. Rinse twice with pre-warmed (70°C) hybridization solution (*see* **Note 12**).
2. Wash twice for 30 min at 70°C with prewarmed hybridization solution.
3. Wash 20 min at 70°C with prewarmed 1:1 mixture of hybridization solution and TBST.
4. Rinse twice with TBST at RT.
5. Wash twice for 30 min with TBST at RT.
6. Rinse twice with MABT.
7. Preincubate for more than 1 h in fresh MABT with 10% heat-treated sheep serum.
8. Incubate overnight at 4°C in MABT with 10% heat-treated sheep serum and anti-DIG antibody diluted 1:1000.

3.3.4. Postantibody Staining: Day 3

1. Rinse three times with MABT.
2. Wash six times for 1 h with 2 mL of MABT rolling. Wash overnight with MABT (*see* **Note 13**).

3.3.5. Color Development: Day 4

1. Wash twice for 10 min with NTMT.
2. Incubate with 1.5 mL of NTMT with NBT and BCIP. Add 50 µL of NBT and 37.5 µL of BCIP to 10 mL NTMT. Rock for the first half hour, then transfer to a 24-well plate for observation. Keep dark during color development.
3. Change stain solution each day until color has developed to the desired extent (30 min up to days). Wash at least three times with PBT. Store in PBT. It is possible to use the embryos for further applications (*see* **Note 9**).

3.3.6. Clearing of Embryos

Both BABB and glycerol render embryo tissue clear which may improve images of stainings. Although embryos can be stored dark at 4°C for some time it is advisable to take photographs as soon as possible since alkaline phosphatase is water soluble.

1. BABB. Incubate in a series of methanol preferably 25, 50, 75% methanol:PBT. Change to BABB for 10 min.
2. Glycerol. Simply wash embryos 15 min each in increasing concentrations of glycerol, preferably 25, 50, 75%, and store in 87% glycerol.

4. Notes

1. Because RNases can destroy the whole experiment by degrading the riboprobe or the RNA in the tissue sample, it is important that all steps until the hybridization is finished are performed strictly RNase free. Gloves should be used at all steps. Glass wear are made RNase free by baking them at 160°C for at least 9 h. Plastics, such as tubes, are considered RNase free when taken directly from an unopened box. For solutions included in the protocol, we have found it enough to prepare them from RNase free chemicals and RNase free H_2O. However, they can also be treated by DEPC by dissolving 500 µL of DEPC in 1000 mL of solution. The solution is then incubated at 37°C for 2 h and autoclaved. Other things, such as working areas or humified boxes, can be made RNase free by RNase ZAP (Ambion) if necessary.
2. It is also possible to use polymerase chain reaction (PCR) products as templates for the in vitro transcription. However, the PCR fragments must contain the RNA polymerase promoter sequences as well. This can be achieved by designing PCR-primers which contain a promoter sequence 5' of the template-specific primer sequence *(5)*. It is advisable to gel purify the PCR product before in vitro transcription.

3. To check the labeling efficiency, the probe and dilutions of this can be spotted on a positively charged nylon membrane. After fixing the RNA probe to the membrane by crosslinking or baking, the membrane is washed and incubated with antibody solution before development with NBT/BCIP. This procedure is performed within a couple of hours and more details can be found in the instruction manual for the DIG-High Prime DNA Labeling and Detection Starter Kit from Roche.
4. When possible, we use fresh sections and proceed directly to the hybridization protocol for maximal sensitivity. However, we have successfully used sections which have been stored in –80°C for several weeks. Add some silica gel in the slide box in the freezer to keep sections dry. If sections are used from the freezer, they should be defrosted in RT for 30 min up to 3 h before starting the hybridization protocol. It is important that the sections are allowed to dry as fast as possible, that is, not to keep them in a box, which will prevent moisture from disappearing.
5. For many probes, an overnight hybridization is sufficient to get a strong signal. The hybridization temperature and formamide concentration of the hybridization solution may need to be optimized for each probe. Also the amount of probe added can be altered, depending on the efficiency of the in vitro transcription and the labeling of the probe, particularly because a longer probe gets more DIG-labeled nucleotides.
6. An additional washing step including RNase A is added in our protocol in order to eliminate unspecifically or incompletely hybridized probes, because we have found that this reduced background. However, it may not be necessary for all ISH applications. We use 5 µg/mL as suggested by others *(**6**)*. To avoid contamination, separate glass cuvettes are used for the steps before and after the hybridization.
7. We add levamisole to Buffer 2 to inhibit the endogenous alkaline phosphates of the sample. This step may not be necessary for all kinds of tissue.
8. The sections are studied by light microscopy to determine the length of the developmental reaction. We usually develop for 1–2 d, with addition of new Buffer 2 containing NBT and BCIP each day. Although a longer development may enhance the specific signal, according to our experience it also makes the sections more fragile.
9. We have successfully used sections after ISH for antibody staining. First, the ISH signals are fixed by incubating it in 4% PFA for 10 min. However, the signal from the ISH may still decrease during the immunohistochemistry protocol. Next, the section is incubated with H_2O_2, and an ordinary immunohistochemistry protocol, including the antibodies of interest, should be followed from there onward. Also, whole mounts can be used further after ISH. For sectioning or whole mount staining with antibodies, post-fix the embryos 30 min with 4% PFA. Then, continue with the appropriate staining protocol. However, because proteinase K, which is included in the WISH protocol, cleaves protein antigens the subsequent antibody staining might becomes weaker.

10. Use fresh proteinase K or a stock stored at –20°C. Duration of proteinase K treatment vary depending on batch and freshness. Thus, incubations have to be optimized.
11. Generally less amount of probe gives cleaner result, but sometimes it may be feasible to increase the amount.
12. Hybridization mix containing probe can be saved at –20°C for several months. Usually, reused probe gives cleaner results.
13. The longer wash the lower the background.

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7

In Situ Hybridization of Whole-Mount Embryos

Murray Hargrave, Josephine Bowles, and Peter Koopman

Summary

The ability to visualize the expression of a gene in both time and space is an essential tool of developmental biology. Here, we detail a robust method for *in situ* hybridization of RNA probes to whole pieces of fixed tissue. This method has been optimized for reliable and sensitive visualization of the spatial patterns of gene expression in mouse embryo tissue.

Key Words: *In situ* hybridization; whole mount; gene expression.

1. Introduction

Since the early analyses of gene expression in the *Drosophila* embryo *(1)*, whole-mount *in situ* hybridization has become one of the most powerful and versatile tools in developmental biology. The ability to visualize a gene's expression both in time and space is a necessary first step in investigating the roles of that gene in cell differentiation and morphogenesis in the developing embryo. Unlike conventional *in situ* hybridization to tissue sections, the whole-mount procedure provides a three-dimensional readout of the sites of gene expression. This readout, combined with sequence analysis, allows an initial prediction of gene function and provides the basis for further investigation. Unique patterns of gene expression have also been used to define regions of developing tissue within areas that otherwise appear anatomically uniform. Increasing sophistication of the technique, by the use of dual hybridizations visualized with different colors or by combining hybridization with immunohistochemistry, has now made it possible to observe simultaneously two or more gene products in the one tissue.

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The protocol described in this chapter is similar to that described by Christiansen et al. *(2)* and Wilkinson and Nieto *(3)* and has been optimized for analysis of vertebrate embryos, particularly those of mice. Embryos are particularly well suited to this technique as the result of their small size, permeability, and translucency compared with adult tissues. The whole-mount procedure is robust and reliable, and once some experience has been gained, is easy to modify for use with different developmental systems.

2. Materials

2.1. Transcription of Labeled Probe

1. A high purity preparation of a plasmid DNA vector containing the gene fragment of interest and RNA polymerase priming sites (T3, T7, or SP6).
2. Appropriate restriction enzymes and their buffers.
3. Sterile, RNase-free, high-purity water.
4. Phenol buffered to pH 7.5 with Tris-HCl.
5. Chloroform.
6. RNase-free 3 *M* NaOAc, pH 4.8.
7. RNase-free absolute ethanol.
8. Sp6, T7, or T3 RNA polymerases.
9. 5X RNA polymerase transcription buffer: 200 m*M* Tris-HCl, pH 7.9, 30 m*M* $MgCl_2$, 10 m*M* spermidine, 50 m*M* NaCl, or as supplied with polymerase enzymes.
10. 0.1 *M* dithiothreitol (DTT).
11. Digoxygenin (DIG) RNA labeling nucleotide mix (10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, 6.5 m*M* UTP, 3.5 m*M* DIG-11-UTP; Roche Diagnostics, Mannheim, Germany *see* **Note 1**).
12. RNase inhibitor (Roche Diagnostics).
13. Agarose.
14. Tris-acetate-ethylene diamine tetraacetic acid agarose gel running buffer.
15. Ethidium bromide (*see* **Note 2**).

2.2. Production of Embryo Powder

1. Acetone.
2. Filter paper.
3. Mortar and pestle.

2.3. Preparation and Hybridization of Embryos

1. Phosphate-buffered saline (PBS; sterile, RNase-free phosphate buffered saline, pH 7.4).
2. 4% paraformaldehyde (PFA) in PBS. Dissolve powder in PBS by heating at 65°C in a closed container, freeze in small volumes and thaw immediately before use (*see* **Note 2**).

3. PBTX: PBS with 0.1% Triton X-100 detergent.
4. Methanol/PBTX series (25, 50, 75, 100% methanol in PBTX).
5. 20 mg/mL solution of proteinase K in RNase-free water.
6. 25% glutaraldehyde solution frozen in small aliquot amounts.
7. Prehybridization/hybridization solution (50% formamide, 5X standard saline citrate (SSC; 20X SSC is 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0), 2% blocking reagent for nucleic acid hybridization and detection (Roche Diagnostics), 0.1% Triton X-100, 0.5% CHAPS (Sigma), 1 mg/mL Torula yeast RNA (Sigma), 5 m*M* ethylene diamine tetraacetic acid, and 50 µg/mL heparin sodium salt).
8. DIG-labeled RNA probes.

2.4. Posthybridization Washes

1. Posthybridization wash Solution 1: 50% formamide, 5X SSC, 0.1% Triton X-100, 0.5% CHAPS.
2. Series of Solution 1/2X SSC washes (75% Solution 1/25% 2X SSC, 50% Solution 1/50% 2X SSC, 25% Solution 1/75% 2X SSC).
3. 2X SSC, 0.1% CHAPS.
4. 0.2X SSC, 0.1% CHAPS.
5. TBTX: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.1% Triton X-100.
6. Preblocking solution (10% sheep serum, 2% bovine serum albumin [BSA], fraction V in TBTX).

2.5. Preabsorption of Antibody

1. Sheep anti-Digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics; *see* **Note 3**).

2.6. Postantibody Washes and Staining

1. NTMT: 100 m*M* NaCl; 100 m*M* Tris-HCl, pH 9.5, 50 m*M* $MgCl_2$, 0.1% Tween-20. NTMT must be made fresh (from stock solutions) on the day of use.
2. 100 mg/mL NBT (nitroblue tetrazolium) in dimethylformamide (Roche Diagnostics).
3. 50 mg/mL BCIP (5-bromo-4-chloro-3-indolyl-phosphate) in dimethylformamide (Roche Diagnostics).
4. PBS with 1% Triton X-100.
5. Sodium azide, 10% stock solution.
6. Glycerol.

3. Methods

All steps up to and including hybridization are carried out in RNase-free conditions, using RNase-free solutions and wearing gloves. Glassware can be rendered RNase-free by baking at 180°C and solutions should be made with DEPC-treated water and chemical stocks that are kept separate from those for

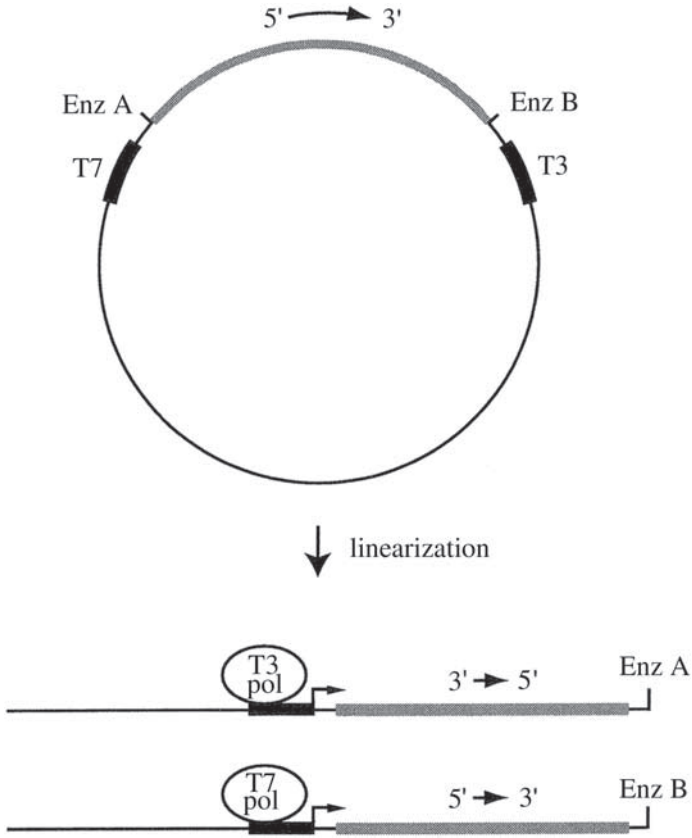

Fig. 1. Production of RNA probes from a plasmid clone. Linearization with restriction enzyme A and transcription with T3 RNA polymerase would give a single stranded antisense probe that will hybridize to mRNA. Similarly linearization with restriction enzyme B and transcription with T3 RNA polymerase would give a single stranded sense probe, which should be used as a control.

general use. Disposable plastic ware is usually RNase-free and is ideal for many steps in the protocol.

3.1. Transcription of Labeled Probe

1. Obtain a clone of the gene of interest (*see* **Note 4**).
2. Select two restriction enzymes, each with a unique site at one end of the cloned fragment and linearize 10–20 µg of plasmid with each (*see* **Fig. 1**). Enzymes that produce 3' overhangs (such as *Apa*I, *Bgl*I, *Kpn*I, *Pst*I, *Pvu*I, *Sac*I, S*ac*II, and *Sph*I) should not be used as the RNA polymerases can false prime at these sites

(*see* **Note 5**).

3. Check that the plasmid preparations are completely linearized by running an aliquot of each on an agarose gel (*see* **Note 5**).
4. Phenol/chloroform extract the DNA, add one tenth volume of RNase-free 3 *M* NaOAc and 2.5 vol of RNase-free absolute ethanol, and precipitate at –20°C for 30 min.
5. Pellet the DNA in a refrigerated microfuge for 15 min.
6. Wash the pellet in ice-cold RNase-free 70% ethanol and air-dry.
7. Resuspend the DNA in RNase-free water at a final concentration of 1 µg/µL. The linearized plasmid can be stored at –20°C and used for multiple probe synthesis reactions.
8. Select an appropriate RNA polymerase for each of the linearized constructs (*see* **Fig. 1**).
9. Mix the following in a 1.5-mL RNase-free tube:
   a. Sterile RNase-free water 8.5 µL
   b. 5X transcription buffer 4 µL
   c. Linearized plasmid (1 µg/µL) 1 µL
   d. 0.1 *M* DTT 2 µL
   e. 10X DIG RNA labeling mix 2 µL
   f. Placental ribonuclease inhibitor (40 U/µL) 1.5 µL
   g. SP6, T7, or T3 RNA polymerase (20 U/µL) 1 µL
10. Incubate at 37°C (or 40°C for SP6) for 1 h, then add another 20 U of SP6 RNA polymerase.
11. Incubate for a further hour at 37°C or 40°C.
12. Remove a 1-µL aliquot and run on a 1% agarose/tris-acetate-ethylene diamine tetraacetic acid gel to estimate the amount synthesized (*see* **Note 5**). An ethidium bromide-stained RNA band of many fold greater intensity than the plasmid band should be noted. Estimate the amount of probe produced. For example, an RNA band of approximately 10-fold greater intensity than the plasmid band indicates that 10 µg of probe has been synthesized.
13. Dilute the probe to 50 µL with DEPC milliQ H_2O, add 5 µL of RNase-free 3 *M* NaOAc, mix and add 2.5 vol of RNase-free absolute ethanol.
14. Incubate at –20°C for 30 min to precipitate the RNA and spin down in a refrigerated microfuge for 15 min.
15. Wash the pellet well (twice) with RNase-free 70% ethanol to remove of any unincorporated nucleotides.
16. Redissolve pellet in RNase-free water to a final concentration of 1.0–0.1 µg/µL and store at –20°C.

3.2. Production of Embryo Powder

1. Homogenize advanced-stage embryos (e.g., 12.5–14.5 d post coitus [dpc] mouse embryos, or Hamburger-Hamilton stage 30–32 chick embryos) in a small volume of chilled PBS.

2. Add 4 vol of ice-cold acetone, mix well and place on ice for 30 min.
3. Spin at 10,000*g* for 10 min and discard supernatant.
4. Wash the pellet with ice-cold acetone and spin again.
5. Air-dry the pellet on a piece of filter paper and grind into a fine powder with a mortar and pestle.
6. Air-dry the powder and store in an air-tight tube at 4°C.

3.3. Embryo Preparation and Hybridization

All steps are conducted on a rocking platform in filled, 2-mL round-bottom tubes to ensure thorough mixing without damage to the embryos, and unless otherwise stated, at room temperature.

1. Dissect embryos in ice-cold PBS (*see* **Note 6**).
2. Fix the embryos in 4% w/v PFA in PBS at 4°C from 3 h to overnight.
3. Wash twice with PBTX for 10 min each at 4°C.
4. Wash with 25, 50, 75% methanol/PBTX, then twice with 100% methanol for 20 min each. The embryos can be stored at 4°C at this point, for up to a few months. Alternatively they can be stored in prehybridization solution at –20°C (*see* **step 11**).
5. Rehydrate by taking the embryos back through the methanol/PBTX series in reverse. The dehydration and rehydration series are essential, even if the embryos are not being stored but are to be used immediately (*see* **Note 7**).
6. Wash three times with PBTX for 10 min each.
7. Incubate with 10 µg/mL proteinase K in PBTX at room temperature (make a fresh dilution of proteinase K from stock solution). The length of this treatment depends on the size of the sample and the batch of proteinase K. Each batch should ideally be tested. As a rough guide, use 5 min for 7.5 dpc mouse embryo, 10 min for 8.5 dpc, 15 min for 9.5 dpc, 20 min for 10.5 dpc, 25 min for 11.5 dpc, and 30 min for 12.5 dpc (*see* **Note 8**).
8. Wash twice with PBTX for 5 min each. Wash carefully as the embryos can be fragile at this point.
9. Refix the embryos in 0.2% glutaraldehyde–4% PFA in PBTX for 20 min (make a fresh dilution of glutaraldehyde and Triton X-100 from stock solutions into freshly thawed 4% PFA).
10. Wash twice with PBTX for 10 min.
11. Place the embryos in prehybridization solution and allow to sink. The embryos can be stored in this solution at –20°C.
12. Incubate at 65°C for at least 2 h, although it is often convenient to perform this step overnight. For this, and the subsequent washes at 65°C, it is suitable to use a heater block placed on its side on a rocking platform or a hybridization oven with rotating cylinders.
13. Remove prehybridization solution and add hybridization solution including 1.0 µg/mL DIG-labeled RNA probe (*see* **Note 9**). If high background is observed, probe concentration can be decreased to 0.5 µg/mL. The tube needs to be full of hybridization solution, otherwise background problems may occur.

14. Incubate at 65°C overnight. If the probe is short or heterologous, 55°C can be used for prehybridization, hybridization and stringency washes.

3.4. Posthybridization Washes

1. Wash with 100% Solution 1 for 5 min at 65°C. From this point on, RNase-free conditions are no longer necessary.
2. Wash with 75% Solution 1/25% 2X SSC for 5 min at 65°C.
3. Wash with 50% Solution 1/50% 2X SSC for 5 min at 65°C.
4. Wash with 25% Solution 1/75% 2X SSC for 5 min at 65°C.
5. Wash with 2X SSC/0.1% CHAPS twice for 30 min at 65°C. During these washes, start pre-absorbing the antibody as described in **Subheading 3.5.**
6. Wash with 0.2X SSC/0.1% CHAPS twice for 30 min at 65°C.
7. Wash with TBTX twice for 10 min at room temperature.
8. Preblock the embryos with 10% sheep serum and 2% BSA in TBTX for 2–3 h at room temperature.
9. Remove the 10% sheep serum, 2% BSA from the embryos and replace with the pre-absorbed antibody (*see* **Subheading 3.5.**) and incubate on a rocker overnight at 4°C.

3.5. Preabsorption of Antibody

1. During the washing of the embryos (*see* **Subheading 3.4.5.**), weigh out 3 mg of embryo powder into a 1.5-mL tube, add 0.5 mL of 10% sheep serum, 2% BSA in TBTX and 1 µL of anti-DIG-AP Fab fragment. Embryo powder should match the species being studied.
2. Rock gently at 4°C for 3 h or longer.
3. Spin in a microfuge for 10 min at 4°C.
4. Remove the supernatant without disturbing the pellet and dilute to 2 mL using 10% sheep serum, 2% BSA in TBTX.
5. Store at 4°C until use.

3.6. Postantibody Washes and Staining

1. Remove the antibody solution and wash the embryos at least five times with TBTX containing 0.1% BSA for 1 h at room temperature The antibody solution can be kept at 4°C and reused up to four times.
2. Wash overnight at 4°C with TBTX containing 0.1% BSA. This wash is optional but usually convenient.
3. Wash twice with TBTX for 15 min.
4. Wash three times with NTMT for 10 min.
5. Incubate with NTMT including 3.5 µL NBT and 3.5 µL BCIP/mL. Rock for the first 20 min then transfer the embryos to a glass embryo dish or scintillation vial (*see* **Note 10**).
6. When the color has developed to the desired extent, wash with NTMT for 10 min then with PBTX for 15 min.
7. Wash several times in PBS with 1% Triton X-100. This will blue the stain and decrease background and signal (*see* **Note 11**).

8. Fix the stain by incubating the embryos in 4% PFA in PBS overnight at 4°C.
9. Photograph embryos as soon as possible as the signal can fade or the entire embryo can turn blue upon storage (*see* **Note 12**).
10. If the embryos are to be stored for extended periods, use PBS containing 0.05% w/v sodium azide, or take them through a PBTX/glycerol series into 100% glycerol.

4. Notes

Although the whole-mount protocol may at first seem complex, it can be broken down into four general parts: preparation of the samples, hybridization, application of antibody, and visualization of the staining. All of the steps in between are aimed at producing the greatest signal to background ratio and although the technique is fairly robust, some adjustment for the particular system of interest may be required. Embryo treatments and washing may need to be changed to allow the processes of hybridization and antibody binding to occur with greatest efficiency. Likewise, changes to the hybridization conditions may be necessary for atypical experiments or when ideal probes are not available. Such changes should be made with the general principles of hybridization science in mind. Most of the common considerations are outlined below however some more detailed discussions of certain aspects can be found in the references at the end of the chapter ***(4,5)***.

1. A number of options exist for both probe labeling and color development. UTP labeled with biotin or fluorescein and the corresponding antibodies, conjugated to either alkaline phosphatase, peroxidase, or fluorescent markers, are commercially available ***(6)***. Additionally, a number of different substrate systems are available that allow staining with different colors. Using these options it is possible to stain for two different transcripts ***(7)***. Furthermore, *in situ* hybridization can be used in conjunction with immunohistochemistry to analyze the distribution of an mRNA and a protein product within the one sample ***(6)***.
2. A number of the reagents in this protocol are known to be irritants or toxins (e.g., PFA, formamide, glutaraldehyde, CHAPS) whereas the safety status of others is unclear. The use of a fume hood and protective clothing is necessary in a number of cases.
3. Endogenous phosphatase enzyme may lead to nonspecific staining when using color reaction substrates that react with alkaline phosphatase. Most of the endogenous phosphatases will be rendered inactive by the PFA fix and the high temperatures of hybridization; however, if a problem is encountered, bleach the embryos as described above or add 2 m*M* levamisole to the staining solution. The phosphatase enzyme conjugated to the anti-DIG antibody is not affected by levamisole.
4. When designing a probe select a portion of the gene that lacks highly conserved motifs to minimize cross hybridization to related gene transcripts. Similarly a

Fig. 2. Sox9 whole mount. Strong staining can be noted in elements of the developing skeleton, such as the digits, limb bones, scapula and pelvis, vertebrae, and ribs, reflecting a role for this gene in skeletal development (*see* **ref.** ***8***).

probe that contains polyA tracts, repetitive sequences or large A/T rich stretches may bind non-specifically. The size of the probe is ideally approx 1 kb, but can be anywhere between a few hundred basepairs to 2 kb in length. Larger probes may need limited alkali hydrolysis to an average size of 500–700 bp for optimal results. Probes that are smaller than 200 bp may require changes to the hybridization and stringency washes.

5. If the plasmid template has been linearized using a restriction enzyme that produces a 3' overhang (e.g., *Apa*I, *Bgl*I, *Kpn*I, *Pst*I, *Pvu*I, *Sac*I, S*ac*II or *Sph*I), the ends of the template should be blunted before transcription. To do this, set up a transcription reaction but omit the ribonucleotides ("labeling mix") and RNA polymerase. Add 5 U of DNA polymerase Klenow fragment, incubate at room temperature for 15 min, then add the labeling mix and RNA polymerase and carry out the transcription reaction at 37 or 40°C as normal. When assessing the success of the probe transcription reaction by running an aliquot on an agarose gel, it is important that the gel be free of RNases as the probe may degrade while running, leading to the conclusion that the labeling reaction was unsuccessful. Since a standard agarose gel is nondenaturing, the RNA probe may not run at the

size predicted from the insert and may be present as multiple bands. If the majority of product runs at 0–50 bp, then the probe has degraded and will have to be remade.

6. In species such as mice that have thick extra embryonic membranes, tear the membranes or, preferably, remove them completely. Be sure to break the amnion. A number of structures within the embryo can trap reagents which in turn can lead to false staining. These structures, such as the brain ventricles, heart, and otic vesicles, can be punctured with a syringe needle to prevent this.
7. If the species of embryo or the tissue of interest has pigmentation that may interfere with the staining, the embryos can be bleached in 6% hydrogen peroxide in PBTX for an hour after the methanol rehydration steps.
8. At later developmental stages, such as 11.5 dpc and older mouse embryos (**Fig. 2**), the density of tissue and the size of the embryo will prevent penetration of the probe to the deeper structures including most of the internal organs. Care should be taken when interpreting the apparent lack of staining in such structures. This problem can be overcome by dissecting out the tissues of interest and performing *in situ* analysis on them alone. The tissues should be dissected from the embryos before the initial PFA fixation to prevent excessive background. A number of structures can be held under a large cover slip on a microscope slide and viewed at higher magnification than can be achieved on a conventional dissecting microscope. Sectioning of stained embryos may allow better resolution of positive tissues and make hybridization of tissue sections unnecessary. Sectioning can be performed on vibratome with agarose embedded samples or on a microtome with paraffin embedding. The latter should only be performed on strongly stained samples as some stains, including that from BCIP and NBT, are soluble in ethanol and xylene. Tissue morphology is likely to be poorer in sectioned whole mounts than on tissue sections, due to the proteinase K treatment.
9. The hybridization of a sense strand probe should be included as a negative control when investigating the expression pattern of a novel transcript.
10. During the staining reaction avoid using a plastic Petri dish as crystals tend to form. Staining should be monitored frequently but otherwise kept in the dark as much as possible. Allow the color reaction to proceed until signal is strongest without producing background staining. The staining time may vary from a few to 12 h, depending on the expression level of the gene, the specific characteristics of the probe and the optimization of the protocol. If samples are to be sectioned, overstaining is recommended. The staining reaction should not be left to continue overnight as the samples may overstain and the experiment fail. The staining reaction can be stopped by washing the embryos in NTMT then TBTX for 15 min each and storing the embryos in fresh TBTX at 4°C in the dark and started again at **step 4** of **Subheading 3.6.** After staining has been stopped, embryos should not be left in NTMT solution. Even though color reagents may have been substantially diluted the alkaline phosphatase is still active and overstaining will occur. The condition of the NBT and BCIP should be checked before use as using

old stocks may increase background. NBT should be bright yellow and BCIP clear. If either have become brown they should be discarded.

11. Some observation and judgment is required for destaining. For a weak signal, this step can be shortened or omitted. If signal is strong and background is weak, then a total of a few hours is recommended. Overstained or high background samples can be washed for up to several days and the destaining can be stopped and started again with the embryos stored at 4°C in PBTX between destaining treatments.
12. When photographing embryos, position them, immersed in PBS, in grooves cut in a layer of agarose in a Petri dish. The lighting during photography should be adjusted to optimize the translucency of the sample. Transferring embryos into 50% glycerol may help to clear tissue.

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8

In Situ Detection of Epstein–Barr Virus and Phenotype Determination of EBV-Infected Cells

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Summary

Epstein–Barr virus (EBV) establishes a lifelong infection of B cells. Consequently, EBV-carrying B cells are present in the peripheral blood as well as in lymphoid and nonlymphoid tissues of most individuals. As a result, the detection by polymerase chain reaction of EBV genomes in DNA extracts from tumor tissues does not permit conclusions as to the precise cellular source of the virus. For a meaningful analysis of EBV infection, it often is necessary to determine the cellular location of the virus using morphology-based techniques. *In situ* hybridization for the detection of the small EBV-encoded RNAs (EBERs) has become the standard method for the detection of latent EBV infection. Owing to their abundance, the EBERs represent ideal targets for *in situ* hybridization using radiolabeled or nonradioactive probes. EBV has been detected in tumors of various lineages, and proliferation of nonneoplastic B cells may occur in the background of EBV-negative tumors. Thus, the assignment of EBV infection to a specific cell type may require double labeling techniques for the simultaneous detection of the virus and of cell lineage-specific gene products. Because of the heterogeneous composition of many EBV-associated tumors, gene expression analysis of EBV-infected cells in tissue sections also may require double labeling techniques. Here, methods are described for the *in situ* detection and phenotypic characterization of EBV-infected cells in the authors' laboratories.

Key Words: *In situ* hybridization; Epstein–Barr virus; B cells.

1. Introduction

Primary Epstein–Barr virus (EBV) infection is followed by a lifelong persistence of the virus in the B-cell compartment of the host *(1)*. Small numbers of EBV-carrying B cells have been identified in the peripheral blood as well as in lymphoid and nonlymphoid tissues of chronic virus carriers *(2,3)*. As a consequence, the detection of EBV by polymerase chain reaction, for example, in

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DNA extracts from a tumor, usually does not permit conclusions as to whether this is the result of the presence of the virus in the tumor cell population or to the presence of EBV-carrying "bystander" B cells in the tissue. For a meaningful analysis of EBV infection it is, therefore, in many instances necessary to establish the cellular location of the virus using morphology-based techniques.

In this chapter, we describe *in situ* hybridization (ISH) techniques for the detection of EBV as used in our laboratories *(4–7)*. Further characterization of the type of EBV infection, i.e., latent vs lytic infection, as well as of the prevalent types of viral latency, is achieved routinely by immunohistochemistry and has been described elsewhere (*see* **refs. 5** and *8*).

1.1. ISH

The first reproducibly successful attempts to localize the virus in tissue sections were carried out using ISH for the detection of EBV DNA (for review, *see* **ref. 4**). Most workers used the cloned *Bam*HI W fragment of the EBV genome as a probe for this purpose. Because this fragment is repeated up to 10 to 15 times in the viral genome, this approach promised greater sensitivity than the use of single copy gene probes. These probes can be labeled with a variety of radioactive (e.g., ^{35}S, ^{33}P, ^{3}H) and nonradioactive (e.g., biotin, digoxigenin [DIG], bromodeoxyuridine, fluorescein isothiocyanate) reporter molecules *(4,7)*. In our hands, ^{35}S-labeled probes have proved to be consistently more sensitive than nonradioactive probes. For practical purposes, this means that if latent EBV infection is to be detected by DNA ISH, ^{35}S-labeled probes should be used. Nonradioactive DNA probes are sufficient for the detection of replicative EBV infection, for example, in oral hairy leukoplakia. However, virus replication is more appropriately and more conveniently demonstrated by simpler immunohistological assays *(8)*.

In recent years, ISH for the detection of the small EBV-encoded RNAs (EBERs), EBER-1 and -2, has become the standard method for the detection of the latent EBV infection *(9)*. The EBERs are small (approx 170 bases) nonpolyadenylated nuclear RNAs of unknown function that are expressed in all known forms of EBV latency at very high copy numbers (as many as 10^7 copies per cell *[10]*). Owing to their abundance and their relative stability compared to mRNA, they represent ideal targets for ISH studies. ISH for the detection of the EBERs is applicable to frozen sections as well as formalin-fixed paraffin-embedded tissue sections and has even been used successfully on post mortem tissues *(9)*. Several types of probes are available for the detection of the EBERs. Oligonucleotides labeled with nonradioactive tags are available from several commercial sources. These probes are adequate for most routine applications. Alternatively, RNA probes derived from transcription vectors can be used, and this approach is described here. These probes can be labeled with

radioactive nucleotides or with nonradioactive compounds (*see* above). In most circumstances, for instance, in the analysis of tumors, the use of nonradioactive probes yields satisfactory results. However, for the detection of rare latently infected B cells in chronic virus carriers, we prefer radioactive probes because of their higher sensitivity. In most laboratories, EBER ISH has replaced DNA ISH as the standard method for detecting latent EBV infection. It has to be kept in mind, however, that the use of EBER ISH relies on the active expression of the viral genome. Although expression of the EBERs has been demonstrated in all known forms of EBV latency, an EBER-negative viral latency remains at least a theoretical possibility.

1.2. Double Labeling Techniques

EBV has been found in normal and neoplastic cells of various lineages, including malignant lymphomas of B- and T-cell type, Hodgkin lymphoma, infectious mononucleosis, carcinomas, neoplasms of follicular dendritic cells, and leiomyosarcomas ***(10,11)***. However, there are examples of cell types that were suspected to harbor EBV but were not found to be infected when applying the appropriate methodology, examples comprising seminoma cells or macrophages in EBV-associated hemophagocytic syndrome ***(12,13)***. Finally, the proliferation of non-neoplastic B cells has been observed in the background of T-cell lymphomas ***(14)***. Thus, confident assignment of EBV infection to a specific cell type may require the application of double labeling techniques for the simultaneous detection of viral DNA or viral gene products on the one hand and of cell lineage-specific gene products on the other hand. Depending on the type of latency, EBV infection may induce phenotypic changes in infected cells such as morphological alterations as well as alterations in the phenotypic make-up of cell surface, cytokine expression profile, and in signal transduction pathways ***(10,15)***. Because of the heterogeneous composition of many EBV-associated lesions, gene expression analysis of EBV-infected cells in tissue sections is, again, best achieved with double labeling techniques. Moreover, simultaneous detection of EBV and immunoglobulin light chain gene transcripts of kappa or lambda types, for instance, not only provides a phenotypic marker but also may yield information as to the clonal composition of these cells.

Various combinations of immunohistology (IH) and ISH with either radioactive or nonradioactive probes (IH/ISH), ISH with a mixture of radioactive and nonradioactive probes (ISH-ISH), and even triple labeling procedures (IH/ISH-ISH) have been used with success ***(16–19)***.

For nonradioactive procedures, two major approaches are possible, double immunofluorescence and double immunoenzymatic techniques. Because of the considerable drawbacks of immunofluorescence, particularly when applied to

formalin-fixed tissue sections, only immunoenzymatic procedures are described here. To achieve proper signal discrimination, the choice of visualization systems and chromogens is important. For immunoenzymatic double labeling, various different color combinations based on the use of different marker enzymes have been described, and numerous reviews and application handbooks exist for those techniques (*see* **Note 19**). In this chapter, we will restrict ourselves to the following procedures: (1) sequential IH/ISH for the detection of differentiation antigens by IH and cellular or viral nucleic acids by radioactive or nonradioactive ISH, and (2) the application of nonradioactive EBER-specific ISH in combination with radioactive ISH for the detection of cellular RNA (e.g., cytokine or immunoglobulin transcripts, ISH-ISH). The sequential procedure has previously been adapted to combinations of ISH with classical enzyme histochemistry, such as visualization of acid phosphatase or esterase activities ***(20)***.

2. Materials

2.1. Slides, Cover Slips, Tissue Sections, and Cytospin Preparations

1. Domestic tinfoil.
2. Decon 90 (Prochem, Wesel, Germany).
3. Acetone.
4. 3-Aminopropyltriethoxysilane (APES; Sigma, St. Louis, MO).
5. 0.2 *N* HCl.
6. Ethanol.
7. Silicon solution (Serva, Heidelberg, Germany).
8. 10X phosphate-buffered saline (10X PBS): 1.3 *M* NaCl, 70 m*M* Na_2HPO_4, 30 m*M* NaH_2PO_4, pH 7.0.
9. 4% paraformaldehyde (PFA).

2.2. Probe Labeling

2.2.1. General

1. Appropriate restriction enzymes with 10X buffer.
2. 3 *M* sodium acetate.
3. Ethanol.
4. Distilled H_2O (ddH_2O).
5. Deionized formamide pH 7.0.
6. 100 m*M* dithiothreitol (DTT).

2.2.2. RNA Probes

1. DIG RNA labeling mix (Roche Diagnostics, Mannheim, Germany).
2. Diethylpyrocarbonate (DEPC, Sigma).
3. DEPC–H_2O (*see* **Note 1**).

4. RNase inhibitor (40 U/µL).
5. RNA polymerases (SP6, T3, T7, 20 U/µL) and appropriate buffer.
6. α-^{35}S-UTP (>1250Ci/mmol; 12.5 Ci/mL).
7. NTP mix (100 m*M* ATP/100 m*M* CTP/100 m*M* GTP).
8. Yeast tRNA (50 mg/mL).
9. DNase I (RNase-free, 10 U/µL).
10. Phenol/chloroform/isoamylalcohol (25/24/1 v/v) pH 4.7 (Sigma).
11. 1 *M* $NaHCO_3$.
12. 1 *M* Na_2CO_3.
13. Acetic acid.

2.2.3. DNA Probes

1. α-^{35}S-dCTP (>1250Ci/mmol; 12.5 Ci/mL).
2. Nick translation kit (e.g., Amersham, Uppsala, Sweden).
3. Nick columns (Amersham).

2.3. ISH

1. Xylene.
2. Ethanol.
3. 0.2 *N* HCl.
4. DEPC–H_2O.
5. 20X Standard saline citrate (20X standard saline citrate [SSC]): 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.0.
6. Triton X-100.
7. Pronase (Roche Diagnostics, *see* **Note 2**).
8. 10X PBS.
9. 0.1 *M* Glycine in 1X PBS.
10. 4% PFA.
11. Triethanolamine.
12. Acetic anhydride.
13. Deionized formamide.
14. Dextran sulfate.
15 100 m*M* DTT.
16. Carrier DNA (1 mg/mL, e.g., calf thymus or herring sperm DNA).
17. 90°C heat block with plane surface.
18. Yeast tRNA (50 mg/mL).
19. Parafilm.
20. RNase A (10 mg/mL)
21. Photographic emulsion (e.g., G5; Ilford, Dreieich, Germany).
22. Light-proof slide boxes.
23. Domestic tinfoil.
24. Kodak D19 developer (Kodak, Stuttgart, Germany).
25. Sodium thiosulfate.

26. Monoclonal mouse anti-DIG antibody (Roche Diagnostics).
27. Biotinylated rabbit antibodies against mouse immunoglobulins (Dako, Glostrup, Denmark).
28. Streptavidin-biotinylated alkaline phosphatase complex (Dako).
29. Naphthol AS-MX phosphate.
30. *N*,*N*-dimethylformamide.
31. 0.1 *M* Tris-HCl buffer pH 8.2.
32. 1 *M* Levamisole.
33. Fast Red TR salt (Sigma).
34. Hematoxylin (Meyer's).
35. Eosin.
36. Glycerol gelatin.

2.4. Immunohistochemistry and Double Labeling

1. 4% PFA/PBS.
2. Xylene.
3. Acetone.
4. Ethanol.
5. Tris-hydroxyminomethane (Tris) base and -hydrochloride.
6. Tris-buffered saline (TBS): 0.05 *M* Tris-HCl, 0.15 *M* NaCl, pH 7.6.
7. Pronase.
8. 0.1 *M* Glycine in 1X PBS.
9. 0.1 *M* Citrate buffer pH 6.0.
10. Domestic microwave oven.
11. Bovine serum albumin (BSA).
12. Sodium heparin.
13. 10X RPMI 1640 medium (without Ca and Mg).
14. Yeast transfer (t)RNA (50 mg/mL).
15. Normal human serum.
16. Biotinylated rabbit antibodies against mouse immunoglobulins (Dako).
17. Monoclonal mouse antirabbit antibody (Dako).
18. Alkaline phosphatase antialkaline phosphatase (APAAP) complex (Dako).
19. 0.2 *M* 2-amino-2,2-methyl-propan-1,3-diole (propandiole).
20. 1 *M* Levamisole.
21. TBS, pH 9.7.
22. Naphthol AS-BI phosphate.
23. *N*,*N*-dimethylformamide.
24. $NaNO_3$.
25. New fuchsin.
26. 2 *N* HCl.
27. 0.1 *M* Tris-HCl buffer, pH 8.2.
28. 30% Hydrogen peroxide (H_2O_2).
29. Peroxidase-conjugated anti-DIG-Fab fragment.

30. 3,3-Diaminobenzidine tetrahydrochloride (DAB).
31. 100X Denhardt's solution (freeze-dried): 2% BSA, 2% Ficoll 400, 2% polyvinylpyrrolidone.
32. 0.5 *M* ethylene diamine tetraacetic acid, pH 8.0.
33. Alkaline phosphatase-conjugated anti-DIG-Fab fragment.

3. Methods

3.1. Preparation of Glassware, Slides, Cover Slips, Sections, and Cytospins

3.1.1. Glassware

For RNA ISH, glassware has to be RNase-free. To achieve this, clean glassware is wrapped in tin foil and baked at 250°C for 6 h.

3.1.2. Slides

Slides are coated with APES to improve adhesion of cells and sections:

1. Wash glass slides in 2% Decon 90.
2. Rinse thoroughly in tap water.
3. Briefly rinse in deionized water.
4. Air-dry for approx 15 min.
5. Place into acetone for 3 min at room temperature (RT).
6. Incubate in 2% APES/acetone for 3 min at RT.
7. Briefly rinse in ddH_2O and air-dry.

3.1.3. Cover Slips

Glass cover slips are siliconized to avoid sticking of probe DNA or RNA to the glass and to allow easy removal after hybridization:

1. Clean cover slips in 0.2 *N* HCl for 20 min.
2. Rinse in deionized water.
3. Briefly dip into 100% ethanol.
4. Air-dry for 15 min at RT.
5. Bake at 250°C for 5 h.
6. Allow to cool.
7. Dip into silicon solution.
8. Bake at 100 to 115°C for 2 h.

3.1.4. Paraffin Sections

Tissues are fixed in neutral buffered formalin and embedded in paraffin wax using routine histological procedures. Approximately 5-µm thick sections (3–5 µm for immunohistochemistry 5–8 µm for ISH) are cut from paraffin blocks and mounted onto APES-coated slides. Dry at 60°C for 1 h.

3.1.5. Frozen Sections

Fresh tissue samples are placed into plastic vessels, covered with physiological saline solution, snap-frozen in liquid nitrogen, and stored at –70°C. Cut 6-µm frozen sections on a cryostat and mount them onto APES-coated slides. For use in immunohistochemistry, air-dry sections overnight. If the slides are not used for immunohistochemistry immediately, store unfixed at –70°C. For use in ISH, sections are dried on a 60°C hot plate for 3 min and fixed immediately in 4% PFA for 20 min at RT. Wash slides twice in 3X PBS and twice in 1X PBS for 1 min each at RT. Dehydrate sections through a series of graded ethanol dilutions (70, 90, 96, 100%), air-dry briefly and store at –70°C.

3.1.6. Cytospin Preparations

Cells are washed and resuspended in PBS. Cell concentration is adjusted so that approx 5×10^4 to 1×10^5 cells are added per slide. After centrifugation, cytospins are treated as described for frozen sections.

3.2. Probe Labeling

3.2.1. RNA Probes

Single-stranded RNA probes are generated from plasmids with the specific insert located between two bacteriophage-derived DNA-dependent RNA polymerase promoter sites to allow the transcription of probes in sense and antisense direction. To ensure transcription of the insert only, linearized plasmid template is used. The method described is for the generation of ^{35}S-labeled probes. DIG-labeled probes are generated using a commercially available kit (DIG RNA labeling mix, Roche Diagnostics) according to the manufacturer's instructions.

1. Linearization of plasmids: mix 10 µg plasmid DNA, 40 U restriction enzyme and 4 µL of appropriate 10X restriction enzyme buffer, make up to 40 µL with ddH_2O. Incubate at 37°C overnight.
2. Precipitation of linearised plasmid DNA: add 4 µL of 3 *M* sodium acetate and 80 µL of ethanol to the linearization mix. Incubate at –70°C for 30 min. and centrifuge at 15,700*g* in a microcentrifuge. Air-dry pellet for 30 min and resuspend in 12.5 µL of DEPC–H_2O.
3. Verify linearization by running a 0.5-µL aliquot of the digestion mixture on a 0.5% agarose gel.
4. For the generation of ^{35}S-labeled probes, the transcription reaction is conducted as follows (*see* **Note 3**):

Mix	50 µCi	α-^{35}S-UTP (*see* **Note 3**)
	0.5 µL	RNase inhibitor
	2 µL	5X transcription buffer
	1 µL	100 m*M* DTT
	1.5 µL	NTP mix

1 µL Linearized plasmid
1 µL RNA polymerase (SP6, T3, or T7).

Make up to 10 µL with DEPC–H_2O and mix, incubate at RT for 60 min, and add another 0.5 µL of RNA polymerase and incubate for another 30 min.

5. DNase digestion of plasmid DNA: to the reaction mix, add 5 µL of yeast tRNA solution, 0.5 µL of RNase inhibitor, and 0.5 µL of DNase I (RNase-free) and incubate at 37°C for 8 min.
6. Phenol/chloroform extraction: add 10 µL of 3 *M* sodium acetate, 74 µL of DEPC–H_2O, and 100 µL of phenol:chloroform:isoamylacohol (25:24:1 v/v, pH 4.7). Centrifuge at 15,700*g* in a microcentrifuge and save upper phase (~ 100 µL).
7. Precipitation: ethanol precipitate with 1/10 vol 3 *M* sodium acetate and 2 vol ethanol as described previously, centrifuge and air-dry pellet. Resuspend in 100 µL DEPC–H_2O.
8. Repeat ethanol precipitation and continue with **step 10,** or
9. Subject labeled probes to alkaline hydrolysis (*see* **Note 4**):
   - Add an equal volume of hydrolysis buffer (80 m*M* $NaHCO_3$/120 m*M* Na_2CO_3 [pH 10.2]/10 m*M* DTT).
   - Incubate the mixture at 60°C.
   - Terminate reaction by adding an equal volume of stop solution (0.2 *M* sodium acetate, 1% acetic acid, 10 m*M* DTT).
   - Ethanol precipitate with 1/10 vol 3 *M* sodium acetate and 2 vol ethanol, air-dry pellet.
10. Resuspend pellet in 12.5 µL of DEPC–H_2O. Take a 0.5-µL aliquot for scintillation counting (*see* **Note 5**). To the remaining 12 µL, add 3 µL of 100 m*M* DTT and 15 µL of deionized formamide.

3.2.2. DNA Probes

For the detection of EBV DNA, a plasmid harboring the *Bam*HI W internal repetitive fragment of the EBV genome is used. Total plasmid DNA is labeled using nick translation with ^{35}S-labeled dCTP. Nick translation requires a balanced mixture of DNase I and DNA polymerase. If this reaction is not conducted frequently, the use of kits is advised, which are available from several suppliers. After the nick translation reaction, labeled DNA is separated from unincorporated nucleotides using Sephadex columns (e.g., Nick columns, Amersham) according to the supplier's instructions. Alternatively, DNA probes can be labeled using random primer labeling kits (e.g., Roche).

3.3. ISH

3.3.1. Prehybridization

Prehybridization treatment is essentially the same for DNA and RNA ISH. Paraffin sections are dewaxed in xylene (two changes, 10 min each) and rehy-

drated through a series of graded ethanols (100, 96, 70, 50%, DEPC–H_2O). Frozen sections or cytospin preparations are taken out of the –70°C freezer and thawed for at least 1 h (keep wrapped to avoid condensation). Prehybridization treatment is then carried out as follows:

1. Incubate in 0.2 *N* HCl for 10 min at RT.
2. Rinse with 2X SSC.
3. Incubate in 0.01% Triton X-100 for 90 s at RT (*see* **Note 6**).
4. Rinse with 2X SSC.
5. Digest with Pronase (0.125–1 mg/mL) in 1X PBS (*see* **Note 7**).
6. Block pronase with 0.1 *M* glycine in 1X PBS for 30 s at RT.
7. Rinse with 1X PBS for 30 s.
8. Postfix in ice-cold 4% PFA/PBS, pH 7.0, for 20 min.
9. Rinse thoroughly with 2X SSC.
10. Acetylate sections with 0.1 *M* triethanolamine (pH 8.0)/0.25% acetic anhydride for 10 min at RT (*see* **Note 8**).
11. Rinse with 2X SSC.
12. Dehydrate sections through graded ethanols.

3.3.2. EBV DNA ISH

1. Make up the hybridization mix to give the following end concentrations (*see* **Note 9**):
   - 50% deionized formamide.
   - 2X SSC.
   - 10% dextran sulfate (*see* **Note 10**).
   - 10 m*M* DTT (*see* **Note 11**).
   - 30 µg/mL carrier DNA.
   - 20 to 40 ng/mL labeled probe.
2. Add 25 µL of hybridization mix to each slide.
3. Cover sections with siliconized cover slips.
4. Denature probe and cellular DNA by placing slides onto a 90°C heat block for 3 min.
5. Hybridize overnight at 37°C in an atmosphere of 50% formamide (*see* **Note 12**).
6. Remove cover slips and wash in 50% formamide/0.1X SSC/10 m*M* DTT at 37°C for 4 h with hourly changes of the wash solution.
7. Rinse in 2X SSC/10 m*M* DTT for 30 min at RT.
8. Rinse in 0.1X SSC/10 m*M* DTT for 30 min at RT.
9. Dehydrate sections through graded ethanols and air-dry.
10. Continue with **Subheading 3.3.3.**

3.3.3. EBER ISH

1. Make up the hybridization mix to give the following end concentrations (*see* **Note 13**):
   - 50% deionized formamide.
   - 2X SSC.

- 10% dextran sulfate (*see* **Note 9**).
- 10 m*M* DTT (*see* **Note 10**).
- 250 µg/mL Yeast tRNA.
- 50,000–200,000 cpm ^{35}S-labeled probe per section (*see* **Note 13**) or DIG-labeled probes to a final dilution of between 1:25 to 1:200 (*see* **Note 13**).
- DEPC–H_2O to final volume.

2. Place approx 25 µL of hybridization mix on each section.
3. Cover sections with a piece of parafilm cut to size (*see* **Note 14**).
4. Hybridize overnight at 50°C in an atmosphere of 50% formamide (*see* **Note 12**).
5. Remove parafilm and wash slides in 50% formamide/1X SSC/10 m*M* DTT at 52°C for 4 h with hourly changes of the wash solution.
6. Wash in 2X SSC/10 m*M* DTT at 37°C for 30 min.
7. Incubate in 20 µg/mL RNaseA/2X SSC/10 m*M* DTT at 37°C for 30 min.
8. Wash in 2X SSC/10 m*M* DTT at RT for 10 min.
9. Wash in 0.1X SSC/10 m*M* DTT at RT for 10 min.

Sections subjected to ISH with ^{35}S-labeled probes are then dehydrated through graded ethanols and air-dried. Autoradiographic detection of bound probes is described in **Subheading 3.3.4.**

10. Sections exposed to DIG-labeled probes are transferred to TBS. Bound probes are detected using immunohistochemistry as described in **Subheading 3.3.5.**

*3.3.4. Darkroom Procedure (*see *Note 15)*

1. Melt Ilford G5 photographic emulsion at 42°C.
2. Prepare a 1:2 dilution of the emulsion in ddH_2O (*see* **Note 15**).
3. Dip slides into the emulsion (*see* **Notes 15** and **16**).
4. Dry slides in an upright position for 1 h (*see* **Note 15**).
5. Place slides into a light proof box and wrap box in tin foil (*see* **Note 15**).
6. Expose slides at 4°C for 3–20 d (*see* **Note 17**).
7. Remove slide box from refrigerator and allow to adjust to room temperature for at least 30 min.
8. Prepare a 1:2 dilution of Kodak D19 developer.
9. Prepare a 25% (w/v) solution of sodium thiosulfate.
10. In complete darkness remove slides from the box and place into the developer for 3 min.
11. Wash in cold tap water.
12. Fix in 25% sodium thiosulfate for 3 min.
13. Rinse thoroughly under running cold tap water.
14. Counterstain with hematoxylin and eosin (filter before use).

3.3.5. Detection of DIG-Labeled EBER-Specific Probes

1. Apply DIG-specific mouse monoclonal antibody diluted in TBS/1% BSA and incubate for 60 min. at RT.

2. Rinse with TBS.
3. Apply appropriate secondary antibody diluted in TBS and incubate for 30 min at RT.
4. During the incubation, prepare streptavidin-biotinylated alkaline phopshatase complex (StreptABC-AP) according to the manufacturer's instructions. Note that the complex should be prepared 30 min before use.
5. Rinse with TBS.
6. Incubate with StreptABC-AP for 30 min at RT.
7. While incubation takes place, prepare substrate/chromogen solution (*see* **Note 18**):
   - Dissolve 2 mg of Napthol AS-MX phosphate in 0.2 mL of *N*,*N*-dimethylformamide.
   - Add 9.8 mL 0.1 *M* Tris buffer, pH 8.2.
   - Add 10 µL of 1 *M* levamisole.
   - Just before use, dissolve 10 mg of Fast Red TR salt the solution and filter through ordinary filter paper.
8. Rinse in TBS.
9. Apply chromogen/substrate solution for 30 min at RT.
10. Rinse with TBS.
11. Counterstain with hematoxylin and mount using an aqueous mounting medium (glycerol gelatin).

3.4. Double Labeling Methods

For a discussion of general aspects, *see* **Note 19**.

3.4.1. Sectioning, Fixation, and Antigen Retrieval

3.4.1.1. Frozen Sections, Cytological Prepraration

1. Cryostat sections of 6-µm thickness and cytocentrifuge slides are prepared immediately prior to use. Air-dry; do *not* use a hot plate.
2. Fix in ice-cold 4% PFA/PBS, pH 7.0, for 20 min. (*see* **Notes 20** and **21**).

3.4.1.2. Paraffin Sections

1. Dewax in xylene (two changes) for a minimum of 30 min and in acetone for 10 min, all at RT. Rehydrate through graded ethanols (5 min each in 100, 96, 70% ethanol and TBS) at RT.
2. Depending on the antibody, predigest the section with pronase (approx 0.01 mg/mL PBS; *see* **Note 7**).
3. Use 0.1 *M* glycine/TBS for 30 s to inactivate pronase. Transfer to TBS.
4. Alternatively or additionally, some antibodies require epitope unmasking by heat-treatment: place sections into a beaker with 1000 mL 0.1 *M* citrate buffer, pH 6.0, made up with DEPC–H_2O. Irradiate in a domestic microwave oven (750 W) at maximum power for up to 60 min (*see* **Note 22**). Allow to cool down and transfer to TBS.

3.4.2. Antibody Reactions

1. Apply the appropriately diluted primary antibody in IH/ISH dilution buffer (1X RPMI-1640, 1% BSA (w/v), 5000 U/mL heparin, 2.5 mg/mL yeast-tRNA, pH 7.5) and incubate for 20 min at RT (***[7]***; *see* **Notes 23** and **24**).
3. Flush the slides with 1X TBS. Take care to remove buffer as completely as possible to avoid diluting subsequently applied reagents, but do not let the section dry out.
4. Apply appropriate bridging antibody (e.g., rabbit anti-mouse immunoglobulin) diluted in IH/ISH dilution buffer including 1/8 vol of heat inactivated (*see* **Note 24**) normal serum for 20 min at RT.
5. Flush the slides with 1X TBS.
6. Apply secondary bridging antibody, if appropriate (*see* **Note 24**).
7. Flush the slides with 1X TBS.
8. Apply APAAP complex (diluted 1:20 ***[8]***) in IH/ISH dilution buffer for 20 min at RT. Save a few drops of the reagent to control the substrate solutions (*see* **Note 18**).
9. **Steps 2–7** may be repeated for increased sensitivity (*see* **Note 24**).

3.4.3. Alkaline Phosphatase Development

The new fuchsin alkaline phosphatase-substrate solution (*see* **Note 25** ***[8]***) is prepared from the solutions A, B, and C, which are made up before use in the following order:

3.4.3.1. Solution A

1. Mix APAAP development buffer (TBS = 0.05 *M* Tris/0.15 *M* NaCl, pH 9.7) and 0.2 *M* propandiole (2-amino-2,2-methyl-propan-1,3-diole, 21 g/1000 mL H_2O, store at 4°C in a dark bottle) in a baked glass beaker, and dissolve the appropriate amount of levamisole:

for	50	100	150	200	250	300	350	400	450	500	mL
TBS pH 9.7	35	70	105	140	175	210	245	280	315	350	mL
Propandiole	12.5	25	37.5	50	62.5	75	87.5	100	112.5	125	mL
Levamisole	20	40	60	80	100	120	140	160	180	200	mg

3.4.3.2. Solution B

1. Under a fume hood, dissolve naphthol-AS-Bi-phosphate in DMF (*N*,*N*-dimethylformamide) in a baked glass beaker:

for	50	100	150	200	250	300	350	400	450	500	mL
Na-AS-Bi-P	25	50	75	100	125	150	175	200	225	250	mg
DMF	0.3	0.6	0.9	1.2	1.5	1.8	2.1	2.4	2.7	3.0	mL

3.4.3.3. Solution C

1. Immediately before use, prepare a solution of the appropriate amount of sodium nitrite by dissolving the substance in bidistilled water.
2. Add the appropriate volume of 5% new fuchsin solution (dissolve 5 g in 100 mL 2 *N* HCl, keep in a dark bottle at 4°C), and
3. Allow the solution to react under vigorous agitation for exactly one min (use a stopwatch!):

for	50	100	150	200	250	300	350	400	450	500	mL
$NaNO_3$	10	20	30	40	50	60	70	80	90	100	mg
H_2O	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0	2.25	2.5	mL
New fuchsin	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	mL

4. Combine the appropriate amounts of solutions A and B, mix well.
5. Add solution C, mix well, adjust to pH 8.8 by dropwise adding 2 *N* HCl, filter, control the pH and, if necessary, adjust again to pH 8.8.
6. Control the AP–substrate solution by mixing of an aliquot with a minute amount of APAAP complex solution saved from the APAAP complex solution (*see* **Note 18**).
7. Use the solution immediately, i.e., within less than 5 min, for developing of sections under constant agitation.
8. Wash three times with 1X TBS, control the staining result by microscopy, and proceed with prehybridization.

3.4.4. Hybridization Pretreatment

1. Incubate in 0.2 *N* HCl for 10 min at RT.
2. Rinse with DEPC–H_2O and 1X PBS for 30 s each.
3. Incubate in 0.01% Triton X-100 for 90 s at RT (for DNA probes, *see* **Note 6**).
4. Rinse in 1X PBS.
5. Digest with pronase (approx 0.125 mg/mL in 1X PBS, pH 7.2) at RT, (*see* **Note 7**).
6. Block pronase with 0.1 *M* glycine in 1X PBS for 30 s at RT.
7. Rinse with 1X PBS for 30 s.
8. Postfix in ice-cold 4% PFA/PBS, pH 7.0, for 20 min.
9. Rinse with 1X PBS for 3 min.
10. Acetylate sections with freshly prepared 0.1 *M* triethanolamine (pH 8.0)/0.25% acetic anhydride (v:v) for 10 min at RT (*see* **Note 8**).
11. Rinse with 1X PBS.
12. Dehydrate sections through graded ethanols with less than 10 s for each step (*see* **Note 26**).
13. Continue with application of the appropriate probe mixture.

3.4.5. RNA ISH

Follow the procedure outlined in **Subheading 3.3.** Sections subjected to ISH with [^{35}S]-labeled probes are then rapidly dehydrated through graded ethanols and air-dried (*see* **Note 25**).

3.4.6. Autoradiography/Darkroom Procedure

Follow the procedure described in **Subheading 3.3.4.** If desired, use Meyer's hematoxylin for nuclear counterstaining and mount in glycerol gelatin.

3.4.7. Detection of Immobilized Digoxigenated Probe

Typically, this applies to the detection of highly abundant transcripts such as EBER or some lytic cycle (e.g., BHLF1) RNA that are localized virtually exclusive to the nucleus except for mitotic figures. When alkaline phosphatase has been used as a tag for the antibody used in the preceding steps of the IH/ISH procedure, peroxidase and DAB chromogen-substrate are a good choice for visualizing the bound digoxigenated probe.

1. Block endogenous peroxidase using 3% hydrogen peroxide (H_2O_2) in ddH_2O for 5 min at RT probe (*see* **Note 27**).
2. Rinse three times for 2 min each with 1X TBS.
3. Apply peroxidase-conjugated anti-DIG-Fab fragments in a dilution of up to 1:1000 and incubate for 20 min at RT.
4. Rinse three times for 2 min each with 1X TBS.
5. Dissolve 6 mg of DAB in 10 mL of 0.05 *M* Tris buffer, pH 7.6, add 0.1 mL of 3% H_2O_2, filter, apply to the section, and incubate for 5 min at RT probe (*see* **Note 27**).
6. Rinse with TBS.
7. Counterstain nuclei with Meyer's hematoxylin, if desired, and mount with glycerol gelatin.

3.5. Combined ISH-ISH Labeling

For this procedure, sectioning, fixation, labeling of RNA probes, prehybridization, and hybridization are conducted as described for simple ISH. However, the preparation of the hybridization mixture and the washing sequence differ.

The probe mixture is made up with [^{35}S]-labeled and DIG-labeled probes keeping the volume as described for [^{35}S]-labeled probes as described previously. Alternatively, a hybridization mixture consisting of 50% formamide, 10 m*M* Tris-HCl, pH 7.5, 10 m*M* sodium phosphate, 1X Denhardt's solution, 1 mg/mL yeast tRNA, 5 m*M* EDTA, 10 m*M* DTT, 10% dextran sulfate, 8×10^6 cpm/mL [^{35}S]-labeled RNA probe (equivalent to approx 6 ng/mL), and 100 ng/mL DIG-labeled EBER probe may be used.

1. After hybridization, washes including the RNase A step are performed up to the 0.1X SSC step as described in the previous chapter for ISH.
2. Slides are then transferred to TBS.
3. DIG-labeled EBER probe is detected in a single step using an AP-conjugated anti-DIG-Fab fragments (diluted in TBS up to 1:1000) for 20 min at RT.

4. Wash with TBS three times for 5 min each at RT.
5. Visualize alkaline phosphatase with new fuchsin as previously described.
6. Dehydrate through graded ethanols, taking care not to dissolve the new fuchsin precipitates.
7. The slides are ready for autoradiography (*see* **Subheading 3.3.4.**; for two-color detection of two nonisotopically labeled probes, *see* **Note 28**).

4. Notes

1. Glassware and solutions used for RNA ISH have to be treated to inactivate RNases that are ubiquitous. For Glassware, this is achieved by baking at 250°C for 6 h. All solutions that can be autoclaved are treated with diethylpyrocarbonate. Add 1 mL of DEPC per liter, shake well, incubate for at least 1 h to overnight, and autoclave. Autoclaving leads to the decomposition of DEPC into ethanol and CO_2. Therefore, pH has to be adjusted after autoclaving. Solutions that cannot be autoclaved are prepared using DEPC-treated water (e.g., Tris-containing solutions, dextran sulfate).
2. Stock solutions of pronase are prepared by dissolving commercially available freeze-dried pronase in ddH_2O, The solution is incubated at 37°C for 4 h to digest contaminating enzymes such as nucleases.
3. For the generation of radiolabeled probes with high specific activity, no unlabeled UTP is added to the transcription reaction. In our hands, probes generated using a mixture of ^{35}S-labeled and unlabeled UTP result in weaker ISH signals. To obtain probes of even higher specific activity, a second labeled nucleotide (e.g., ^{35}S-CTP) can be added to the transcription reaction. If the volume of the radioactive nucleotide is too large, use a speed-vac or a similar centrifuge to freeze-dry the labeled NTP solution.
4. To increase penetration into tissues and cells, it has been recommended that RNA probes should have an average length of between 50 and 150 bases ***(21)***. This is achieved by controlled alkaline hydrolysis of labeled probes. The hydrolysis time is calculated according to the equation $t = (L_0 - L_f)/(k \cdot L_0 \cdot L_f)$, with t = hydrolysis time (min), L_0 = initial transcript length, L_f = final transcript length, and the constant $k = 0.11\ kb^{-1} \cdot min^{-1}$. However, use of nonhydrolyzed, longer probes has been reported to improve signal-to-noise ratio ***(22–24)*** and, in our hands, the elimination of the alkaline hydrolysis step has at least not led to a loss of signal intensity for most probes.
5. Probes are used if scintillation counting of a 0.5-µL aliquot yields an activity of at least 5×10^5 cpm.
6. Treatment with Triton-X is optional and in our laboratories is used only in conjunction with DNA ISH.
7. A variety of proteases have been recommended for ISH. In our hands, pronase has given reliable and reproducible results over many years. The concentrations given are meant as a guideline only. It is important to titrate the pronase to establish the conditions that will give the best signal without disrupting tissue morphology. It is possible to vary the pronase concentration, the duration of the

digestion, and the temperature. In particular, when the ambient temperature in the laboratory may be variable, it is advised to use an incubator, for instance, at 37°C. Factors that may affect the outcome of the pronase digestion are type of fixative, length of fixation, and other variables of the embedding procedure. Nevertheless, once the optimal pronase digestion conditions have been established, there is very little variation necessary for blocks from the same institution. Currently used concentrations in our laboratories are 0.5 mg/mL for paraffin sections and 0.125 mg/mL for frozen sections and cytospins. Higher concentrations may be required for DNA ISH. To identify an appropriate pronase concentration, digest tissue sections with different pronase concentrations keeping temperature and incubation time constant. Counterstain sections and identify the pronase concentration, which visibly starts to damage the tissue. Then, use half that concentration.

8. The mixture of triethanolamine and acetic anhydride must be prepared freshly immediately before adding it to the sections. Acetylation is believed to reduce background by decreasing nonspecific probe binding to glass *(25)*. Whether this actually works is uncertain. However, it is included in our protocol for ISH with ^{35}S-labeled probes. We usually omit this step when working with nonradioactive probes.
9. Calculations are based on the assumption that 25 µL of hybridization mix are required for the average slide. The amount of DNA probe added per section is calculated assuming that the nick translation reaction does not produce a net change of the amount of DNA included in the reaction.
10. The dextran sulfate solution is viscous and difficult to pipet, however, this procedure may be facilitated by warming the solution. The following procedure also has proved useful. Set the pipet to the required volume and fit appropriate tip. Draw the required volume of water into the pipet tip and mark the level. Eject the water again, increase the volume setting of the pipet, and draw dextran sulfate solution into the tip up to the marked level.
11. DTT is added to hybridization mix and washing solutions only when using ^{35}S-labeled probes.
12. We add a 50% formamide solution to the incubation chamber. In our experience, making a humid chamber just by adding water will lead to probe dilution.
13. Calculations are based on the assumption that 25 µL of hybridization mix are required for the average slide. Radioactive probe is included in the mix so that between 50,000 cpm and 250,000 cpm are added per slide. The amount of radioactive probe necessary may vary depending on the abundance of the target, the individual probe and the labelling reaction. For the EBERs, usually between 50,000 cpm and 100,000 cpm per slide suffice. Note that if the EBER1 and EBER2 probes are used as a mixture, 50,000–100,000 cpm per slide are added for each probe. Dilutions of DIG-labeled probes have to be titrated using appropriate positive control sections. Note that before adding to the hybridization mix, RNA probes are heated at 80°C for 30 s and put on ice to remove secondary and tertiary structures.

14. Alternatively, siliconized glass cover slips may be used and this may be more appropriate for DNA ISH, which requires heating the slides to denature the target DNA. However, for RNA ISH, the use of parafilm is perfectly adequate.
15. Before entering the dark room, slides are sorted into a rack to facilitate dipping and to make sure that the slides are put into the correct light proof boxes in complete darkness. Photographic emulsion is diluted in the dark room using a low energy red light only. To facilitate this procedure, before entering the dark room 10 mL of ddH_2O are filled into a 50-mL plastic vial and the 20-mL mark is highlighted with a marker pen so that it can be seen using only a dim red light. Ten milliliters of the melted emulsion are carefully poured into the water and mixed so as to avoid formation of froth. For dipping the sections, plastic slide containers for mailing glass slides have proved useful. These hold a volume of approx 20 mL. The diluted emulsion is carefully poured into the slide container, and the slides are dipped into the emulsion. If a larger number of slides are to be coated with emulsion, it is advisable to keep the emulsion in a 42°C water bath. Coated slides are placed onto an absorbent paper towel in an upright position to allow draining of excess emulsion and air-dried for 1 h in complete darkness. Still in complete darkness, the slides are then placed into a light proof box together with a drying agent, wrapped in tin foil and placed into a 4°C refrigerator.
16. In our experience, it is safe to reuse diluted emulsion once. For this purpose, the container with the emulsion is tightly wrapped with tin foil and stored at 4°C. To reuse the emulsion, place the container into a 42°C water bath and proceed as described.
17. Exposure times will vary depending on the abundance of the target. Three to seven days usually are sufficient for the EBERs, whereas detection of viral DNA and of low copy mRNAs may require longer exposure. For every experiment it is advisable to prepare at least two or three sets of slides to allow development after different exposure times. For low copy number targets, preparation of more sets is recommended, and exposure times for up to 6 wk have been used successfully in our laboratories *(26)*.
18. The solution consisting of Naphthol AS-MX phosphate, dimethylformamide and Tris buffer can be stored at 4°C for several weeks. Fast Red TR salt is added immediately before use. To make sure that the substrate/chromogen solution is working before adding it to the slides, a small aliquot of the StreptABC-AP reagent can be mixed with an aliquot of the substrate/chromogen solution in a reaction tube. The solution should turn red quickly.
19. In our experience, autoradiographic silver grains are best documented against the background of a red chromogen. Substrates producing a red colored product at the site of enzymatic activity are available for both alkaline phosphatase and peroxidase, the most frequently used reporter enzymes in IH. AEC (3-amino-9-ethylcarbazole) as a substrate for peroxidase results in a bright red product which, similar to fast red for alkaline phosphatase visualization is easily dissolved in ethanol and other solvents, whereas new fuchsin is more stable and permits dehydration through graded ethanols. We are thus providing a protocol for detection

of alkaline phosphatase activity with new fuchsin. When detecting two targets by nonradioactive methods, the two colors should give a good contrast and should be distinguishable when colocalized. A good contrast is obtained for alkaline phosphatase/new fuchsin (red signal) and peroxidase/DAB (brown signal). However, if the antigen is detected by immunostaining and the nucleic acid is visualized by nonadioactive methods, a mixed color product may arise which is difficult to distinguish if both targets localize to the same cellular compartment. From the technical point of view, this combination has a number of advantages over other substrate combinations, and the problem of mixed colors is not important for transcripts with predominantly nuclear localization, such as EBER and BHLF1 RNA. This combination is also best for triple labeling procedures combining IH with radioactive as well as nonradioactive ISH (ISH-ISH) labeling procedures. Moreover, it is compatible with the use hematoxylin for nuclear counterstaining. We are therefore restricting ourselves to peroxidase and DAB as a second substrate for two-color nonisotopic immunohistochemistry. The most commonly applied procedure is the application of ISH subsequent to IH, which requires precautions to be taken to prevent loss of cellular RNA targets (*see* **Note 23**). In principle, it is possible to perform the ISH in sequence prior to IH. Many epitopes, however, even when resistant to formalin-fixation and paraffin embedding, do not tolerate the denaturing conditions with high formamide concentrations present during hybridization, and the heat denaturation when detecting DNA or using DNA probes. This results in reduced or, often in case of monoclonal antibodies, loss of reactivity.

20. Label the baked and APES-coated glass slides as comprehensively as possible on their frosted area with a lead pencil to avoid unnecessary handling later on. Use gloves and take care to remove talcum powder with a sterile towel and DEPC–H_2O, because the powder may disturb the immunoenzymatic reactions and may interfere with microscopy, particularly with darkfield illumination. Prepare a minimum of three (serial) sections for immunostaining and ISH with the antisense probe, at least one section for immunostaining and ISH with the sense (control) probe, and at least one slide for ISH with the antisense probe without IH. The latter are developed with the last set of immunostained/anti-sense hybridized slides and permit estimating the background (sense probe) and the loss of cellular target RNA (anti-sense probe) during the course of the immunostaining procedure.
21. Frozen sections should be used immediately for best results. If this is not possible, fix them in ice-cold acetone for 10 min, air-dry, and store at –80°C in air-tight boxes. Boxes (and their contents) are brought to RT, and sections are fixed immediately in ice-cold 4% PFA/PBS for 20 min. Best results are obtained with PFA-fixed frozen sections. However, this fixation restricts the spectrum of antibodies to those reacting with formalin-resistant epitopes. Some antibodies require extended PFA-fixation such as the CD68-antibody PG-M1. When detecting antigens/epitopes sensitive to formalin or PFA, sections may be precipitation fixed sequentially in acetone and chloroform for up to 30 min each. The sections are then ready for application of the primary antibody and should never dry out in

subsequent immunostaining steps. PFA fixation may then be carried out after the enzymatic development. Unlike PFA, acetone and chloroform do not reduce the endogenous RNase activity, so this procedure is not suitable for the subsequent ISH for RNA transcripts with the exception of the highly abundant and stable EBER molecules. The same considerations apply for cytospin preparations.

22. Irradiation of formalin-fixed paraffin sections in citrate buffer using a microwave oven has proved useful for the detection of many antigens *(27)*. A variety of microwaving conditions have been described, including variations in energy, time, or buffer used. Domestic pressure cookers may be used instead microwave ovens. Optimum conditions have to be determined individually in every laboratory. In our hands use of a large volume of citrate buffer (1 L) has proved useful. It requires longer microwave irradiation time than smaller quantities but makes substitution of volume lost through evaporation unnecessary. When placing slides into a beaker for microwave irradiation, make sure they are not stacked too tightly to ensure even distribution of heat.
23. When IH precedes the ISH labeling step, it is important to protect the cellular RNA from endogenous and exogenous RNases. This step is achieved by the addition of yeast-tRNA and heparin to all antibody preparations *(28)*. Moreover, it is equally important to reduce all incubation times to an absolute minimum. In most cases, incubation times of less than 20 min will suffice. If the APAAP procedure is repeated, the incubation periods may be further reduced to 5–10 min. IH/ISH dilution buffer is made up under sterile conditions with 10X RPMI 1640 (with phenol red serving as a useful pH indicator, similar buffer solutions will suffice as well), sterile filtered BSA stock solution, heparin, and yeast tRNA. The pH is adjusted to pH 7.5 before reaching the final volume with DEPC–H_2O. Aliquots may be kept frozen. The optimal concentration of the primary antibody in IH/ISH dilution buffer (including heparin, but without the expensive tRNA) has to be determined in preliminary experiments, because heparin nonspecifically binds to and inhibits not only RNase, but also immunoglobulins. For alkaline phosphatase reactions, TBS is used instead of PBS because phosphate ions inhibit the enzyme.
24. We recommend diluting secondary antibodies in TBS containing heat-inactivated serum obtained from the species that the sectioned tissue was derived from. This procedure prevents nonspecific reactivity of secondary antibodies with serum proteins absorbed by the tissue before or during fixation. Inactivation is achieved by incubating the serum at 56°C for 30 min. If the primary antibody is not a murine monoclonal antibody, but, e.g., a goat antibody, an additional incubation step ("mousification") is required using, for this example, mouse anti-goat immunoglobulin before proceeding with rabbit anti-mouse immunoglobulin and APAAP complex. If the primary antibody is from the rat, commercially available rabbit anti-rat immunoglobulin and rat APAAP complex (Dako) may be used.
    The APAAP procedure may be repeated to enhance the sensitivity of the IH procedure by increasing the amount of immobilized enzyme at the site of primary antibody binding. This is performed by repeated incubation of the slides with the

anti-mouse immunoglobulin and, after washing, with APAAP for 5–10 min each. However, this extends exposure of the sections to endogenous and exogenous RNases and may result in weaker autoradiographic signals.

25. The preparation of the new fuchsin substrate as outlined below is more laborious than the preparation of the fast red substrate previously described, but it results in a more intensively colored product. Moreover, new fuchsin is less readily soluble in ethanol and xylene, permitting rapid dehydration through graded ethanols.
26. Concentrated ethanol may remove the azo-dye precipitate, some other substrates are even more sensitive to alcohol. In this case, remove as much of the PBS as possible and proceed directly with the application of the appropriate probe mixture. Once ISH and washing have been completed, it is also possible to air-dry slides directly from the finally washing step and eliminate the dehydration through graded ethanols. This is advisable when using nonalcohol-resistant chromogens.
27. Alternatively, use a commercial blocking agent. Do not use methanol as this may dissolve the chromogen of the previous immunolabeling steps. DAB is a possible carcinogen. The brown color can be enhanced by treatment with nickel sulfate. Buffers must not contain sodium azide because it will interfere with the enzymatic reaction.
28. When attempting to detect two transcripts localizing to the same cellular compartment, the probe detecting the transcript with the higher copy number should be labeled with DIG, and the other one should be isotopically labeled. If both targets are of sufficiently high copy number to be detected by nonradioactive techniques, two-color immunostaining may be used to detect the immobilized probes if they do not localize to the same cellular compartment. Target transcripts with predominantly nuclear localization such as EBER or BHLF1 are therefore well suited for two-color techniques in conjunction with probes detecting cellular RNA transcripts. The cellular RNA with the least abundance should be detected by the sensitive APAAP method and new fuchsin as substrate, and the EBERs are then visualized in a single step reaction with peroxidase-conjugated Fab fragments specific for DIG. If the detection of both probes involves the application of primary or secondary antibodies of the same animal species, the peroxidase step is conducted with DAB development, the precipitates of which provide sufficient shielding of immune complexes from detection in the subsequent immunostaining procedures. The peroxide has to be washed out completely prior to the akaline phosphatase substrate reaction.

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9

PNA–*In Situ* Hybridization Method for Detection of HIV-1 DNA in Virus-Infected Cells and Subsequent Detection of Cellular and Viral Proteins

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Summary

We describe *in situ* hybridization protocols using peptide nucleic acid (PNA) as a probe for detecting HIV-1 DNA in virus-infected cells and the subsequent detection of cellular and/or viral proteins. Because a PNA probe of approx 20 bases was sufficiently long to detect a specific target sequence, a conserved sequence of such a short length was easily identified. Therefore, this probe is valuable even to identify quasi-species of HIV-1. In addition, we adopted a catalyzed signal amplification method to amplify weak viral DNA signals; thus, stringent washing was crucial for eliminating false-positive signals. Our double-staining method using PNA–*in situ* hybridization and subsequent immunostaining enabled the active and inactive proviruses to be distinguished.

Key Words: *In situ* hybridization; peptide nucleic acid; catalyzed signal amplification; HIV-1 provirus; CD4-positive T lymphocytes; p24; HLA-DR.

1. Introduction

In situ hybridization (ISH) is now popularly used in cytogenetic studies to determine the localization of a specific gene on a chromosome and to detect mRNA expression and viral infection within cells using DNA or RNA probes. Nonradioactive ISH methods using fluorescence or visible light to visualize signals generally are used for the detection of target nucleic acid sequences. In general, probes of more than 500 bp in length are required in such studies *(1)*.

Until recently, the detection of HIV-1 has been performed by using autoradiography, using probes labeled with radioisotopes, such as ^{35}S *(2–4)* and ^{125}I *(2,5)*. Although the use of radioactive ISH for the detection of HIV-1 is time consuming and not very convenient, the small copy number of HIV-1 in

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infected cells has hindered the development of a conventional nonradioactive detection system. In addition, designing a suitable proper probe with a length of more than 150 bp is very difficult because of the general lack of long-conserved DNA sequences in viruses, such as HIV-1, that have error-prone reverse transcriptase without any repair activity. To bypass this problem, we developed a peptide nucleic acid (PNA) that mimics the DNA configuration ***(6–8)***. PNA, being electrically neutral, can penetrate into cells more easily than DNA and, in addition, PNA can more strongly hybridize with DNA than DNA ***(9,10)***. For these reasons, a PNA probe of approx 20 bases in length is long enough to detect a specific target sequence ***(11)***.

To overcome the weak viral DNA signal, one of the following methods for signal amplification can be used: one is *in situ* polymerase chain reaction (PCR ***[12]***), and the other is a catalyzed signal amplification (CSA) using biotinyl tyramide ***(13)***. *In situ* PCR is quite powerful for amplification of rare target DNA within cells; therefore, *in situ* PCR-driven ISH would be suitable for detecting low copy number DNA sequences. However, this method carries the risk of amplicons synthesized *in situ* diffusing and resulting in false-positives ***(14)***. On the other hand, a single copy of the HPV-16 virus was detected successfully using the CSA method ***(14)***. Therefore, we adopted the CSA method for detecting HIV-1 DNA ***(15)*** and incorporated a crucial stringent washing step to eliminate nonspecific signals that arise from the CSA procedure.

Here, we describe the procedures for detecting HIV-1 DNA in infected CD4-positive T lymphocytes, and the phenotypic determination of HIV-1 DNA-positive cells by a double staining method.

2. Materials

2.1. Cell Lines

1. ACH2: positive control. Human lymphoid cell line latently infected with HIV-1.
2. MOLT4-IIIB: positive control. Human leukemic cell line persistently infected with HIV-1 strain IIIB.
3. MOLT4: negative control.

2.2. Sample Preparation

2.2.1. CD4-Positive T-Lymphocytes Smears

1. StemSep column chromatography (Stem Cell Technologies, Inc., Vancouver, BC, Canada; cat. no. STS-14032).
2. Phosphate-buffered saline (PBS) with 3% fetal bovine serum.
3. 4% paraformaldehyde (PFA) containing 0.1 *M* sodium phosphate buffer, pH 7.4.
4. Ethanol.
5. Silane-coated slides.

6. Wax pen (DakoCytomation A/S, Glostrup, Denmark).
7. Staining racks and containers.
8. Dryer.

2.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Tissue blocks of 20% formalin-fixed or 4% PFA-fixed, paraffin-embedded samples.
2. Silane-coated slides.
3. Staining racks and containers.
4. Xylene.
5. Rehydration series: 100, 95, 90, and 70% ethanol.

2.3. Pretreatment

2.3.1. CD4-Positive T Lymphocytes Smears

1. Tris-buffered saline containing Tween-20 (TBST): 50 m*M* Tris-HCl, 300 m*M* NaCl, 0.1% Tween-20, pH 7.6.
2. Target retrieval solution (DakoCytomation A/S; cat. no. S1700).
3. Methanol containing 0.3% H_2O_2.
4. Water bath.
5. Staining racks and plastic containers.

2.3.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. The same as **Subheading 2.3.1.**, **items 1–**5.
2. Proteinase K (DakoCytomation A/S, S3004).

2.4. Preparation of PNA Probe

1. PNA probe: the structure of the probe is as follows: FITC·HN-GCAGCTTCCT-CATTGATGG-$CONH_2$ (FASMAC Co. Ltd., Kanagawa, Japan; *see* **Note 1**).
2. DNA ISH solution (DakoCytomation A/S; cat. no. S3305).
3. Cover slips.

2.5. Heat Denaturation

1. Hotplate.

2.6. Hybridization

1. Stringent wash solution (DakoCytomation A/S; cat. no. K5201).
2. TBST.
3. Incubator.
4. Plastic containers.
5. Water bath.
6. Moist chamber.

2.7. PNA Probe Detection by CSA

1. Horseradish peroxidase (HRP)-conjugated anti-FITC antibody (DakoCytomation A/S; cat. no. P5100).
2. Biotinyl tyramide solution (DakoCytomation A/S, GenPoint kit K0620).
3. HRP-conjugated streptavidin (DakoCytomation A/S, GenPoint kit K0620).
4. Alexa Fluor 488-labeled streptavidin (Invitogen, Carlsbad, CA).
5. 4,6-diamino-2-phenylindole (DAPI) II (Vysis, Inc., Downers Grove, IL).
6. TBST.
7. Staining racks and containers.
8. Cover slips.

2.8. PNA–ISH and Immunohistochemistry (IHC) (Indirect Method)

1. Mouse anti-human CD4 monoclonal antibody (Novocastra Laboratories, Ltd., Newcastle, UK; cat. no. NCL-CD4-1F6) or mouse anti-human HLA-DR monoclonal antibody (DakoCytomation A/S; cat. no. M0746).
2. Alexa Fluor 594-labeled goat anti-mouse IgG antibody (Invitrogen; cat. no. A-11005).
3. DAPI II (Vysis, Inc.).
4. Cover slips.

2.9. PNA–ISH and IHC (Labeled Streptavidin-Biotin Method)

1. Avidin solution (DakoCytomation A/S; cat. no. X0590).
2. Biotin solution (DakoCytomation A/S; cat. no. X0590).
3. Mouse monoclonal anti-HIV-1 p24 antibody (DakoCytomation A/S; cat. no. M0857).
4. Biotinylated goat anti-mouse Ig antibody (DakoCytomation A/S; cat. no. E0433).
5. Alexa Fluor 594-labeled streptavidin (Molecular Probes, Inc.; cat. no. S-11227).
6. DAPI II (Vysis, Inc.).
7. Cover slips.

3. Methods

3.1. Sample Preparation (see Note 2)

3.1.1. CD4-Positive T Lymphocytes Smears

1. Negatively select and purify CD4-positive T lymphocytes by StemSep column chromatography according to the manufacturer's instructions.
2. Spin down the collected cells at 250*g* for 5 min.
3. Discard supernatant and resuspend with PBS.
4. Mark the area for sample-mount on a silane-coated slide with a wax pen. Drop 5 µL of the cell suspension onto the slide and spread it out gently using the pipet tip.
   The area of the sample-mount (15 × 15 mm).
5. Dry the slides using a dryer at a cool setting.
6. Fix the slides with 4% PFA containing 0.1 mol/L sodium phosphate buffer, pH 7.4, at room temperature for 60 min or at 4°C overnight.

7. Rinse the slides in PBS (3 min, three times).
8. Dehydrate the slides in absolute ethanol and then store at –20°C until use.

3.1.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Place a 4- to 5-µm section onto a slide. Heat the slide to melt the paraffin in a 60°C oven for 15 min and dry at 37°C overnight.
2. Deparaffinize sections in fresh xylene (3 min, three times) and rehydrate in graded (100, 100, 95, 90, and 70%) ethanols and autoclaved water.

3.2. Pretreatment

3.2.1. CD4-Positive T-Lymphocyte Smears

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C, and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Immerse the slides in methanol containing 0.3% H_2O_2 for 20 min.
5. Wash the slides in autoclaved water for 1 min.
6. Briefly immerse slides in 95% ethanol and allow to air dry.

3.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Digest sections with proteinase K for 10 min at room temperature (*see* **Note 3**).
5. Wash the slides in autoclaved water (1 min, three times).
6. Treat the slides with methanol containing 0.3% H_2O_2 for 20 min.
7. Wash the slides in autoclaved water for 1 min.
8. Briefly immerse the slides in 95% ethanol and allow to air-dry.
9. Mark the area of the section with a wax pen.

3.3. Preparation of PNA Probe

1. Dilute FITC-conjugated PNA probe in hybridization solution to a final concentration of between 0.2 and 0.5 µg/mL.
2. Apply 25 µL of hybridization solution containing PNA probe to the marked area of the slide.
3. Carefully apply the cover slip, avoiding the introduction of air bubbles.

3.4. Heat Denaturation

1. Heat the slides at 93°C for 5 min on a hotplate to denature the double-stranded DNA.

3.5. Hybridization

1. Incubate the slides with the PNA probe at 45°C for 60–90 min in a moist chamber.
2. After hybridization, immerse the slides in TBST and gently remove the cover slips.

3. Wash the slides in prewarmed stringent wash solution at 57°C (20 min, twice).
4. Immerse the slides in TBST at room temperature for 5 min.

3.6. PNA Probe Detection by CSA (see Notes 4 and 5; Fig. 1)

1. Incubate HRP-conjugated rabbit anti-FITC antibody (1:500 dilution) for 60 min (*see* **Note 6**).
2. Wash the slides in TBST (3 min, three times).
3. Incubate biotinyl-tyramide for 15 min.
4. Wash the slides in TBST (3 min, three times).
5. Incubate HRP-conjugated streptavidin (1:600–800 dilution) for 15 min.
6. Wash the slides in TBST (3 min, three times).
7. Incubate biotinyl-tyramide for 15 min.
8. Wash the slides in TBST (3 min, three times).
9. Incubate 0.5 μg/mL Alexa Fluor 488-labeled streptavidin for 15 min in the absence of light.
10. Wash the slides in TBST in the absence of light (3 min, three times).
11. Immerse the slides in distilled water.
12. Apply DAPI II and mount cover slip.

3.7. Fluorescence Microscopy

The slides were examined under a fluorescence microscope (BX50 and BX-FLA, Olympus Corp., Tokyo, Japan) equipped with appropriate filter sets (61002, Chroma Technology Corp., Rockingham, VT). Photographic images of the fluorescent signals were taken with a CCD camera (SenSys 0400, Photometrics Ltd., Tucson, AZ) and were uploaded to a microcomputer using IPLab software (Scanalytics, Inc., Fairfax, VA). The stored images were merged to reveal various aspects (*see* **Note 7**; **Fig. 2**).

3.8. Subsequent Determination of Phenotypes After PNA–ISH Treatment

We describe here a double staining method by PNA–ISH and IHC methods for the detection of both HIV-1 DNA and a protein in the same cell, respectively. Proteins can be detected with one of two methods with IHC: an indirect method and a labeled streptavidin-biotin (LSAB) method. For the detection of CD4 or HLA-DR molecules, the conventional indirect method was used. On the other hand, the LSAB method that has higher sensitivity than the indirect method was performed to detect the p24 HIV-1 capsid protein.

Fig. 1. (*opposite page*) Schematic representation of the procedures for using the PNA-probe in the ISH method. The hybridized probe was detected by sequential reactions of the following antibodies and reagents: HRP-conjugated anti-FITC antibody, biotinylated tyramide (first amplification), HRP-labeled streptavidin, biotinylated tyramide (second amplification), and streptavidin-conjugated Alexa 488.

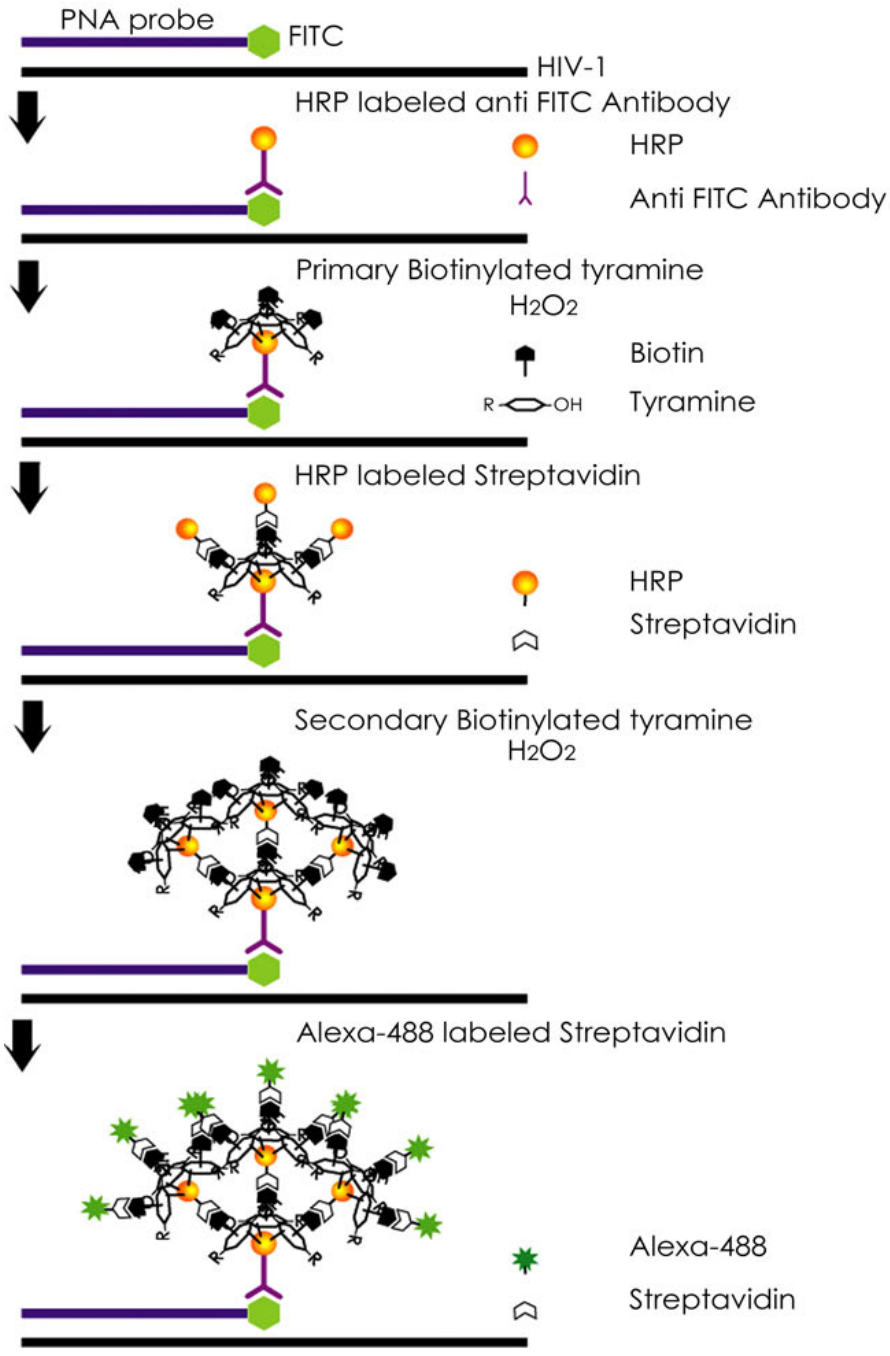
PNA probe
FITC
HIV-1
HRP labeled anti FITC Antibody
HRP
Anti FITC Antibody
Primary Biotinylated tyramine
H2O2
Biotin
R
OH
Tyramine
HRP labeled Streptavidin
HRP
Streptavidin
Secondary Biotinylated tyramine
H2O2
Alexa-488 labeled Streptavidin
Alexa-488
Streptavidin

Fig. 1

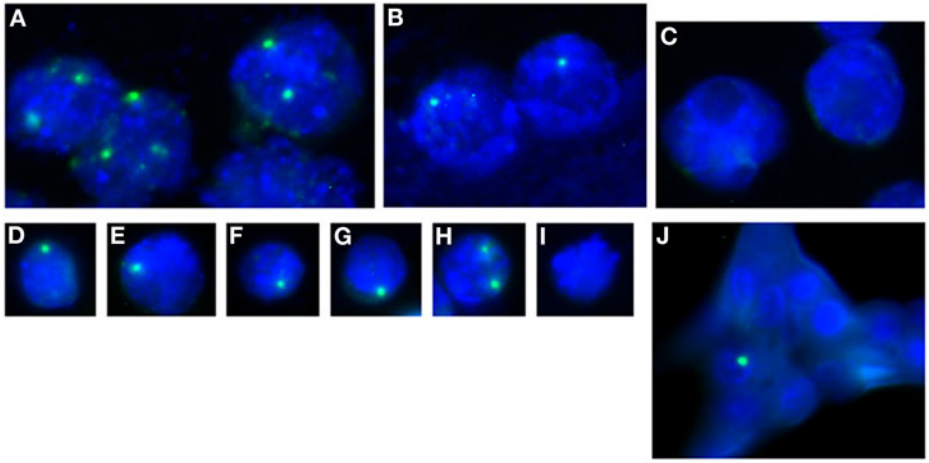

Fig. 2. Detection of HIV-1 provirus by PNA–ISH. Signals detecting HIV-1 proviruses were observed as green dots in a nucleus stained blue by DAPI. **(A)** MOLT4-IIIB. One to four proviruses per cell. **(B)** ACH2. One provirus per cell. **(C)** Negative control (MOLT4). No provirus. **(D–H)** CD4-positive T lymphocytes from HIV-1-infected patients. Most provirus-positive cells contained one provirus. Positive cells rarely contained more than two proviruses. **(I)** Negative control (CD4-positive T lymphocytes from a HIV-1-negative volunteer). No provirus. **(J)** Paraffin-embedded section of bone marrow from an AIDS patient. Positive cells contained one provirus in a nucleus.

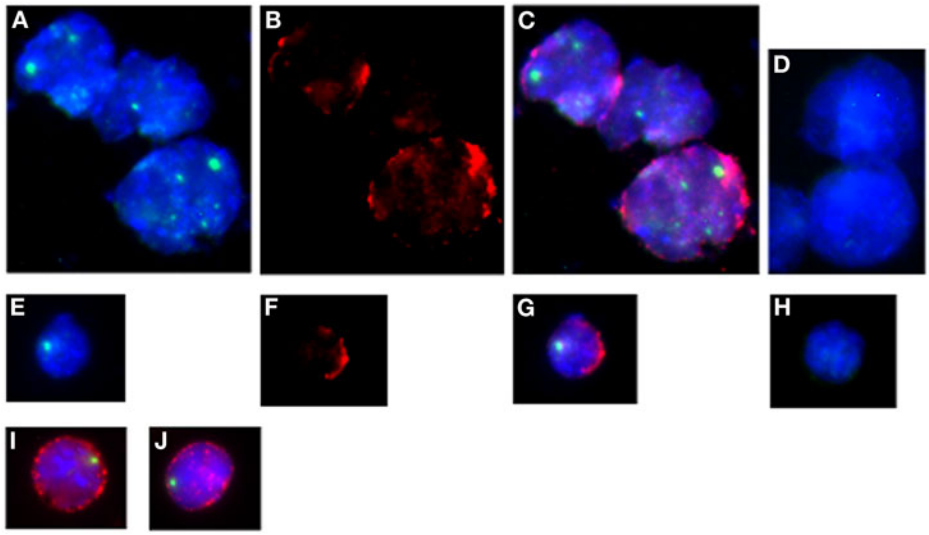

Fig. 3. Subsequent determination of phenotypes after PNA–ISH treatment. Proteins were stained red and localization of the proteins can be observed. **(A–C)** p24/HIV-1 provirus of MOLT4-IIIB. **(D)** Negative control (MOLT4). **(E–G)** p24/provirus

3.8.1. PNA–ISH and IHC (Indirect Method)

1. Follow **steps 1–10** inclusive for PNA Probe Detection by CSA (**Subheading 3.6.**).
2. Incubate with mouse anti-human CD4 monoclonal antibody or mouse anti-human HLA-DR monoclonal antibody at 4°C overnight.
3. Wash in TBST (3 min, three times).
4. Incubate with Alexa Fluor 594-labeled goat anti-mouse IgG antibody for 30 min.
5. Wash in TBST (3 min, three times).
6. Apply DAPI II and mount a cover slip.

3.8.2. PNA–ISH and IHC (LSAB Method)

Biotin blocking of biotinyl-tyramide after PNA–ISH staining is recommended before the reaction with primary antibody for the target protein.

1. Follow **steps 1–10** inclusive for PNA Probe Detection by CSA (**Subheading 3.6.**).
2. Incubate with avidin for 10 min.
3. Wash in TBST (3 min, three times).
4. Incubate with biotin for 10 min.
5. Wash in TBST (3 min, three times).
6. Incubate with mouse monoclonal anti-HIV-1 p24 antibody at 4°C overnight.
7. Wash in TBST (3 min, three times).
8. Incubate with biotinylated goat anti-mouse Ig antibody for 30 min.
9. Wash in TBST (3 min, three times).
10. Incubate with Alexa Fluor 594-labeled streptavidin for 15 min.
11. Wash in TBST (3 min, three times).
12. Apply DAPI II and mount a cover slip.

3.8.3. Fluorescence Microscopy of Double-Stained Sample

The slides were examined under a fluorescence microscope with appropriate filter sets. Photographic images of the fluorescent signals were taken with a CCD camera, and were uploaded to a microcomputer using IPLab software. The stored images were merged to reveal various aspects (**Fig. 3**).

Fig. 3. *(continued from opposite page)* of CD4-positive T lymphocytes from HIV-1-infected patients. **(H)** Negative control (CD4-positive T lymphocytes from a HIV-1-negative volunteer). **(A**,**E)** provirus (green) and nucleus (blue) were merged. **(B**,**F)** p24 (red). p24 HIV-1 capsid proteins were observed in the cytoplasm of HIV-1 provirus positive cells. **(C**,**G)** provirus (green), p24 (red) and nucleus (blue) were merged. **(I**,**J)** CD4-positive T lymphocytes from a HIV-1-infected patient; HIV-1 provirus (green) was seen in the cell nucleus. In contrast, CD4 molecules (red) were seen in the outer-membrane **(I)** Membrane-bound or cytoplasmic HLA-DR molecules (red) were detected in HIV-1 provirus positive cells **(J)**.

4. Notes

1. The base sequence corresponds to the region from 1379 to 1397 of the HIV-1 gag gene. As the melting temperature of the anti-parallel probe is higher than that of the parallel, the use of anti-parallel type is recommended. PNA probe can be dissolved with DEPC-treated water to a concentration of 100 µg/mL, aliquotted, and stored at –20°C. A 0.01% trifluoroacetic acid can be replaced instead of water.
2. Wear gloves throughout the steps until the hybridization step is completed. Use the dry-sterilized glassware equipment and those made by stainless steel (200ºC, 2 h). Use autoclaved water (121ºC, 15 min) through the hybridization step.
3. To obtain an optimal concentration of proteinase K, treat fixed specimens with three different concentrations between 2 and 7 µg/mL of the enzyme.
4. This amplification method is based on the binding reaction of biotinylated tyramine to a phenol derivatives of a protein by peroxidase. This step sometimes gives nonspecific signals, therefore thorough pretreatment of specimens with methanol containing 0.3% H_2O_2 is essential to diminish the endogenous peroxidase activity. Also, it is important to stain two kinds of negative control to allow the identification of nonspecific signals: (1) HIV-1 DNA negative-specimen with a PNA probe and (2) HIV-1 DNA positive-specimen without a PNA probe (**Fig. 1**).
5. A single amplification method was successfully applied for HIV-1 RNA detection ***(16)***.
6. Considerable amounts of endogenous biotin is contained in liver, kidney, mucosa of digestive tract, and brain. Even in other organs, endogenous biotin becomes exposed through an activation step (*see* **Subheading 3.2.2.**, **steps 2** and **4**). A biotin blocking of the endogenous biotin is recommended between the steps of stringent wash and HRP-FITC antibody reaction. Biotin blocking system (DakoCytomation A/S, X0590): (1) Incubate with avidin solution for 10 min, (2) wash in TBST three times for 3 min, (3) Incubate with biotin solution for 10 min, (4) wash in TBST three times for 3 min.
7. To measure the positivity of HIV-1 provirus in the CD4-positive T lymphocytes, we count 500 cells and calculate. The positivity of the HIV-1 provirus among 62 HIV-1-infected patients ranged between 0.3% and 7.9% (average of 2.7).

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10

Electron Microscopy and *In Situ* Hybridization

Expression of P2Y$_2$ Receptor mRNA in the Cerebellum

Andrzej Loesch and Rainer Glass

Summary

This chapter describes a pre-embedding *in situ* hybridization method utilizing an immunogold-silver intensification step to identify P2Y$_2$ receptor mRNA transcripts in the adult rat cerebellum. The method was applied for ultrastructural (electron microscopic) examination. Transcripts for P2Y$_2$ receptors, marked by the gold-silver grains, were revealed in Purkinje cells. Transcripts were essentially localized in the cytoplasm although they also appeared to be specifically associated with granular endoplasmic reticulum. This suggests that Purkinje cells may produce functional P2Y$_2$ receptors involved in the ATP-related regulatory role in the cerebellum. The *in situ* hybridization method that was applied enabled simultaneous preservation of tissue ultrastructure and localization of labeled mRNA transcripts. This method may be adapted and used to study various tissues.

Key Words: *In situ* hybridization; ultrastructure; cerebellum; P2Y$_2$ receptor.

1. Introduction

In situ hybridization techniques to detect mRNA transcripts in morphological studies are mostly used at the light microscope level (e.g., **ref.** ***1***). These techniques also are used in electron microscopic (EM) studies, when more precise intracellular–ultrastructural localization of mRNA transcripts is desirable ***(2)***. Examples of such applications are studies of the expression of Xcat$_2$ mRNA in germinal granules during oogenesis ***(3)*** or P2Y$_2$ receptor mRNA in rat thymus ***(4)***. The method described in this chapter, which enables detection of transcripts for P2Y$_2$ receptors in rat cerebellum, is based on our original *in situ* hybridization study of the localization of the transcripts of P2Y$_2$ receptor in immune and vascular components of rat thymus at ultrastructural level ***(4)***. It should be mentioned that P2Y receptors, together with P2X receptors, com-

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prise the family of P2 receptors associated with the extracellular action of ATP *(5)*. These receptors play roles in various organs and tissues of the body, including the central nervous system (for review, *see* **refs.** *6* and *7*). The presence of these receptors in the central nervous system also has been demonstrated using immunocytochemical techniques at EM level *(8–12)*. The present contribution provides an ultrastructural image of the localization of transcripts for $P2Y_2$ receptors in rat cerebellum, using a pre-embedding *in situ* hybridization technique for EM. In addition to the presentation of the relevant methodological protocol, here we also suggest the involvement of purinergic–$P2Y_2$ receptor-associated signaling in Purkinje cells in the cerebellum.

2. Materials

2.1. Animals

Sprague–Dawley 3-mo-old male rats ($N = 6$). Breeding, maintenance, and killing of the rats used in this study followed the principles of good laboratory animal care and experimentation in compliance with UK national laws and regulations.

2.2. Chemicals: Reagents

1. Sodium pentobarbitone (Sagatal; RMB Animal Health Ltd; Harlow, UK).
2. Paraformaldehyde (PFA) EM-grade (TAAB, Aldermaston, Berkshire, UK).
3. 25% Glutaraldehyde EM-grade (Agar Scientific, Stansted, Essex, UK).
4. 0.2 *M* Phosphate buffer, pH 7.4.
5. Sterile 0.1 *M* Tris-buffered saline (TBS), pH 7.4.
6. Ethanol.
7. Sterile phosphate buffered-saline (PBS; Sigma, St. Louis, MO).
8. Formamide (molecular biological grade) (Sigma).
9. Saline sodium citrate buffer (SSC; Invitrogen, Carlsbad, CA).
10. Denhardt's (BFP; Sigma).
11. Sheared and denatured salmon sperm DNA (Sigma).
12. Transfer (t)RNA type X from bakers yeast (Sigma).
13. Prehybridization buffer: 50% formamide, 2X SSC, 1X Dennhardt's, 1 mg/mL sheared and denatured salmon sperm DNA, and 1 mg/mL tRNA type X from baker's yeast (*see* **Note 1**). This buffer is freshly made before use.
14. Digoxigenin (DIG) oligonucleotide tailing kit (Roche Diagnostics GmbH, Penzberg, Germany).
15. DIG-labeled probe; it is rat $P2Y_2$ antisense oligonucleotide probe of 44 nucleotides: 5'-ATGGCGTTGAGGGTGTGGCAACTGAGGTCAAGTGATCGGAAGGA-3' for rat $P2Y_2$ receptor (MWG Biotech AG, Ebersberg, Germany). It was labelled at its 3'-end with the DIG oligonucleotide tailing kit (Roche Diagnostics) according to the manufacturer's instructions (*see* **Note 2**). The specificity of the probe was checked by comparison of the sequence with the Swissprot-database and was found to be highly specific for rat $P2Y_2$ receptors.

16. Hybridization buffer consisting of DIG-labeled probe; the concentration of the probe was 1 ng per 1 µL of hybridization buffer.
17. Sheep anti-DIG antibody conjugated to 1-nm gold particles (Roche Diagnostics).
18. Sodium azide (Sigma).
19. Silver enhancing kit (British Biocell Int.; Cardiff, Wales, UK).
20. Deionized distilled water.
21. 0.2 *M* sodium cacodylate buffer, pH 7.4.
22. 1% osmium tetroxide in 0.1 *M* sodium cacodylate buffer.
23. Araldite (standard mixture of: Araldite CY212 + DDSA + BDMA and dibutyl phthalate; Agar).

2.3. Facilities: Equipment: Others

1. Fridge.
2. Electric oven (60–65°C) for polymerization.
3. Vibratome (Agar).
4. Humidified chamber.
5. Ultramicrotome, e.g., Ultracut E (Reichert-Jung).
6. Transmission electron microscope (TEM), e.g., JEM 1010 (JEOL, Tokyo, Japan).
7. Melinex sheets, e.g., Type 400, 75 µm (ICI, UK).
8. Copper grids (Athene 300-mesh) for EM (Agar).

3. Methods

The procedures for the *in situ* hybridization method for TEM are described in **Subheadings 3.1.–3.5.**). The procedures enable subcellular localization of mRNA transcripts within well-preserved ultrastructure of cells examined (**Figs. 1–3**).

3.1. Fixation and Cutting (First and Second Day)

1. Anaesthetize rat(s) with sodium pentobarbitone (60 mg/kg i.p.).
2. Perfuse rat through the heart left ventricle with fixative containing 4% PFA and 0.2% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 15 min at room temperature (*see* **Note 3**).
3. Dissect the brain and place it in the same fixative for 5 h at 4°C.
4. Transfer the brain to stock of the phosphate buffer and stored overnight at 4°C.
5. The following day, cut sections of 50–60 µm through the cerebellum (transverse section through a folium) on a vibratome and collect in 0.1 *M* sterile TBS or PBS in culture dish (*see* **Note 4**).
6. Process the sections for the pre-embedding *in situ* EM hybridization technique, as exemplified for the detection of $P2Y_2$ receptor mRNA (*see* **Subheading 3.2.**).

3.2. In Situ *Hybridization as a Pre-Embedding Technique for EM (Second and Third Day;* see *Note 5)*

1. Expose the sections to 30% ethanol for 15 min at room temperature (this is to improve the infiltration of reagents into the tissue).

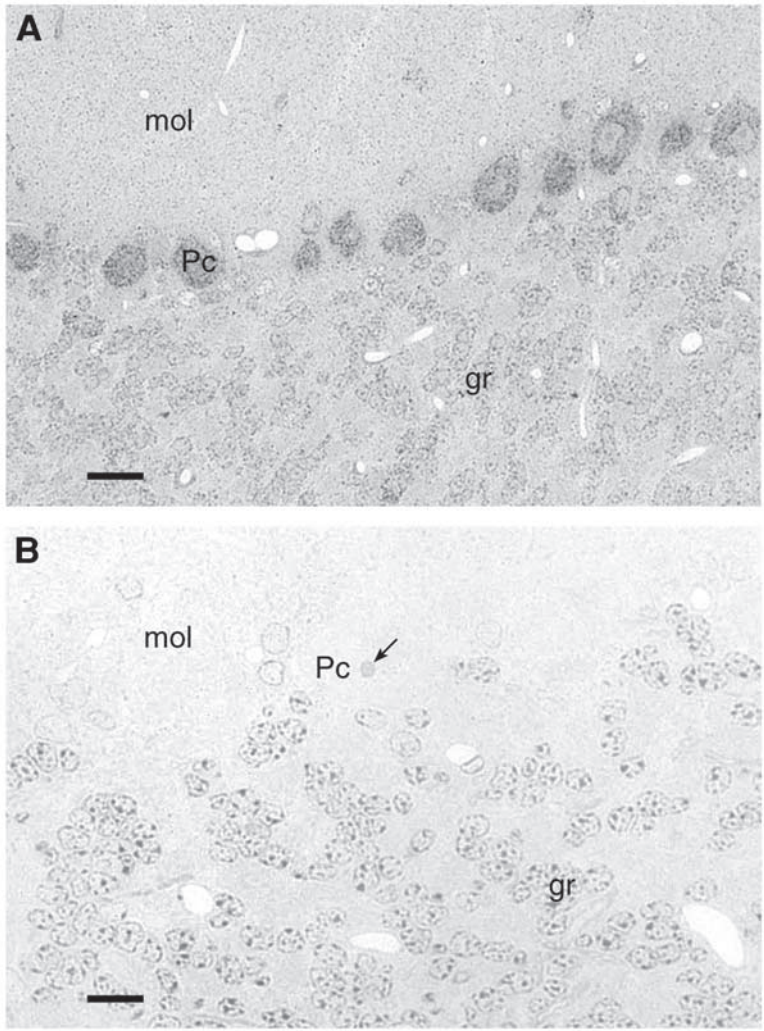

Fig. 1. Light microscopic overview of semithin (1-µm thick) sections of rat cerebellum processed for *in situ* hybridization of $P2Y_2$ receptor mRNA; sections were lightly stained with toluidine blue. (**A)** Demonstrates a specimen hybridized to the DIG-labeled rat $P2Y_2$ receptor antisense oligonucleotide probe. Note the dominant expression of $P2Y_2$ receptor mRNA (greyish–brownish label in original preparation) in characteristically positioned Purkinje cells (Pc). Molecular layer (mol) and granular layer (gr) with granule cells can be distinguished. Bar: 25 µm. (**B)** A control specimen in which the labeled probe was competed with an excess of the unlabeled probe. No labeled Purkinje cells are present; a nucleolus of the nucleus of a Purkinje cell is visible (arrow). In granule layer, nuclei of granule cells can be seen (due to toluidine stain of nuclear chromatin). Bar: 12.5 µm

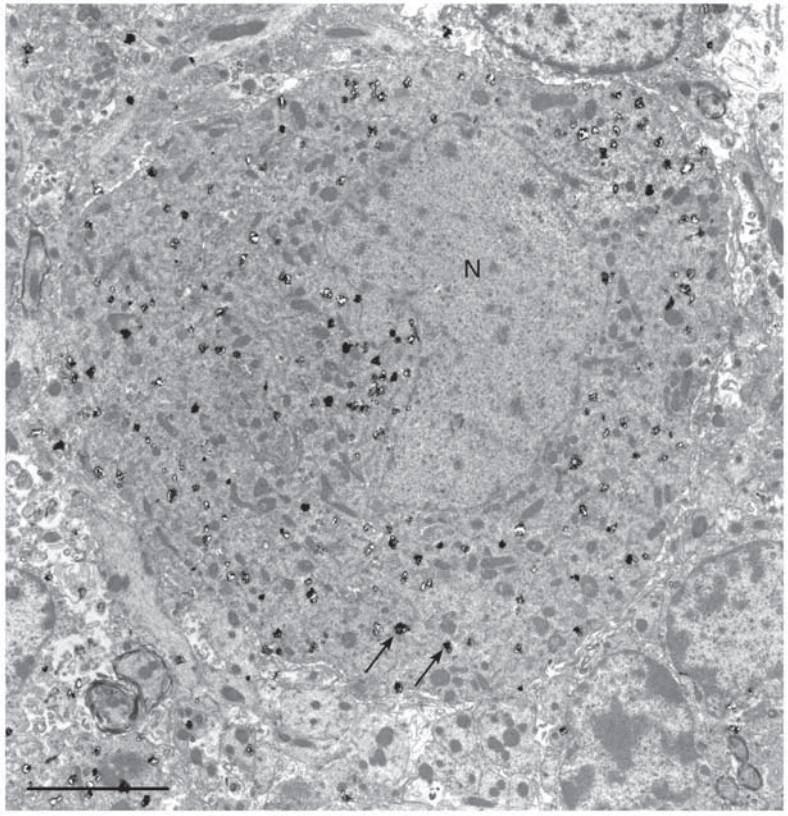

Fig. 2. Electron microscopic (EM) overview of an ultrathin section (90 nm) of rat cerebellum specimen processed for *in situ* hybridization showing a Purkinje cell expressing P2Y$_2$ receptor mRNA. The ultrastructural expression of P2Y$_2$ receptor mRNA is marked by numerous black gold–silver labels (arrows) located in the cytoplasm of a Purkinje cell body. The cell nucleus (N) is essentially free of labels. Bar: 4 µm.

2. Rinse sections in PBS at room temperature (three rinses for 5 min each).
3. Incubate sections in prehybridization buffer (consisting of 50% formamide, 2X saline SSC, 1X Denhardt's, 1 mg/mL sheared and denatured salmon sperm DNA, and 1 mg/mL tRNA type X from baker's yeast) for 1 h at 37°C in a humidified chamber (*see* **Note 6**).
4. Incubate sections in hybridization buffer (consisting of 1 ng of DIG-labeled probe in 1 µL of prehybridization buffer) at 37°C overnight.
5. Wash sections twice for 5 min in 2X SSC at room temperature (washing out of unhybridized probe; *see* **Note 7**).
6. Wash sections twice for 15 min in 2X SSC at 37°C (*see* **Note 8**).
7. Wash sections twice for 15 min in 1X SSC at 37°C (*see* **Note 9**).

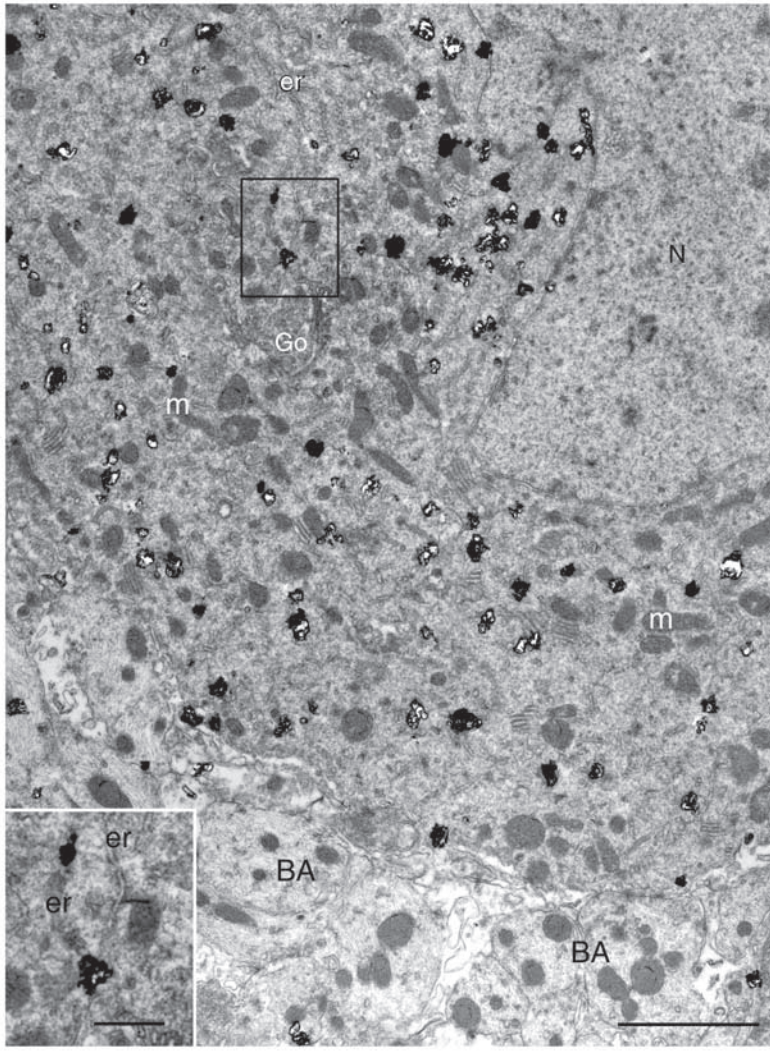

Fig. 3. Magnified fragment of the Purkinje cell illustrated in **Fig. 2** showing black gold–silver grains indicating cytoplasmic sites of $P2Y_2$ receptor mRNA. Note that the size and shape of the grains vary; this is related to the duration of silver intensification and corrosion of some grains (*see* **Note 12**). Details of cell ultrastructural organization are preserved: Golgi apparatus (Go), granular endoplasmic reticulum (er), mitochondria (m) and nucleus (N) are clearly seen. The profiles of descending basket axons (BA) contacting the Purkinje cell, show no expression of $P2Y_2$ receptor mRNA. Bar: 2-µm **Inset** is an enlargement of the area of the Purkinje cell indicated with a box. It demonstrates an association of two labels with granular endoplasmic reticulum. Bar: 0.5 µm. The good structural preservation of tissue observed here is largely owing to

8. Wash sections of twice for 30 min in 0.5X SSC at 37°C. (*see* **Note 10**).
9. Incubate sections, initially 1 h at room temperature, then at 4°C overnight, with the sheep anti-DIG antibody conjugated to 1-nm gold particles diluted 1:30 in PBS containing 0.1% sodium azide. This step is to detect the DIG-labeled probe.

3.3. Washing, Fixation, and Silver Enhancement (Fourth Day)

1. After hybridization, wash sections several times, e.g., four times for 15 min in PBS. Use a slow motion shaker to perform this step.
2. Fix sections for 10 min at room temperature with 1% glutaraldehyde (in 0.1 *M* phosphate buffer, pH 7.4).
3. Wash sections in the phosphate buffer.
4. Wash sections 6–10 times for 5 min in deionized distilled water (*see* **Note 11**).
5. Expose sections for 10–16 min to components of a silver enhancing kit (*see* **Note 12**).
6. Wash sections in deionized distilled water three times for 5 min.
7. Wash sections in distilled water once for 5 min.
8. Wash sections in 0.1 *M* sodium cacodylate buffer (pH 7.4) for 10 min.

3.4. Postfixation, Dehydration, Embedding, and Polymerization (Fourth and Fifth Day)

1. Transfer sections to 10-mL glass pots.
2. Postfix sections for 10 min (at 4°C) with 1% osmium tetroxide (in 0.1 *M* sodium cacodylate buffer).
3. Wash sections for 10 min in stock of sodium cacodylate buffer.
4. Dehydrate sections in a graded series of ethanol (25% once for 5 min, 50% once for 5 min, 70% once for 5 min, 90% once for 10 min, 100% three times for 10 min, propylene oxide three times for 10 min).
5. Infiltrate sections for 1 h at room temperature with a mixture (1:1) of propylene oxide and Araldite (*see* **Note 13**).
6. Place sections in pure Araldite resin overnight (***see* Note 14**).
7. Flat-embed sections in Araldite using Melinex sheets (*see* **Note 15**).
8. Place flat-embedded sections/specimens into oven (60–65°C) for about 24 h for polymerization (*see* **Note 16**).
9. Remove specimens from oven and let them cool down at room temperature.

Fig. 3. (*continued*) the omission of proteolytic digestion of the specimens (with proteinase K) before *in situ* hybridization processing. The digestion step is usually used for the *in situ* hybridization protocol at the light microscope level, where preservation of subcellular details is not required. The pre-embedding *in situ* hybridization methodology for the EM is believed to preserve mRNA(s) better than the post-embedding *in situ* hybridization method ***(2)***.

3.5. Mounting, Cutting of Semithin and Ultrathin Sections, Staining (Sixth Day)

1. From polymerized specimens dissect the area of interest (approx 1–2 mm^2; *see* **Note 17**).
2. Mount-flat the dissected area on an Araldite block using superglue (*see* **Note 18**).
3. The Araldite block with mounted/attached specimen is now ready for cutting using an ultramicrotome.
4. For general observations, semithin section (e.g., 2-µm thick) may be cut and collected on glass slides, lightly stained with toluidine blue, cover slipped, and examined with a light microscope.
5. The ultrathin sections, for ultrastructural examination, are collected on 300-mesh copper grids and dried (*see* **Note 19**).
6. Grids with ultrathin sections are then: (1) stained for 3 min at room temperature with 2% agueous uranyl acetate, (2) washed carefully in several changes of distilled water, (3) stained for 3 min at room temperature with lead citrate, and then rinsed in several changes of distilled water (*see* **Note 20**).
7. Grids are now ready to be examined with a TEM (e.g., JEM-1010).

3.6. Controls for In Situ Hybridization

For negative controls, follow the protocols described previously but with the following differences: (1) omit the use of labeled probe (*see* **Subheading 3.2.4.**), (2) use a labeled "sense" probe instead, and (3) compete labeled "antisense" probe with a 75-fold excess of unlabeled "antisense" probe.

3.7. Expression of mRNA $P2Y_2$ Receptor

Figures 1–3 demonstrate the effects of the pre-embedding *in situ* hybridization procedures with immunogold-silver intensification described previously. The semithin sections of *in situ* hybridized preparations of rat cerebellum show a general picture of the locati on of $P2Y_2$ receptor mRNA expression/labeling at the light microscopic level. This labeling appears as a grayish–brownish stain in original preparations. **Figure 1A** presents the expression of $P2Y_2$ receptor mRNA, predominantly associated with the Purkinje cells. In contrast, no $P2Y_2$ receptor mRNA labels are observed in control preparations in which a sense probe was used or a step with labeled antisense probe was omitted. **Figure 1B** shows another control in which the signal also is reduced greatly after competing the labeled probe with the unlabeled probe on the tissue sections. EM of ultrathin sections reveals both details of the expression of $P2Y_2$ receptor mRNA, and good morphological–ultrastructural preservation of the tissue. The gold-silver grains of various sizes indicative of the sites of $P2Y_2$ receptor mRNA, are primarily present within Purkinje cells (**Fig. 2**), essentially in the cell cytoplasm. This can be clearly observed at higher magnification (**Fig. 3**).

However, localization of transcripts to granular endoplasmic reticulum (the Nissl body) also was noted (**Fig. 3, Inset**).

4. Notes

1. Pre-hybridization buffer is freshly made before use.
2. It is important to check the quality of the labeling reaction after each new labeling procedure because it does not always work with the same efficiency. If the labeling efficiency is found to be different, it is best to repeat the labeling (e.g., several labeling reactions may be made in parallel and only probes labelled with similar efficiency may be used). In some applications, it may be sufficient to compensate for inefficient/overefficient labeling by using more or less of the labeled probe. However, using excess amounts of weakly labeled probe may affect the specificity of the hybridization procedure.
3. Fixative is made up in a standard way, for instance, by heating up 8% PFA in water to 65°C (use a heater with magnetic stirrer), and then adding a few drops of NaOH (or one pellet of NaOH/200 mL) to clarify solution; cooling it down quickly; and then adding (1:1) 0.2 *M* phosphate buffer, pH 7.4 to obtain 4% PFA in 0.1 *M* phosphate buffer. Then, add 0.8 mL of 25% glutaraldehyde per 100 mL of 4% PFA solution and adjust pH to 7.4. This fresh fixative is ready to be used.
4. It is really important to work under the cleanest conditions possible. Otherwise degradation of the mRNA content on the slices may be induced.
5. All glassware, buffers, and substances in the following steps (**steps 1–4** inclusive) should be RNase free. The substances and buffers used for the hybridization proper are usually offered RNase-free by the manufacturer. Glassware should be baked (220°C overnight).
6. This step works in analogy to the blocking procedure before immunostaining. In the prehybridization mixture, there are DNA fragments, tRNA fragments, and it contains Denhardt's solution, which consists of serum albumin, Ficoll and polyvinyl–pyrrolidine. The DNA and tRNA quench nonspecific binding of the probe to cellular DNA and tRNA. The serum albumin quenches nonspecific interaction with cellular proteins. The Ficoll and the polyvinyl–pyrrolidine make the mixture more viscous. The concentrations of the SSC and of the formamide are very important. Formamide decreases the melting temperature of DNA (which means it separates the two complementary DNA strands from each other). Without this substance, there would not be any possibility for specific interactions between the cellular mRNA and the probes. Salt content (delivered by the SSC buffer) of the hybridization solution also is highly important. Divalent cations decrease the stringency of the solution (they increase binding of the probe to the mRNA, but therefore also decrease the specificity of the reaction). Monovalent cations work vice versa. Normally, the salt content (SSC concentration) of the hybridization solution (and later of the wash-buffers) should be determined empirically for hybridization reactions with different probes. However, our protocol indicates at least good starting conditions.

7. Wash on a shaker, for example, in the same glassware used for washing or slides in histochemistry. At this point, it is not so important anymore to work under very clean conditions. Here, we handle double-stranded templates (consisting of mRNA hybridized to our probe. RNase cannot degrade this).
8. This is a continuation of washing out of unhybridized probe. Here, the salt content is high, i.e., as high as in the hybridization buffer. Therefore, this is washing at very low stringency.
9. This is a continuation of washing out of unhybridized probe. Here, the washing stringency is increased.
10. This is the final stringency washing out of unhybridized probe (this is the washing step with the highest stringency). If no signal can be detected later on, the wash-steps should be modified, e.g., by using only 2X SSC and 1X SSC, or all steps could be performed at room temperature. If the background is high, then the concentration of the SSC could be decreased further (e.g., to 0.25X) and then stringent washing could be prolonged. Other possibilities are to decrease/increase the concentration of the probe and to check the specificity of the probe. This can be performed with a computer program, Primer 3, which is available free of charge on the following website http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. The same program can be used to *create* a probe.
11. This step is to remove ions, especially chloride. Otherwise, these may interfere with the silver intensification reaction.
12. Perform silver intensification step at room temperature in the dark, e.g., cover culture dish with aluminium foil. It is recommended that this step is carried out in a glass pot rather than the culture dish. The speed of the enhancement reaction is temperature dependent; the longer the enhancement is applied, the bigger the size of the final label. The enhancement step is necessary because 1-nm gold particles conjugated to anti-DIG antibody are too small to be detected at even high primary magnifications. Our own study of thymic immune tissue demonstrated that the specimens exposed to silver-enhancement for shorter time showed more precise, pin-pointed, localization of the *in situ* hybridization labels ***(4)***. For more details on the silver enhancement (*see* **ref. *13***).
13. For better infiltration of Araldite-propylene oxide mixture into specimens, place glass pots on rotator at room temperature.
14. Place glass pots on a rotator at room temperature; this will help Araldite infiltration into the specimens.
15. Use Melinex sheets, e.g., 9×9 cm, to sandwich the sections between the sheets. This sandwiching will allow viewing the sections under the light/dissecting microscope and to select the area of interest for further examination once sections are polymerised. During embedding, use one or two drops of Araldite per a tissue section.
16. Before placing specimens into the oven, place a few glass slides (e.g., 5×7.5 cm) over the melinex-sandwiched specimens so that the embedded sections will remain flat. If more than one melinex-sandwiched specimen is to be polymerized, they can be placed on a top of each other, but we recommend putting glass slides between individual sandwiches.

17. Use a razorblade or any relevant razor for dissection; remove melinex from the dissected area, at least from the site being mounted on Araldite block.
18. Wait a few minutes until glue has bonded the specimen to the Araldite block. To speed up bonding, blocks can be placed in oven (60–65°C) for a few minutes.
19. Before collecting sections on grids, avoid stretching (flattening) ultra-thin sections, e.g., with xylene, as this may loosen the connection of immunogold-silver labels with Araldite.
20. Avoid longer exposure to uranyl acetate and lead citrate because these may remove the label.

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11

In Situ Hybridization Using Riboprobes on Free-Floating Brain Sections

Neil C. Owens, F. Martin Hess, and Emilio Badoer

Summary

A method is described for *in situ* hybridization of riboprobes to free-floating brain sections. Brain sections are hybridized and processed free-floating in buffer, i.e., without attachment to a support such as a slide. To withstand the extra wear compared with sections processed on-slide, the brain tissue must be well fixed (4% paraformaldehyde) and sections cut at thickness of typically 40 μm. Sections were exposed to a prehybridization treatment before a riboprobe is added to form the hybridization solution. Riboprobes were prepared from cDNA via an in vitro transcription reaction and are labeled with digoxigenin. The sections are subsequently processed to remove nonspecific binding and the digoxigenin label detected via an antibody conjugated to alkaline phosphatase. This method may be readily combined with neuronal tracing and is ideal for further processing by immunohistochemistry to detect specific proteins.

Key Words: Free-floating; brain; riboprobes; digoxigenin.

1. Introduction

In situ hybridization has been used widely for the detection of mRNA in brain tissue. Traditionally, the process usually uses thin frozen sections (5–10 μm) mounted onto slides that are then subjected to the hybridization protocol. An alternative to the traditional "on-slide" method is outlined here, in which sections are not adhered to any support and processed "free-floating" in the buffer. The method described uses a nonradioactive riboprobe, labeled with digoxigenin (DIG), to detect the target mRNA. To accommodate the extra wear on the sections bought about by the processing, thicker sections (typically 40 μm) are used. Additionally, the sections need to be well fixed (e.g., 4% paraformal-

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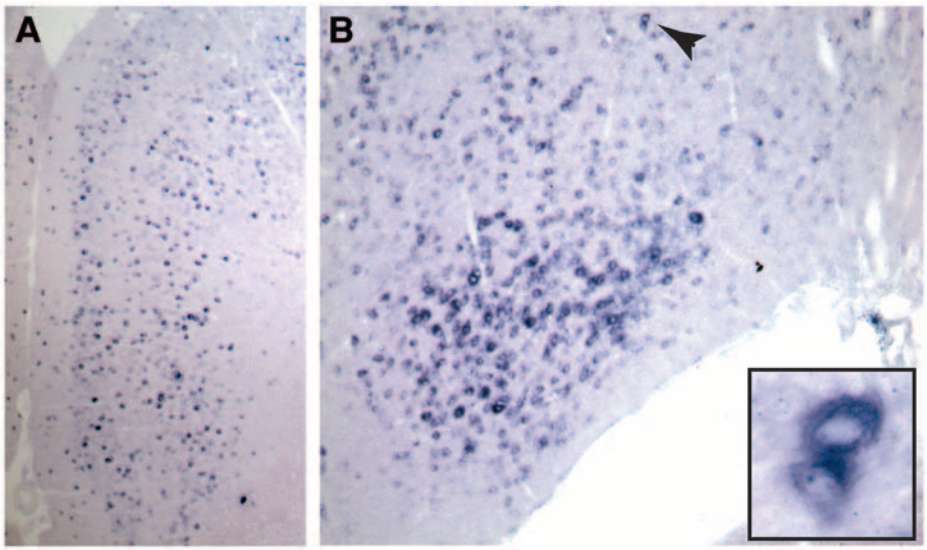

Fig. 1. Photomicrographs showing GAD67 mRNA containing neurons in a coronal section from a rat brain. The neurons were detected using an antisense riboprobe labeled with DIG and detected via an alkaline phosphatase reaction using nitroblue tetrazolium as the chromogenic substrate. **(A)** Low-power image (×4) of a forebrain section showing the distribution of GABAergic (GAD67 positive) neurons in the motor cortex. **(B)** GABAergic neurons in the anteroventral preoptic nucleus (×4) with an insert showing two positive neurons in the dorsal (top) part of the section (arrow head) at higher magnification (×20). Note that the lower neuron appears slightly out of focus due to the thickness of the section (40 µm).

dehyde [PFA]) for the process to be successful because fresh or poorly perfused tissue simply falls apart under "free-floating" conditions. Using the free-floating approach, it appears that mRNA molecules are more accessible because, in general, much higher antibody (anti-DIG) dilutions may be used, typical dilutions being in the range of 1:1000 to 1:10,000 (or higher). For a number of applications, processing sections free-floating has become a method of choice, but until recently it has been difficult to combine this method with *in situ* hybridization. The free-floating method described uses a riboprobe to detect the mRNA of interest (e.g., **Fig. 1**). This approach has been useful in determining markers for neurons that have been electrophysiologically identified ***(1)*** or that project to a particular region of the central nervous system ***(2)***. The latter is achieved by pre-microinjecting the animals with a retrograde tracer, a summary of which is given below.

2. Materials

2.1. Sectioning and Staining Equipment

1. Fluorescent microspheres (LumaFluor, New York, NY; *see* **Note 1**).
2. 4% PFA in phosphate buffer, pH 7.4.
3. RNase-free 20% sucrose in phosphate buffer, pH 7.4 (*see* **Note 2**).
4. Freezing microtome (cryostat).
5. Microtome chucks.
6. 24-well plastic culture dishes.

2.2. Solutions for Free-Floating In Situ Hybridization

2.2.1. DIG Labeling

1. RNA dilution buffer (6X SSC, 0.2% formaldehyde in diethylpyrocarbonate treated water [DEPC–H_2O]).
2. DIG-UTP labeling kit containing labeled UTP and unlabeled nucleotides (Roche Diagnostics, Mannheim, Germany).
3. 10% (w/v) Blocking solution (Roche).
4. Fast Red detection tablets (Sigma, St. Louis, MO).
5. Hybond-N^+ nylon membrane (Amersham).
6. Buffer 1 solution (B1): 0.1 *M* maleic acid, 0.15 *M* NaCl, pH 7.5.
7. Blocking solution made from powder (Roche).
8. SYBR green (Molecular Probes; *see* **Note 3**).
9. Agarose.
10. Ultraviolet (UV) transilluminator.

2.2.2. Hybridization

1. Free-floating salts (for 1 mL of 5X free-floating salts): 600 µL 5 *M* NaCl, 50 µL 1 *M* Tris, pH 7.5. 50 µL ethylene diamine tetraacetic acid (EDTA), 200 µL of DEPC-treated-H_2O.
2. 50X Denhardt's (Sigma).
3. DEPC-treated saline (0.9% NaCl).
4. DEPC-treated H_2O.
5. Formamide (Molecular Biology grade, Sigma).
6. 1 *M* Dithiothreitol (DTT).
7. 50X Dextran (Sigma, dissolved in DEPC–H_2O).
8. 10 mg/mL tRNA (Sigma).
9. 20X SSC: 3 *M* NaCl, 0.3 *M* NaCitrate.
10. RNase reaction buffer: 5 m*M* Tris-HCl, pH 7.5, 0.5 *M* EDTA, pH 8.0, 0.5 *M* NaCl.
11. RNase A (Sigma).

2.2.3. DIG Detection

1. TBS: 0.9% NaCl, 50 m*M* Tris-HCl, pH 7.5.
2. NT: 0.1 *M* NaCl, 0.1 *M* Tris-HCl, pH 9.5.
3. 5-bromo,4-chloro, 3-indolyphosphate/nitroblue tetrazolium (BCIP/NBT; Roche).

3. Methods

3.1. Tissue Preparation and Sectioning

3.1.1. Preparation of Brain Tissue

Adult male rats are pretreated by injecting small quantities (50–100 nL) of retrograde tracer (5% in sterile saline; LumaFluor) into a region of interest (e.g., the spinal cord). The rats are then housed for a period of several days to weeks to allow the tracer time to be transported to the soma of the neuron (at ca. 1–2 mm/d). For fluorescent microspheres this process takes approx 1–2 wk, depending on the distance that needs to be traveled. Rats are then deeply anesthetized, exsanguinated by transcardially perfusing phosphate-buffered saline (PBS) and fixed with 4% PFA (500 mL in PBS). A good quality perfusion/fixation is required for the success of the method; therefore, any perfusions that are not of high quality (in particular good fixative infiltration) should be treated with suspicion. The brain tissue and spinal cord are rapidly removed and placed in 20% RNase-free sucrose (in PB) until they sink. It is essential that this solution be RNase-free (*see* **Note 2**). The brains are now ready to be cut, or they may then be stored frozen over liquid nitrogen and maintained at –80°C.

3.1.2. Sectioning

We routinely use a cryostat to cut sections. Transverse (coronal) sections are cut at 40 µm and collected into RNase-free cyropreservative solution (15% [w/v] RNAse-free sucrose [Sigma], 30% [v/v] ethylene glycol [Sigma] in RNAse-free water). Individual sections are placed separately into wells of a culture plate (either 24- or 96-well) in serial order and stored at –20°C. The sections are catalogued so that separate series of sections (e.g., 1 in 3) may be examined from the same brain. In this manner we maintain an archive of "good experiments" that may be used for different purposes. Brushes, cryostat chucks and all solutions are treated with DEPC and gloves are changed frequently in a bid to maintain RNase-free conditions.

3.2. Transcription of DIG-Labeled Riboprobe

3.2.1. Preparation of Linear Template

The plasmid containing the cDNA insert of interest (plasmid + insert) is cut with a restriction enzyme (RE) to produce a linear DNA. Usually, a RE is chosen on the basis of having a single cut site (in the plasmid + insert) that is located at the far end of the cDNA insert from the promoter sequence. In this manner, the RNA polymerase can only transcribe the cDNA and the transcription of plasmid sequences is avoided. Thus, the choice of RE and RNA polymerase is critical for producing a sense or antisense probe.

Most commercially available plasmids have transcription promoter sequences that flank the multiple cloning site (where the cDNA is inserted), typical promoter sequences are SP6, T7, and T6 with the RNA polymerase that binds to the sequence sharing the corresponding name (i.e., SP6 polymerase binds to the SP6 promoter).

1. Under RNase-free conditions, add the following components (in order), to a sterile RNase-free microcentrifuge tube.

a. Sterile water (nuclease free)	to give V_T of 20 µL
b. Restriction enzyme 10X buffer	2 µL
c. BSA, Acetylated (1 mg/mL)	2 µL
d. Template (Circular plasmid in water or TE)	5 µg
e. Restriction enzyme	2 µL

2. Mix gently by flicking. Briefly centrifuge (pulse) to collect the contents at the bottom of the tube and incubate at 37°C for 1–4 h.

3.2.2. Purifying the Linear DNA

If the RE digest is with untested substrates then confirmation of linearization (*see* **Subheading 3.2.3.**) is best performed before the following extraction procedure is used. We use Promega Wizard DNA clean-up kit; we have found this extraction method to be superior to the phenol:chloroform method, by being faster, produces better quality riboprobes. We list here a modified version of the manufacturer's protocol. Use one Wizard column for each sample and label both column and syringe barrel.

1. Bring the volume of the digest to 200 µL by adding 180 µL of sterile DEPC–H_2O water (i.e., best quality water).
2. Thoroughly mix the extraction resin before removing an aliquot. If crystals or aggregates are present, dissolve by warming the resin to 37°C for 10 min (cool to 25–30°C before use).
3. To bind the DNA, attach the syringe barrel to the columns. Insert the tip of the column/syringe barrel assembly into a vacuum line. Add 1 mL of resin to the microfuge tube containing the 200 µL of the diluted digest. Mix by gentle inversion.
4. Pipet the resin/DNA mix into the syringe barrel and apply a vacuum to draw the solution into the column.
5. Wash the columns with 2 mL of 80% isopropanol and reapply vacuum to draw the solution through column.
6. Briefly dry the resin by continuing to draw a vacuum then remove the syringe barrel and transfer the column to a 1.5-mL microfuge tube.
7. Centrifuge the column at 10,000*g* in the microfuge for 2 min to remove the residual isopropanol
8. To elute the DNA, transfer the column to a new microfuge tube and add 50 µL of prewarmed (65–70°C) sterile DEPC water, wait for 1 min, then centrifuge the

column for 20 s at 10,000*g* to elute the bound DNA. Discard the column. Note not all of the 50 µL added will be recovered; it will be in the range of 40–45 µL.

9. Aliquot the eluted DNA in four tubes of 10 µL each. The remaining DNA solution is loaded onto a gel to check the efficiency of linearization. The linear DNA can be stored this way for years at –20°C.

3.2.3. Digest Control: Visualizing the Restriction Digest on an Agarose Gel

1. Run 500 ng of linear template on a 0.8 % agarose gel. For each sample: place 2 µL of loading buffer and 9 µL of DNA solution at 65°C for 2 min and chill on ice (to denature the binding of any cohesive ends resulting from the RE enzyme).
2. Add 1 µL of SYBR Green (prediluted 1:100 in TE, pH 8.0; *see* **Note 3**). Run on a 0.8% agarose gel (0.32 g/40 mL 0.5X TBE) at 60 V for 40–60 min. Include uncut plasmid and molecular weight markers as controls.
3. The separated bands are viewed under UV light.

3.2.4. In Vitro Transcription (DIG Labeling)

1. Set up the following reaction in a sterile microfuge tube

a. DEPC–dH_2O	1.5 µL
b. DNA template (from above)	10.0 µL
c. 5X labeling buffer (Promega)	4.0 µL
d. Labeling mix (LM; Promega)	2.0 µL
e. RNasin	0.5 µL
f. RNA polymerase, e.g., SP6 or T7	1.0 µL

2. Incubate at 37°C for 1 h. After 1 h, add a further of 1.0 µL of RNA polymerase (*see* **Note 4**).
3. Stop the reaction by adding 2 µL of 0.2 *M* EDTA, 5 µL of 4 *M* LiCl, and 75 µL of 100% ethanol (ice cold). Mix by gentle inversion and place at –80°C (at least 60 min) or –20°C (overnight) to precipitate.
4. Spin at 10,000*g* for 15 min. Remove supernatant and wash pellet with 70% ethanol.
5. Spin 10,000*g* for 5 min. Remove supernatant and dry pellet (on bench).
6. Resuspend pellet in 50 µL of nuclease free formamide ***(3)***. Store frozen at –20 or –80°C (*see* **Note 5**).

3.2.5. Labeling Control: Dot Blots

To determine the amount of DIG-labeled RNA:

1. Take a small piece of Hybond-N nylon membrane (approx 3 × 4 cm; Amersham) and clip the top off the top left corner (for orientation purposes). Place the membrane on a clean surface such as fresh aluminum foil and label with a pencil.
2. Perform serial dilutions of samples using RNA dilution buffer (e.g., undiluted, 1:20, 1:200, 1:2000, 1:20,000). Also prepare a series of dilutions with a known standard for comparison (e.g., Roche DIG-labeled RNA, supplied at 100 ng/µL).
3. Spot 2 µL onto the nylon membrane (leave room for the solution to spread).

Cover the membrane loosely in aluminum foil (leaving an air gap) and bake at 120°C for 30 min. The membrane can be stored in a dark, cool, dry place.

4. In a clean, appropriately sized tube (e.g., 15- or 50-mL Falcon tube, depending on the membrane size), briefly rinse the membrane in B1 solution (ca. 1 min) (B1: 0.1 *M* maleic acid, 0.15 *M* NaCl, pH 7.5).
5. Replace B1 solution with a 4 mL of fresh B1 and add 400 µL of 10% Blocking solution. Incubate for 30 min at room temperature with gentle agitation.
6. Add 4 µL of anti-DIG antibody (conjugated to Alkaline Phosphatase; 1:1000) for 1 h at room temperature with gentle agitation.
7. Wash membrane 2X with B1 (swirl and discard)
8. To visualize the DIG-labeled RNA, we use the Sigma Fast Red system.
9. To 1 mL dH_2O, add one Tris tablet. Once dissolved, add one Fast Red tablet (Sigma). This solution is then added to the membrane (and approx 4 mL B1). The reaction should be very fast.
10. Wash the membrane once with B1 and then twice with tap water
11. Estimate concentration by comparison with the range of intensities of the control RNA spots.

3.3. Hybridization

3.3.1. Prehybridization and Hybridization

1. In a 24-well titer tray, wash section three times for 5 min with DEPC sterile saline 1 mL/well). Set up prehybridization, adding in order:

Stock solutions	Working concentration	Volume per well (300 µL)
a. Water	–	15 mL
b. 5X FF salts	1X	60 mL
c. Formamide	50%	150 mL
d. 1 *M* DTT	0.01 *M*	3 mL
e. 50X Dextran (500 ng/mL)	10%	60 mL
f. 10 mg/mL tRNA	1X	12 mL

FF, free-floating

2. Remove as much saline as possible from the sections
3. Add 300 µL of prehybridization solution to each well and place at 37°C for 60 min.
4. Add 50–500 ng of labeled probe (using RNase free technique) to each well, mix well using a short but vigorous swirling action. Try to avoid adding the probe directly onto tissue.
5. Incubate at 55°C for 12–36 h in a sealed container (helps avoid loss of formamide).

3.3.2. Washing and Detection

From this step on there is no need to use RNase-free technique.

1. Low stringency wash. With a 1-mL automatic pipettor, remove as much hybridization solution as possible, add 1 mL of 4X SSC, and place at 37°C for 15 min.

2. Repeat twice.
3. RNase digest. Transfer sections to a new well containing 500 µL of RNase reaction buffer per tube. Pre incubate at 37°C for 5 min, then add 1 µL of RNase stock to each tube and incubate at 37°C for 15 min.
4. Midi stringency wash. Remove as much of the RNase buffer as possible, add 1 mL of 2X SSC, and place at 37°C for 30 min.
5. High stringency wash (optional; *see* **Note 6**). Remove as much of the midi stringency wash solution as possible, add 1 mL 0.1X SSC, and place at 55°C for 30 min.

3.3.3. DIG-Detection Protocol (Alkaline Phosphatase)

Following the stringency washes:

1. In a 24 well tray, wash sections TBS three times for 15 min.
2. Block sections by incubating in 10% NHS in TBS (500 µL/well).
3. Primary antibody incubation. Incubate with anti-DIG:alkaline phosphatase (1:5000) in 10% NHS, 0.1% Triton X-100, in TBS. Incubate 12–36 h at 4°C.

3.3.4. Postantibody Washes and Color Reaction

1. Wash sections with TBS three times for 5 min.
2 Transfer sections to a new well containing NT (500 µL/well). Incubate for (10 min at room temperature).
3. Staining reaction: (protect from light at all time). Prepare staining solution just before use; 1X NBT/BCIP diluted in 10% PVA with NT buffer (use 300 µL per well; *see* **Note 7**).
4. Incubate sections in the dark at room temperature until the signal reaches a satisfactory intensity (0.5–3 h; occasionally overnight at 4°C; *see* **Note 8**).
5. When the blue coloration has developed to your satisfaction, stop the reaction by transferring the sections to a new well containing stop buffer (500 µL per well). Wash three times for 10 min (*see* **Note 9**).
6. Mount on gelatin-subbed slides and coverslip using an aqueous mountant (like Crystal [Biomedia]). Do not use DPX (BDH) because xylene will extract the color of the NBT over time. An example is given in **Fig. 1**.

4. Notes

1. The rhodamine-labeled microspheres are sensitive to glycerol and alcohols; therefore, to preserve fluorescence, they must be avoided.
2. To make RNase free 20% sucrose, add 20 g of molecular biology-grade sucrose (DNase and RNase free, Sigma) to an autoclaved bottle containing 100 mL of DEPC (0.1%) pretreated phosphate buffer pH 7.4. (Do not autoclave when the sucrose is in the solution.)
3. SYBR Green is pre-diluted 1:100 in TE (pH 8.0). The SYBR green is pH and heat sensitive (or the pH changes that accompany heating Tris buffers) it is therefore added to the DNA sample after warming and chilling to remove secondary structures (65°C for 2 min, then chilled in ice).

4. The addition of another microliter of RNA polymerase is particularly useful for SP6 because it is rather a "weak" polymerase.
5. We resuspend our newly made RNA in 100% formamide. This process protects the RNA from degradation by RNase *(2)*, but we have found that extra care is needed in resuspending the RNA pellet because it is somewhat more difficult to resuspend in formamide than TE.
6. High-stringency wash may not be needed. The decision to include it is based on the signal-to-noise ratio and the strength of the observed signal. Generally, it is best to include it if possible.
7. We use a staining buffer that does not contain Mg^{2+} (NT rather than NMT), because in the presence of Mg^{2+} a black precipitate tends to form over the sections that is difficult to wash off.
8. Because it is possible to continue the alkaline phosphatase reaction for long periods of time, it is possible to overdevelop the reaction and risk false-positive results. Therefore, it is important to develop criteria for correct staining so that the reaction may be stopped appropriately. For example neurons in the piriform cortex and motor neurons in the medulla tend to develop such background staining.
9. The reaction product from the development of the NBT/BCIP color reaction is soluble in water; therefore, if the sections are processed further care should be taken so as not to unduly wash out the staining.

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12

An *In Situ* Hybridization Technique to Detect Low-Abundance Slug mRNA in Adherent Cultured Cells

Changsun Choi, Laurie G. Hudson, Pierre Savagner, and Donna F. Kusewitt

Summary

In this chapter, we describe a simple and relatively rapid technique for detecting low-abundance slug mRNA in cultured cells. The procedure uses nonradioactive digoxigenin-labeled RNA probes that are more sensitive than deoxyribonucleic probes and simpler to detect than radioactively labeled probes. Cells are grown in glass chamber slides, fixed in an acidic fixative, dehydrated through ethanol and xylene, permeabilized in pepsin, and post-fixed. Slides are then incubated overnight at 37°C in a buffer containing 50% formamide and 5–10 ng/μL probe. Excess probe is removed by washing at high temperature in low-salt buffer and by treatment with RNase. Probe is detected immunohistochemically with an anti-digoxigenin Fab fragment, using a tyramide amplification kit to enhance signal and Fast Red for visualization. The technique has the advantages of probe stability and sensitivity, hybridization at low temperature, rapidity and sensitivity of probe detection, and the production of permanent specimens.

Key Words: *In situ* hybridization; cell culture technique; immunohistochemistry; transcription factor.

1. Introduction

Principles and applications of *in situ* hybridization techniques for detecting mRNA in cultured cells and tissue sections have been described in many review articles ***(1–11)***, as well as in a variety of texts and laboratory manuals (e.g., **refs.** ***12–17***). These *in situ* hybridization techniques use DNA or RNA probes, riboprobes, containing either radioactive or nonradioactive labels. In general, RNA probes offer increased sensitivity and specificity compared with DNA probes, although RNA probes are more difficult to produce and more labile

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than DNA probes. It is widely believed, perhaps erroneously, that radioactively labeled probes provide increased sensitivity compared with nonisotopically labeled probes. However, nonradioactive probes have many advantages over radioactive probes, including greater probe stability, lower cost of reagents and materials, greater speed and ease of use, improved tissue morphology, and better localization of target sequences *(18–24)*. Nonradioactive RNA probes often are produced by in vitro transcription reactions that incorporate digoxigenin-labeled nucleotides; digoxigenin is then detected immunohistochemically. Digoxigenin is a steroid hapten derived from digoxin, a cardiac glycoside. Digoxigenin-labeled nucleotides, unlike biotinylated nucleotides, are stable in basic conditions; thus, digoxigenin-labeled probes can be cleaved by alkaline hydrolysis to yield a mixture of shorter probe fragments. An additional advantage of digoxigenin over biotin is the fact that digoxigenin is not found in mammalian tissue; this absence markedly reduces background staining during immunohistochemical detection of bound probe. The biotinylated tyramine (tyramide) signal amplification system can increase the sensitivity of nonradioactive *in situ* hybridization by 2- to 100-fold *(25–31)*. Tyramine is a phenolic compound that binds to electron-rich proteins found at or very near sites of horseradish peroxidase activity. Thus, tyramide is deposited at sites where horseradish peroxidase has been localized by immunohistochemical detection of digoxigenin; this, in turn, localizes biotin to these sites. The biotin is readily detected by virtue of its affinity for avidin.

The technique described herein is based closely on the *in situ* hybridization technique described by Raap et al. *(32,33)*. For these studies, we used nonradioactive riboprobes and tyramide amplification to detect low-abundance mRNA for the slug transcription factor in cultured human keratinocytes. We have found that this technique reliably and reproducibly detects slug mRNA in transfected keratinocytes that modestly overexpress exogenous slug. Using this technique, we have also shown that slug mRNA is undetectable in confluent SCC 12F keratinocytes but is increased at wound margins in vitro for 24–72 h after wounding (**Fig. 1**).

2. Materials

1. pCRII. M.Slug.P64-41 plasmid *(34)*.
2. Restriction enzymes (*Eco*RV, *Bam*HI).
3. Agarose for gel electrophoresis.
4. Phenol:chloroform (1:1).
5. 3 *M* Sodium acetate (cat. no. S-7899; Sigma; St. Louis, MO).
6. Ethanol.
7. ATP, GTP, CTP, UTP (cat. no. 1 976 290; Roche; Indianapolis, IN).
8. Digoxigenin-labeled UTP (cat. no. 1 209 256; Roche).

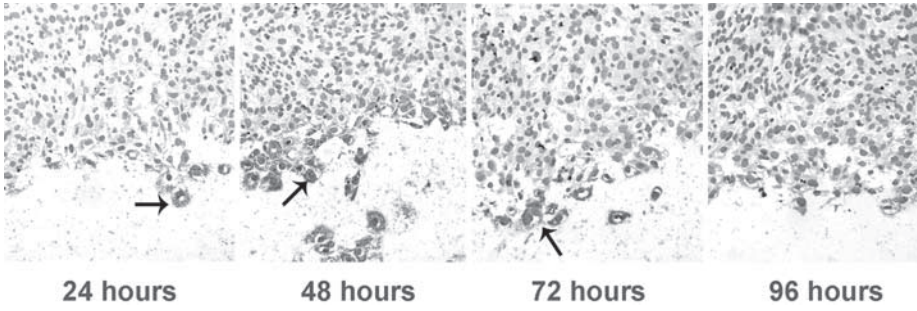

Fig. 1. *In situ* hybridization for slug mRNA at wound margins. Wounds were introduced into confluent cell sheets at the indicated times before harvest. *In situ* hybridization for slug was conducted as described in the text. Slug expression was first observed at 24 h, peaked at 48–72 h, and had diminished by 96 h after wounding. Arrows indicate cells expressing Slug mRNA in the cytoplasm. (Reproduced from **ref.** ***33*** with the permission of Wiley Interscience.)

9. T7 RNA polymerase (cat. no. 600123; Stratagene; LaJolla, CA).
10. SP6 RNA polymerase (cat. no. 600151; Stratagene).
11. RNase inhibitor (RNaseZap: cat. no. 9780 9782; Ambion, Austin, TX).
12. 0.5 *M* EDTA (cat. no. E-7889; Sigma).
13. 4 *M* LiCl.
14. Lab-Tek II Chamber Slide System (cat. no. 154461; Nalge Nunc; Rochester, NY).
15. Formaldehyde.
16. Glacial acetic acid.
17. 5 *M* NaCl.
18. Phosphate-buffered saline (PBS; cat. no. P-3813; Sigma).
19. Xylene.
20. Pepsin (cat. no. 2629-57; Mallinckrodt; Phillipsburg, NJ).
21. Hydrochloric acid.
22. Saline-sodium citrate buffer (SSC; cat. no. S-6639; Sigma).
23. 50% Dextran sulfate.
24. 100X Denhardt's solution (cat. no. 0032 007 104; Eppendorf).
25. Formamide.
26. Transfer (t)RNA (cat. no. R-8508; Sigma).
27. 1 *M* Tris-HCl, pH 7.5.
28. Hybri-slips (cat. no. Z37,027-4; Sigma).
29. Ribonuclease A (cat. no. 78020Y; USB, Cleveland, OH).
30. Ribonuclease buffer; 10 m*M* Tris-HCl, pH 7.5, 0.5 *M* NaCl, 1 m*M* ethylene diamine tetraacetic acid (EDTA).
31. Tween-20.
32. TNT buffer; 0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.05% Tween-20.

33. TSA Biotin System (cat. no. NEL700A; Perkin Elmer Life Sciences, Boston, MA).
34. TNB blocking buffer; 0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 0.5% blocking reagent included in TSA Biotin System kit.
35. Anti-digoxigenin-POD, Fab fragment (cat. no. 1 207 733; Roche).
36. Vector NovaRed substrate kit (cat. no. SK-4800; Vector Laboratories; Burlingame, CA).
37. Mayer's hematoxylin (cat. no. S3309; DAKO, Carpinteria, CA).

3. Methods

The methods in **Subheadings 3.1.–3.5.** describe: (1) production of riboprobes, (2) cell culture techniques, (3) fixation and permeabilization of cultured cells, (4) *in situ* hybridization for low-abundance slug mRNA, and (5) detection of hybridized riboprobe. Probe preparation and evaluation generally requires two half-days. Once the riboprobe and all stock solutions, particularly RNase-free solutions, have been prepared, the technique generally can be completed during the course of one afternoon and the next morning. Technique failure may occur as a result of probe deterioration or inadequate cell permeabilization. There are a number of points at which the technique may be modified to allow it to be applied to other systems, including probe length, probe concentration, hybridization temperature, rinse temperature, antibody concentration, and visualization time.

3.1. Riboprobe Production

Riboprobe production is the most critical factor in determining the success of any *in situ* hybridization procedure. The steps in **Subheadings 3.1.1.** and **3.1.2.** describe enzymatic vector linearization and in vitro transcription to produce digoxigenin-labeled single-stranded sense and anti-sense slug riboprobes. It is critical that all solutions and materials used for the production of riboprobes be RNase-free (*see* **Note 1**).

3.1.1. Vectors

The pCRII. M.Slug.P64-41 plasmid (**Fig. 2**) contains approx 500 bp of mouse slug cDNA inserted into the TOPO TA cloning vector (Invitrogen; Carlsbad, CA ***[34]***). The slug insert is flanked by T7 and SP6 promoters. For production of antisense riboprobe, this vector is linearized with *Bam*HI and RNA is transcribed using T7 RNA polymerase. For the sense probe (negative control), pCRII. M.Slug.P64-41 is linearized with *Eco*RV and transcribed using Sp6 RNA polymerase (*see* **Note 2**).

3.1.2. Linearization

1. Digest 10 µg of pCRII. M.Slug.P64-41 plasmid in a total volume of 20 µL in an RNase-free tube. Use 2 µL of high-concentration restriction enzyme (*Eco*RV or

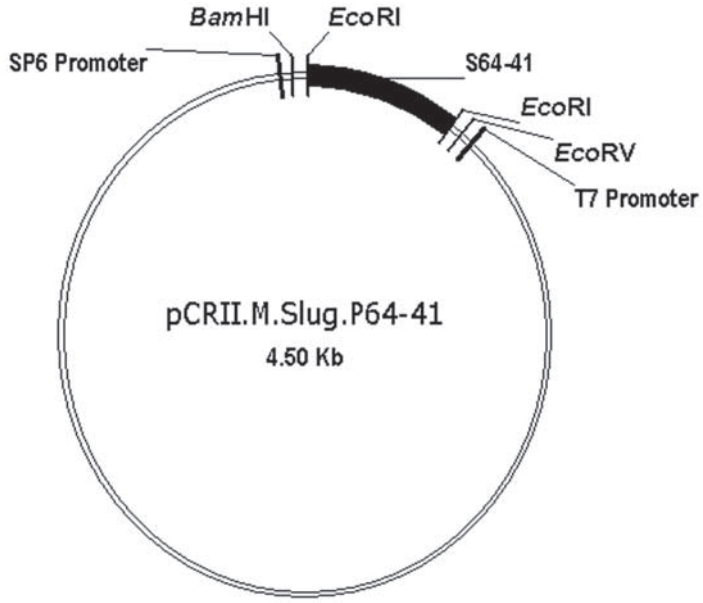

Fig. 2. Map of pCRII. M.Slug.P64-41 plasmid used for in vitro transcription of riboprobes. Antisense riboprobe is produced by linearizing the vector with *Bam*HI and transcribing RNA using T7 RNA polymerase. For the sense probe the vector is linearized with *Eco*RV and transcribed using Sp6 RNA polymerase.

*Bam*HI) and digest for 2 h at 37°C.
2. Run 2 µL of the sample on an agarose gel to verify complete cutting.
3. Extract twice with 1 vol of phenol:chloroform (1:1) each time. For each extraction, vortex vigorously, then centrifuge at 16,000*g* for 1 min and transfer the supernatant to a clean tube.
4. Add 1.8 µL of 3 *M* sodium acetate and 40 µL of 100% ethanol. Store at –20°C overnight to assure complete precipitation.
5. Centrifuge at 16,000*g* for 30 min at 4°C.
6. Discard the supernatant carefully and air-dry for 10 min.
7. Redissolve the plasmid in 45 µL of RNase-free water to give a DNA concentration of 1 µg of linearized plasmid/5 µL.

3.1.3. In Vitro Transcription

1. To make a stock 10X NTP mixture, mix 10 µL of each of ATP, GTP, CTP, 3.5 µL of UTP, and 6.5 µL of digoxigenin-labeled UTP. Store frozen (*see* **Note 3**).
2. For each in vitro transcription reaction, add the following to an RNase-free tube: 1 µg of linearized plasmid, 2 µL of 10X NTP mixture, 4 µL of 5X transcription buffer (supplied with enzyme), 1 µL of RNA polymerase, and 8 µL of distilled water (*see* **Note 4**).

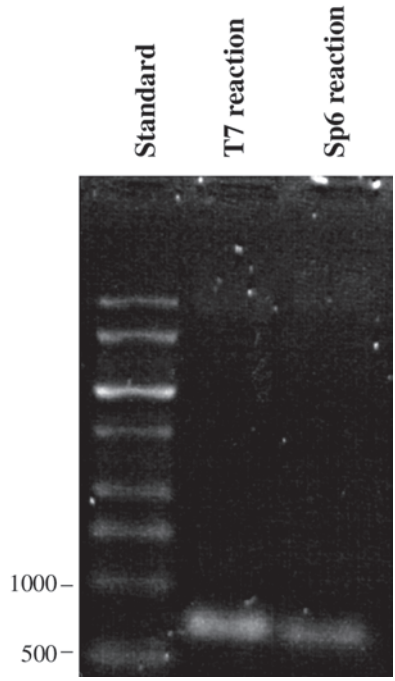

Fig. 3. Gel to quantify riboprobes. The molecular weight standard is labeled to show the size in bases of bands that flank the riboprobes. The T7 reaction is presumed to represent the production of 10 µg of RNA by in vitro transcription. The yield of the Sp6 reaction can be estimated as a fraction of the T7 yield, approx 50% in this instance.

3. Mix gently and spin down contents.
4. Incubate at 37°C for 2 h.
5. Add 2 µL of 0.2 *M* EDTA to each tube to stop the reaction.
6. Reserve 2 µL of each riboprobe for later analysis by electrophoresis (**Fig. 3**; *see* **Note 5**).

3.1.4. Riboprobe Purification

1. Precipitate each riboprobe with 2.5 µL of 4 *M* LiCl and 75 µL of 100% ethanol. Freeze at –20°C overnight.
2. Centrifuge at 6000*g* at 4°C for 30 min, gently remove the supernatant.
3. Add 100 µL of cold 70% ethanol to each tube.
4. Centrifuge at 16,000*g* at 4°C for 30 min. Remove the supernatant gently, being careful not to dislodge the RNA pellet.
5. Air-dry for 10 min then reconstitute the pellet in 20 µL of distilled water. Store riboprobes at –70°C (*see* **Note 6**).

3.1.5. Riboprobe Quantification

The incorporation of digoxigenin into the riboprobe precludes accurate quantification by spectroscopy. We estimate the amount of RNA produced by visual inspection of a formaldehyde-agarose gel, as described in **Note 5**.

3.1.6. Riboprobe Cleavage

We have had similar results using both full-length probe (approx 500 bases) and probe cleaved to an average length of 100–200 bases by alkaline hydrolysis. The technique we use for hydrolysis is described in **Note 7**.

3.2. Cell Culture

The steps in **Subheadings 3.2.1.–3.2.3.** describe culturing SCC 12F cells and in vitro wounding.

3.2.1. Cell Type

The SCC 12F cell line was derived from a squamous cell carcinoma tumor of the facial epidermis *(35)*. This adherent human keratinocyte cell line is maintained in 5% CO_2 at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium:Ham's F12 nutrient mixture (DMEM:F12Ham) containing 5% defined calf serum (cat. no. SH30072; Hyclone, Logan, UT), 1% glutamine, and 0.5% penicillin/streptomycin.

3.2.2. Culture Vessels

Cells are grown in two-chambered coated glass Lab-Tek II chamber slides (*see* **Note 8**). The plastic culture chambers on these slides are easily removed, eliminating the need to culture cells on cover slips and making the slides very convenient for *in situ* hybridization.

3.2.3. Wounding

The wounding technique has been described by McCawley et al. *(35)*. Briefly, cells are grown to confluence, then medium is removed, cells are washed twice with serum-free medium in which calf serum has been replaced by 1 mg/mL bovine serum albumin (fraction V). Mitomycin C (10 µg/mL) may be included in the medium to suppress mitosis, allowing examination of cell migration in the absence of proliferation. After cells have been cultured for 24 h in serum-free medium, wounds are introduced by drawing a sterile plastic pipettor tip (1000-µL size) in a straight line across the cell sheet. Additional wounds are introduced into cell sheets daily, allowing comparison of slug expression at different times after wounding on the same slide (**Fig. 4**).

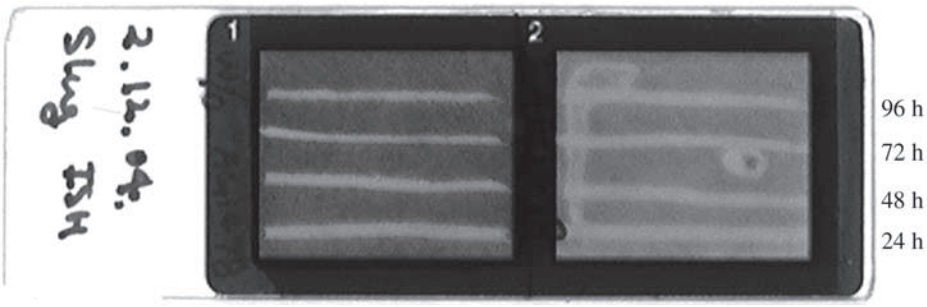

Fig. 4. Chamber slide showing the results of an in vitro wounding study, wounds were introduced at the indicated times before harvest. Cells in the left chamber were grown in serum-free medium; cells in the right chamber were grown in the presence of a receptor tyrosine kinase inhibitor. Notice that there is clear evidence of wound closure at later time points in the chamber on the left, but that wound width has not decreased in the chamber on the right.

3.3. Cell Fixation and Permeabilization

Pretreatment steps include cell fixation, permeabilization, and postfixation. The purpose of these steps is to preserve cellular morphology while allowing penetration of riboprobes through the cell membrane. All steps should be carried out using RNase-free solutions, glassware, and plasticware (*see* **Notes 1** and **9**).

3.3.1. Cell Fixation

1. Rinse cells twice using 2 mL of PBS per well each time.
2. Fix cells for 20 min at room temperature by adding to each well 2 mL of 4% formaldehyde and 5% glacial acetic acid in 0.9% NaCl (*see* **Note 10**).
3. Remove fixative solution carefully and rinse each well with 2 mL of PBS for 5 min.

3.3.2. Cell Permeabilization

1. Dehydrate cells by adding 70, 80, 95, and 100% ethanol sequentially, allowing 5 min for each step.
2. Detach the chamber well carefully using the tool included with the Lab-Tek II chamber slide system.
3. Immerse the slide in xylene for 10 min to remove lipids.
4. Immerse the slide in 100% ethanol for 10 min.
5. Rehydrate slide in PBS for 5 min.
6. Permeabilize the cells in 0.1% pepsin in 0.01 *M* HCl at 37°C for 10 min (*see* **Note 11**).
7. Rinse slides briefly in PBS.

3.3.3. Postfixation

1. Fix cells with 1% formaldehyde in PBS for 10 min (*see* **Note 12**).
2. Dehydrate through 70, 80, 95, and 100% ethanol for 5 min at each step.
3. Air-dry the slide completely before applying the riboprobe.

3.4. In Situ *Hybridization*

The procedure in **Subheadings 3.4.1.–3.4.3.** describes hybridization of riboprobe to target mRNA molecules. All steps in the process should be conducted under RNase-free conditions (*see* **Notes 1** and **9**).

3.4.1. Hybridization Solution

The hybridization solution consists of 4X SSC, 10% dextran sulfate, 1X Denhardt's solution, 50% formamide, and RNase-free water (*see* **Note 13**). The high concentration of formamide in the hybridization solution allows incubation at relatively low temperatures, eliminating much of the evaporation that can be a problem in other techniques (*see* **Note 14**). To reduce nonspecific probe binding, 500 µg/mL tRNA is included in the hybridization mixture.

3.4.2. Probe Concentration

We have found that a final concentration riboprobe concentration of 5–10 ng/µL works best. The probe mixture (riboprobe plus tRNA) should be prepared immediately before use by heating to 80°C for 10 min; the heated probe mixture should then be mixed thoroughly with hybridization buffer maintained at a temperature of 37°C (*see* **Note 15**).

3.4.3. Hybridization

Apply 300 µL of probe mixture to each slide and carefully cover with a Hybri-slip (Sigma) (*see* **Note 16**). Place slides in an RNase-free humidified chamber and incubate at 37°C overnight (*see* **Note 17**).

3.5. Riboprobe Detection

The detection process includes posthybridization washes, immunohistochemical detection of digoxigenin-labeled riboprobes, signal amplification, and visualization. **None of these steps require RNase-free conditions.** Posthybridization washes remove much of the unbound probe from the slide, but subsequent RNase A treatment is essential to eliminate riboprobe binding non-specifically *(see* **Note 18**).

3.5.1. Posthybridization Rinses and RNase Treatment

1. Soak slides in a mixture of 60% formamide in 2X SSC at room temperature until cover slips slide off easily.

2. Rinse slides twice with a mixture of 60% formamide in 2X SSC at 40°C for 10 min (*see* **Note 19**).
3. Rinse in ribonuclease buffer at 37°C for 5 min.
4. Prepare 50 µg/mL RNase A in ribonuclease buffer and preheat to 37°C. Incubate slides in this mixture for 30 min at 37°C (*see* **Note 20**).
5. Rinse slides in Buffer A for 5 min at 37°C.
6. Rinse twice with 60% formamide in 2X SSC for 10 min at room temperature each rinse.
7. Rinse with 2X SSC for 5 min.

3.5.2. Immunohistochemistry

1. Rinse slides in TNT buffer for 5 min.
2. Incubate slides with 300 µL of TNB blocking buffer for 30 min at room temperature in a humidified chamber. Carefully remove blocking buffer, but do not rinse slides.
3. Incubate with 300 µL of anti-digoxigenin-POD diluted 1:50 in TNB blocking buffer for 30 min at room temperature in a humidified chamber (*see* **Note 21**).
4. Rinse three times with TNT buffer for 5 min at room temperature with gentle agitation.

3.5.3. Signal Amplification

1. Apply 300 µL of TSA amplification reagent diluted 1:50 in amplification diluent (supplied with kit) and incubate for 10 min at room temperature in a humidified chamber (*see* **Note 22**).
2. Rinse slides three times with TNT buffer for 5 min each rinse at room temperature with gentle agitation.
3. Apply 300 µL of streptavidin-HRP reagent (supplied with kit) diluted 1:100 in TNB Blocking buffer for and incubate for 30 min at room temperature in a humidified chamber.
4. Rinse slides three times with TNT buffer for 5 min each rinse at room temperature with gentle agitation.
5. Visualize using the NovaRed (Vector) substrate for 5 min (*see* **Note 23**).

3.5.4. Counterstaining and Mounting

1. Rinse slides in distilled water for 5 min.
2. Counterstain very briefly with Mayer's hematoxylin (a few dips).
3. Rinse several times in distilled water.
4. Dehydrate slides through 70, 95, 95, 100, 100% ethanol.
5. Clear twice in xylene for 5 min each time.
6. Cover slip using xylene-based mounting medium.

4. Notes

1. At every step of riboprobe synthesis, it is important to prevent introduction of RNases. All solutions and equipments should be RNase-free, thus the use of dis-

posable RNase-free plasticware, RNase-free or DEPC-treated solutions, and gloves is essential. Complete descriptions of how to prevent RNase contamination in the laboratory are given in general molecular biology technique books such as Sambrook and Russell *(36)*. To save time, we usually purchase RNase-free solutions from a vendor such as Sigma. Ambion and other vendors provide an assortment of RNase inhibitors to add to solutions and supplies to inactivate RNases on solid surfaces that we have found very useful.

2. Many vectors are suitable for in vitro transcription reactions. These vectors generally include a multiple cloning site to accommodate the insert of interest flanked by recognition sites for T3, T7, or Sp6 RNA polymerases. It is essential to have exact vector information so that the proper combination of restriction enzymes for plasmid cleavage and RNA polymerases for riboprobe production can be selected. For RNA transcription, vectors should be linearized downstream of the DNA insert to be transcribed but upstream of any heterologous promoters. The restriction enzyme used to linearize the vector should yield a blunt end or a 5' overhang. For our negative control probe, we use a riboprobe containing the sense mRNA sequence. Other investigators have not found such probes satisfactory, but in our hands these riboprobes work well for negative controls.
3. This mixture can be stored for many months at –20°C. To minimize thawing and refreezing, store the mixture in small aliquots.
4. For the enzymes available from some companies, Sp6 gives less efficient transcription than T7 on a per unit enzyme basis. However, we have found that Stratagene Sp6 and T7 enzymes often give similar efficiency for both reactions, and we routinely use these enzymes.
5. To confirm the quality and estimate the quantity of riboprobe, run 2 µL of the transcription reaction on a formaldehyde agarose gel. The detailed protocol is given below.
   a. Dissolve 0.6 g of agarose in 36 mL of sterile distilled water. Heat until dissolved. Allow agarose solution to cool down to about 70°C.
   b. In a fume hood, add 5.0 mL of 10X MOPS/EDTA (0.2 *M* MOPS, 50 m*M* sodium acetate, 10 m*M* EDTA, pH 7.0; store protected from light) and 8.9 mL of formaldehyde to the cooled agarose solution.
   c. Mix the agarose solution gently and pour gel into a mini gel electrophoresis apparatus. Allow gel to harden for at least 30 min in the fume hood.
   d. To prepare sample, add 8 µL of RNA loading mix (1X MOPS/EDTA, 4.3 *M* formaldehyde) to each 2 µL of riboprobe sample. Mix gently then, incubate at 60°C for 15 min and place on the ice.
   e. Add 1 µL of 10X gel loading dye (50% glycerol: 0.1 *M* EDTA, pH 7.5, 1% sodium dodecyl sulfate, 0.1% bromophenol blue) to each sample. Mix gently and spin down.
   f. Fill gel box with RNA electrophoresis running buffer.
   g. Load the samples and run the gel at 30 V for 4 h.
   h. Rinse the gel in distilled water. Stain for 30 min with 0.5 µg/mL ethidium bromide.

i. Destain the gel in distilled water at 4°C overnight. If bands are visible under the UV illuminator, assume the reaction went to completion and that the T7 in vitro transcription reaction produced approx 10 µg of riboprobe. Estimate the amount of material produced by the Sp6 reaction compared with the T7 reaction. If bands are not seen or if bands are smeared, this suggests a failure of transcription or RNase contamination, respectively.

Concentrations of digoxigenin-labeled ribopobes also may estimated by dotting probe solution on a membrane followed by application of anti-digoxigenin antibodies and colorometric reagents as for a Western blot.

6. Riboprobes are stable for many months at –70°C. For added stability, 1 µL of placental ribonuclease inhibitor (Ambion) can be added to each 10 µL of probe and the probe stored in small aliquots. We have had no problem using probe that has been thawed and re-frozen one to two times, but we have never used probe thawed more than twice.
7. Alkaline lysis technique to reduce probe size:
   a. Calculate the hydrolysis time in min from the following equation:

$$T = \frac{L_0 - L_f}{0.11 \times L_0 \times L_f}$$

   where L_o is the original length of the transcript in kb, L_f is the desired length in kilobases of the probe, and T is the time in min required to hydrolyze the probe to this length.
   b. To the resuspended probe, add 100 µL of hydrolysis buffer (80 m*M* $NaHCO_3$, 120 m*M* Na_2CO_3, 20 m*M* β-mercaptoethanol) made up as follows: make carbonate buffer up in 80% of the final volume of RNase-free water, then autoclave, add β-mercaptoethanol to 20 m*M*, and bring to correct volume with RNase-free water. The pH should be 10.2. Store in small aliquots at –20°C.
   c. Incubate for the calculated amount of time at 60°C.
   d. Add 200 µL of stop buffer (0.2 *M* NaAc, pH 6.0; 1% glacial acetic acid; 10 m*M* DTT, 1 µg of tRNA), 40 µL of 3 *M* NaAc, and 1 mL of ethanol. Precipitate at –70°C or on dry ice for at least 2 h.
   e. Centrifuge at 16,000*g* for 15 min, remove supernatant, and dry pellet.
   f. Resuspend probe at desired concentration and store frozen at –70°C.
8. Nalge Nunc produces two types of Lab-Tek glass chamber slides, Lab-Tek and Lab-Tek II, both of which are suitable for this technique. Lab-Tek II chamber slides are RS-treated; RS treatment is a proprietary glass washing step to enhance cell attachment. Removal of wells from these slides requires the special tool provided with the system. On the other hand, plastic wells and gaskets are removed easily from Lab-Tek slides. For epithelial cells that require special cell substrates like collagen or fibronectin, substrate-coated glass slides (BioCoat culture slides, Becton-Dickinson; Franklin Lakes, NJ) can be used. Cells that can attach only to plastic can be cultured on Permanox slides from Nalge Nunc; however, organic solvents like xylene cause marked deformation of these slides, precluding their use in this technique.

9. For many of the rinse steps, we use organic solvent-resistant plastic Coplin jars. We have found that filling clean Coplin jars with chloroform and allowing them to sit in a fume hood overnight eliminates problems of RNase contamination.
10. Fixative solution should be prepared fresh for each set of slides. Combine 10 mL of 40% formaldehyde, 5 mL of glacial acetic acid, and 3.1 mL of 5 *M* NaCl; add distilled water to a final volume of 100 mL. We have found this method of fixation very satisfactory; however, other fixatives, including paraformaldehyde, alkaline formaldehyde, and alcohol-based fixatives like Histochioice (Amresco; Solon, OH), have been used successfully by other investigators.
11. Permeabilization is a very important step, allowing the riboprobe to penetrate the cell membrane. Proteinase K, pronase, and pepsin are often used for permeablization, while Triton-X is used infrequently. We have determined that freshly prepared 0.1% pepsin solution in 0.01 *M* HCl works very well in this protocol. Pepsin should be added to preheated 0.01 *M* HCl solution before use. Slides should be incubated in this solution at 37°C. It may be necessary to test different concentrations of pepsin and various incubation times to determine which condition provides optimal results. Caution should be exercised in working with pepsin or pepsin solutions because pepsin is a proteolytic enzyme.
12. Some *in situ* hybridization protocols do not require a post fixation step; however, we have found that this step improves signal intensity. The postfixation solution should be freshly prepared as follows: combine 10 mL of 10X PBS and 2.5 mL of 40% formaldehyde; bring to 100 mL with distilled water.
13. This hybridization solution is quite thick, thus careful mixing is required. Prepared solution without tRNA can be stored frozen at –20°C for weeks or months.
14. The kinetics of hybridization depend on both hybridization conditions (salt concentration, formamide concentration, and temperature of buffer) and probe characteristics (GC content and length). For a different riboprobe, hybridization buffer and incubation temperature may require adjustment. A wide variety of different buffers and temperature conditions have been used successfully in other *in situ* hybridization protocols.
15. This high probe concentration increases sensitivity, but it also can lead to high background staining because of the nonspecific binding. For this reason, RNase treatment of slides after hybridization is essential.
16. A variety of techniques for cover slipping during *in situ* hybridization incubations have been described, including no cover slipping and application of silanized glass cover slips or paraffin laboratory film. We have found that Hybrislips from Sigma work best in our hands. They are inexpensive, are supplied RNase-free, and are readily removed following overnight incubation.
17. To prepare an RNase-free humidified chamber, treat the chamber with RNA Zap or RNA Zap wipes (Ambion), rinse several times with RNase-free water, and air dry. Humidify with an RNase-free solution of 60% formamide in 2X SCC rather than with water. This appears to give superior results, although we have no explanation for this observation.
18. The RNA–RNA bond formed between target nucleic acid and riboprobe is stronger than RNA–DNA or DNA–DNA bonds, and the RNA–RNA duplex is resis-

tant to RNase A digestion. Other techniques use combinations of RNase A and RNase T1.

19. For most purposes, 60% formamide in 2X SSC at 40°C is adequate for posthybridization washing. However, to increase the stringency of the washes, salt concentration may be reduced or temperature may be increased.
20. Any nondisposable items used in the RNase A treatment step should be used only for this step.
21. In our studies, we employ the anti-digoxigenin POD antibody from Roche at a relatively high concentration (1:50 dilution in TNB blocking buffer). However, for different combinations of probe and target, it will be necessary to determine optimal antibody concentration and incubation time. To increase the sensitivity of detection while reducing background, overnight incubation at 4°C may be useful.
22. For very abundant mRNA species, the tyramide amplification step can be eliminated and peroxidase activity can be detected using a suitable chromogen.
23. NovaRed substrate solution (Vector) should be prepared fresh according to the manufacturer's instructions for each use. Although we generally notice results in 5–15 min, much longer time incubation times may be required for other applications.

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13

Identification of Transplanted Human Cells in Animal Tissues

Daniel Benten, Kang Cheng, and Sanjeev Gupta

Summary

The potential of cell and gene therapy has generated extensive interest over the past several years. More recently, identification of stem cells of various types, especially embryonic stem cells, reinforced this interest. Systematic studies are now being launched to define the biology of various stem cells, including after transplantation of cells in immunodeficient animals. This requires robust and unequivocal means to identify transplanted cells. Ideally, it should be possible to screen animal tissues for human cells with relatively simpler methods, followed by more precise localization of transplanted cells. We describe the application of conserved primate Charcot-Marie-Tooth disease type 1A repeat element for polymerase chain reaction-based screening of animal tissues for human cells. Similarly, direct polymerase chain reaction labeling of pancentromeric human alphoid sequences with digoxigenin-UTP generates *in situ* hybridization probes for identifying transplanted human cells. This pancentromeric probe identifies human cells irrespective of the original tissue source and can be combined with additional *in situ* methods to analyze cell differentiation. Incorporation of these strategies will facilitate translational studies aimed at understanding mechanisms concerning the trafficking, engraftment, proliferation, differentiation and function of human stem cells in animals.

Key Words: Stem cells; transplantation; *in situ* hybridization; PCR; identification; human; animal.

1. Introduction

The potential of cell therapy has excited widespread interest *(1)*. Recent insights into stem/progenitor cells isolated from specific organs, as well as the derivation of pluripotential embryonic stem cells from rodents and humans, has further bolstered this interest. The characterization of donor cells in intact animals represents a critical component of efforts to establish which cell types will be most effective for cell therapy. Similarly, it is necessary to analyze the

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fate of stem cells in intact animals to demonstrate whether transplanted cells will differentiate into required cell types and whether such differentiated cells will reconstitute deficient function *(2)*. In recent years, animal models have begun to become available for studies of transplanted human cells *(3–6)*. These include immunodeficient mice, as well as fetal sheep capable of tolerating human cells. The use of genetic reporters has been most effective for identifying transplanted cells in animals *(7)*. Measurement of transgene products secreted into the peripheral blood also can help in demonstrating engraftment and proliferation of transplanted cells. However, analysis of cell differentiation after transplantation requires tissue analysis *(2)*. Although human cells could be marked by exogenous genetic reporters, available gene transfer vectors are inefficient for some cell types, promoter extinction during cell differentiation could be a problem and some genetic reporters could perturb cellular behavior *(8)*. Such confounding could be avoided by the use of endogenous markers for identifying human cells in animals. One approach concerns use of fluorescence *in situ* hybridization for X or Y chromosome-specific sequences. However, fluorescence *in situ* hybridization for sex chromosomes generates only one or two hybridization signals in euploid cells, identification of transplanted cells is tedious and background signals may make it even more difficult. Therefore, we consider probes that generate multiple signals in cells and can be simultaneously used for rapid polymerase chain reaction (PCR) screening of tissues to identify transplanted cells as of greatest value *(9)*. For instance, pancentromeric sequences represented in all chromosomes provide such a marker. Here, we further illustrate these molecular assays useful for detecting transplanted human cells in the mouse.

2. Materials

1. DNeasy® Tissue Kit (cat. no. 69504; Qiagen, Valencia, CA) or equivalent to extract genomic DNA.
2. Specific PCR primer pairs synthesized by automated commercial methods (*see* **Subheadings 3.1.** and **3.2.2.**).
3. PCR reagents: 10X PCR buffer, 50 m*M* $MgSO_4$, Platinum Taq DNA Polymerase High Fidelity (cat. no. 11304-029; Invitrogen, Carlsbad, CA), dNTP Mix (cat. no. 18427-013; Invitrogen).
4. Platinum PCR Supermix (cat. no. 11306-016; Invitrogen).
5. Agarose gel electrophoresis apparatus and power supply.
6. Molecular weight marker for DNA size separation (e.g., 1-kb DNA ladder, cat. no. 15615-016; Invitrogen).
7. Genomic human DNA (e.g., from whole blood collected in standard anticoagulants).
8. PCR DIG Probe Synthesis Kit to incorporate digoxigenin-UTP (cat. no. 1636090; Roche Diagnostics, Indianapolis, IN).
9. Methylbutane cooled to –80°C or liquid nitrogen to freeze tissue.

10. Cork sheets (cat. no. 23420-708; VWR Scientific Products, West Chester, PA).
11. HistoPrep™ frozen tissue embedding medium (cat. no. SH75-1251; Fisher Scientific, Pittsburgh, PA) or equivalent.
12. 4% paraformaldehyde in phosphate-buffered saline (PBS; stable at 4°C for up to 2 wk).
13. Acetone cooled to 4°C (Fisher Scientific).
14. Ethanol (50, 75, 90, and 100%).
15. PBS, pH 7.4.
16. Tris-buffered saline (if using alkaline phosphatase-based detection of hybridization signals).
17. 20X standard saline citrate (SSC; 175.3 g sodium chloride and 88.2 g of sodium citrate in 1 L of water) and 2X SSC, pH 7.0.
18. Formamide.
19. Dextran sulfate.
20. Anti-digoxigenin-POD *or* anti-digoxigenin-AP Fab fragments (cat. no. 1 207 733 and 1 093 274, respectively, Roche Diagnostics).
21. DAB+ detection kit (cat. no. K3467, Dako Cytomation, Carpinteria, CA).
22. Sigma Fast™ Fast Red Detection Kit (cat. no. F-4648, Sigma).
23. Harris hematoxylin (cat. no. S212; Poly Scientific, Bay Shore, NY).
24. Glycerol as mounting medium (Sigma).
25. Instruments for animal surgery and tissue harvesting.
26. Cryostat machine with standard tissue sectioning accessories.
27. Precleaned "Colorfrost" Plus microscope slides and cover slips (Fisher Scientific).
28. Hydrophobic slide marker, e.g., Pap Pen (Research Products International, Mount Prospect, IL).
29. Water baths for 37 and 72°C incubations.
30. Humid chamber for slide incubations.
31. Dry incubator for maintaining 37°C temperature.

3. Methods

The procedures described in **Subheadings 3.1.–3.3.** offer a strategy to rapidly screen tissues for transplanted cells by PCR. It uses the conserved primate Charcot-Marie-Tooth disease type 1A repeat (CMT1A-REP) centromeric element, along with an irrelevant mouse gene, *c*-mos, to establish the integrity of the DNA sample. Also, we provide a convenient strategy for generating an *in situ* hybridization probe by direct PCR labeling of pancentromeric human alphoid sequences with digoxigenin-UTP, along with suitable methods to perform *in situ* hybridization with this probe for identifying human cells in tissues of specific animal species.

3.1. Screening of Tissues for Human Cells by PCR Assay

The CMT1A-REP element is composed of two highly homologous 24-kb sequences on human chromosome 17p11.2-12 ***(10)***. The proximal and distal CMT1A-REP sequences, which flank a 1.5-Mb chromosomal segment, share

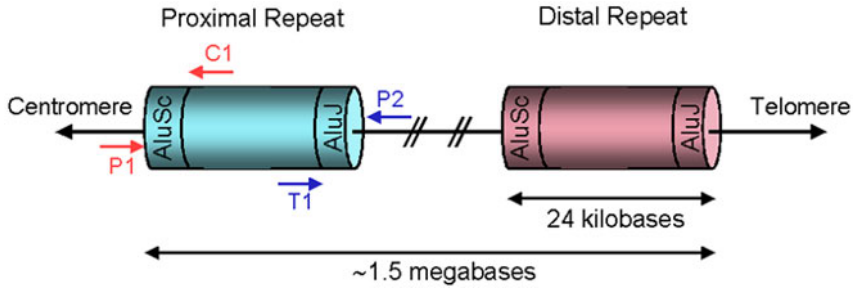

Fig. 1. Schematic organization of the CMT1A-REP element showing centromeric (left) and telomeric (right) orientations. The AluSC and AluJ sequences defining the boundaries of the CMT1A-REP sequences are shown. The position of C1 and P1 primer set and T1 and P2 primer set that amplify the proximal CMT1A-REP element sequences is indicated by colored arrows. (Redrawn and modified from **ref.** ***10***.)

99% sequence similarity, and display an AluSc element defining the centromeric boundary and an AluJ element defining the telomeric boundary in both copies of the repeat (**Fig. 1**). The CMT1A-REP element is present in primates, including humans and chimpanzees (both repeats), as well as the gorilla, orangutan, and gibbon (only one repeat), but is absent in bovine, murine, rabbit, or *Drosophila* genomes ***(11)***. Studies of the CMT1A-REP element in primates further established the presence of unique sequences in the proximal portion of the CMT1A-REP element in only human and chimpanzee genomes. The previously published primer sequences described below amplify either the centromeric (C1/P1 primers) or the telomeric boundary (T1/P2 primers) of the proximal REP element (*see* **Fig. 1** for approximate genome positions ***[10]***). In case of immunodeficient mice, which are used most commonly for human xenografts, PCR amplification of *c*-mos sequences has been effective as an internal control ***(5)***, although many other sequences can be readily substituted for this purpose. Because CMT1A-REP sequences can be amplified to represent individual copies of the genome, the assay can be converted in principle to measure the mass of transplanted human cells in suitable animals, e.g., by utilizing quantitative PCR methods. Such quantitation of transplanted cell mass is not possible by the use of Alu repeat sequences for PCR because Alu sequences are repeated extensively in the human genome.

CMT1A-REP primer sequences:
C1: 5'-GAGTGCACATTCAGACAAGAGCCC-3'
P1: 5'-CCATTAGAGAGCTTTCCTCATTGC-3'
Expected PCR product size: approx 550 bp.
T1: 5'-CGTGTGTTTTTGGTTACTTCTCCCC-3'

P2: 5'-CTTAGCCATTGCCCATTGATGGAC-3'
Expected PCR product size: approx 770 bp.
Murine c-mos primer sequences:
Forward primer: 5'-GAATTCAGATTTGTGCATACACAGTGACT-3'
Reverse primer: 5'-AACATTTTTCGGGAATAAAAGTTGAGT-3'.
Expected PCR product size: approx 430 bp.

3.1.1. DNA Extraction

To extract genomic DNA, tissues from animals subjected to cell transplantation are processed with standard proteinase K digestion followed by DNA extraction using the DNeasy Tissue Kit according to the manufacturer's instructions. Many other kits and protocols are available to isolate genomic DNA of suitable quality. During tissue collection, sample contamination is avoided by using either separate sets of clean surgical instruments for individual animals or by thoroughly cleaning instruments with detergent/ethanol rinses between animals. The PCR assay is best suited for screening tissues for the presence of human genomic DNA and for this purpose measurement of DNA content in the samples is not essential. However, it should be appropriate to standardize PCR conditions with approx 1 µg of genomic DNA.

3.1.2. PCR for Human REP Sequences and Mouse c-mos Sequences

1. Perform PCR in 50-µL reactions using:
   a. 5 µL of 10X PCR buffer.
   b. 15 µL of 50 m*M* $MgSO_4$.
   c. 4 µL of 2.5 m*M* dNTPs.
   d. 1 µL of 15 m*M* specific primers.
   e. 0.5 µL of Platinum Taq DNA Polymerase High Fidelity (Invitrogen).
   f. 1 µg of genomic DNA sample (volume adjusted with clean PCR-quality water).
2. PCR products are resolved by electrophoresis in 1% agarose gels containing 1 µg/mL ethidium bromide (**Fig. 2**).
3. PCR conditions for CMT1A-REP. Denature at 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and final elongation at 72°C for 7 min.
4. PCR conditions for *c*-mos: denaturation at 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and final elongation at 72°C for 7 min.

3.2. Identification of Transplanted Human Cells in Tissues With In Situ *Hybridization*

An excellent probe is obviously necessary to identify cells in tissues. We developed a suitable *in situ* hybridization probe to hybridize with tandemly repeated DNA sequences present in all primate chromosomes ***(12)***. These repeat DNA sequences are referred to as alpha satellite DNA or alphoid DNA

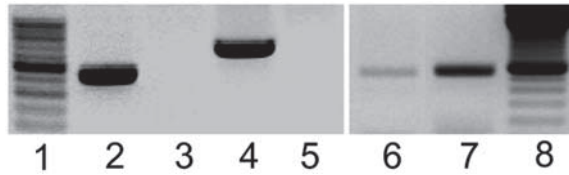

Fig. 2. Identification of transplanted human cells in the mouse with CMT1A-REP PCR. Lanes 1 and 8 contain molecular weight markers; lanes 2 and 4 show products of C1/P1 and T1/P2 primers, respectively, with reference human genomic DNA; lanes 3 and 5 show absence of corresponding PCR products when mouse DNA was analyzed with these primers; and lanes 6 and 7 show the product of C1/P1 primers after amplification of genomic DNA from the liver of two Balb/c-SCID mice transplanted intrasplenically with human liver cells. The integrity of mouse DNA samples analyzed was verified by c-mos PCR (not shown).

and a 170-bp repeat unit constitutes a common feature of alphoid DNA. One of these alphoid DNA sequences, designated the pαH21 clone, hybridizes *in situ* to the centeromeric region of all chromosomes in humans and great apes and to additional noncentromeric sequences on human chromosomes, perhaps representing atavistic centromeric remnants ***(12)***. We used the published sequence of this clone to obtain a digoxigenin-labeled probe by using genomic human DNA isolated from peripheral blood as a template. This convenient step avoids the need for plasmid DNA clones of alphoid human DNA as templates.

3.2.1. Extraction of Human Genomic DNA

1. Five milliliters of venous human blood is collected in ethylene diamine tetraacetic acid and the "buffy coat" containing nucleated cells is separated by centrifugation under 1000*g* for 15 min at room temperature. The buffy coat is located in the middle of the blood column and is identified as a pale band.
2. This band is transferred by pipetting into a clean tube and genomic DNA is extracted by the DNeasy Tissue Kit according to the manufacturer's instructions.

3.2.2. PCR for Amplifying Human Alphoid DNA Sequences

The complete human alpha satellite DNA sequence is available on the worldwide web (GenBank accession number M64321 ***[12]***). We designed the following set of PCR primers to obtain a suitable product from genomic DNA template:

Forward primer: 5'-GAATCTGCAAGTGGATATTAAGACC-3'
Reverse primer: 5'-AACCGGCTCTATCAAAGGAAAG-3'
Expected primary PCR product size: 153 bp.

1. PCR is performed in a final volume of 50 µL containing 45 µL of Platinum PCR supermix, 1.5 µL of each primer (15 m*M*), and 50 ng of genomic human DNA.

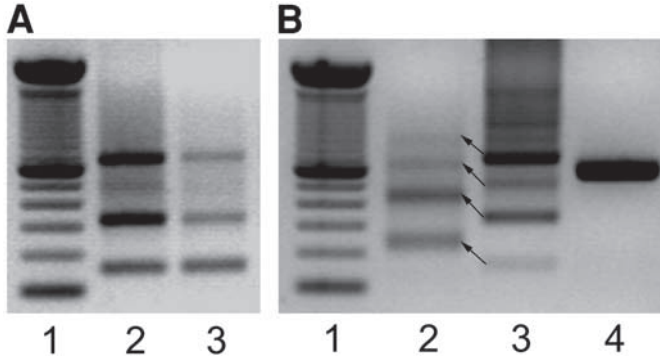

Fig. 3. Generation of *in situ* hybridization probe. **(A)** PCR amplification of human alphoid DNA sequence using genomic DNA template from peripheral blood cells. PCR products were resolved in an agarose gel. Lane 1, molecular weight marker using a 100 basepair ladder; lane 2, PCR products following amplification of genomic DNA template from human blood; lane 3, PCR products using DNA template from human liver cells. The bands at the bottom in lanes 2 and 3 are 153 basepairs in size. **(B)** Digoxigenin labeling of human alphoid DNA by direct PCR. Lane 1, molecular weight marker as in the preceding panel; lane 2, digoxigenin-UTP labeling of DNA as indicated by the upward shift in the position of PCR bands (arrows); lane 3, unlabeled PCR products; lane 4, amplification of control DNA sample provided in the PCR DIG Probe Synthesis Kit.

The conditions were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 2 min, and final elongation at 72°C for 7 min.

2. Analysis of the PCR product in 1% agarose gels reveals the expected band of 153-bp size, as well as additional bands of approx 650 bp and 320 bp sizes, as likely products due to additional cognate alphoid DNA sequences (124- to 786- and 124- to 447-bp positions, GenBank accession number M64321, respectively) or concatameric products (**Fig. 3A**). These additional PCR products do not interfere with the performance of the probe and selection of the 153-bp band is unnecessary.

3.2.3. Direct PCR Labeling of Human Alphoid DNA With Digoxigenin as an In Situ *Probe*

To obtain an *in situ* hybridization probe, human genomic DNA is labeled with digoxigenin directly by PCR using a commercial kit (PCR DIG Probe Synthesis Kit, Roche). The kit contains all reagents required except for specific primers and template genomic human DNA (*see* above). The manufacturer's instructions are followed. In brief:

1. Mix the following reagents for 50-µL reactions:
   a. 50 ng whole blood human DNA.
   b. 5 µL of 10X PCR buffer with $MgCl_2$.

c. 5 µL of PCR DIG Labeling Mix (for control reactions, the PCR DIG Labeling Mix, which contains DIG-11-dUTP, is substituted by 5 µL dNTP stock solution).
d. 1.5 µL of each of the primers.
e. 0.75 µL of Enzyme Mix.
2. The reagents are mixed and PCR is conducted for 30 cycles under conditions described previously (*see* **Subheading 3.2.2.**).
3. Of the PCR product, 7.5 µL is used to resolve in 1% agarose gels (**Fig. 3B**). As a result of DIG incorporation, the size of labeled PCR products is shifted. The DIG-labeled PCR product is aliquoted and stored at –20°C.

3.3. In Situ *Hybridization With Digoxigenin-Labeled Alphoid DNA Probe to Identify Human Cells*

This section describes sampling and preparation of mouse tissues containing transplanted human cells, followed by methods for probe hybridization and detection. Prototype tissue analysis concerns the mouse liver following intrasplenic or intraportal transplantation of human cells ***(2–5)***.

3.3.1. Tissue Sampling and Storage

1. The liver is carefully excised and rinsed briefly in clean PBS to remove blood. The liver lobes of interest are sampled with a clean razor blade (approx 1 cm^3) and placed on 5-mm thick cork sheets (1.75–2 cm^2) after interposing a small bead of HistoPrep™ resin (*see* **Notes 1** and **2**).
2. The tissue and cork are quickly submerged into methylbutane cooled previously to –80°C (liquid nitrogen could also be used for this purpose).
3. After 1 h in methylbutane, tissues are transferred to storage at –80°C.

3.3.2. Tissue Sectioning and Fixation

1. Cryosections are cut to 5-µm thickness at –18 to –20°C and sections are placed on precleaned "Colorfrost" microscope slides.
2. The sections are air-dried for at least 1 h at room temperature (*see* **Note 3**).
3. To fix tissue sections, slides are immersed in fresh ice-cold acetone at 4°C for 10 min followed by drying for 20 min at room temperature. These fixed and air-dried slides can be stored for up to 4 wk at –20°C before further processing.

3.3.3. Preparations for In Situ *Hybridization*

On the day of *in situ* hybridization, the following solutions are prepared approx 1 h before the procedure:

1. Cool freshly prepared ethanol solutions (50, 75, 90, and 100%) to –20°C.
2. Fill two clean Coplin jars with 2X SSC to a level that will cover tissue sections on slides. The Coplin jars are then placed into a 37°C water bath.
3. Another clean Coplin jar is filled with denaturation solution containing 70% formamide/2X SSC and this is brought to 72°C in a water bath (*see* **Note 4**).

3.3.4. Postfixation of Tissue Sections

1. The stored slides with tissue sections are thawed and air-dried for 30 min.
2. Tissue sections are then fixed in 4% paraformaldehyde in PBS for 20 min at 4°C (*see* **Note 5**).
3. After fixation, slides are transferred to a Coplin jar containing 1X PBS and washed three times for 2 min each in PBS.

3.3.5. Preincubation and Denaturation of Tissue DNA

1. Excess PBS is wiped from around tissue sections and slides are immersed in 2X SSC for 20 min at 37°C with intermittent agitation. This process is repeated a second time.
2. The slides are transferred from 2X SSC into the denaturation solution at 72°C for exactly 2 min (*see* **Note 6**). During denaturation, a clean Coplin jar is filled with 50% ethanol cooled to –20°C and placed on ice.
3. After completion of denaturation, slides are transferred rapidly into ice-cold 50% ethanol for 2 min followed by dehydration in ethanol as follows: 75%, 2 min; 90%, 2 min; and 100%, 5 min (*see* **Note 7**).
4. The tissue sections are air-dried for at least 30 min at room temperature (*see* **Note 3**). In the meantime, the probe is prepared (*see* **Subheading 3.3.6.**).

3.3.6. Probe Preparation and Hybridization

1. The DIG-labeled probe (*see* **Subheading 3.2.2.**) is thawed on ice. Each tissue section requires 1 µL of probe mixed with 10 µL of hybridization solution containing 50% formamide, 10% dextran sulphate, and 2X SSC. This probe mix is vortexed gently and spun briefly in a microcentrifuge.
2. The probe mix is denatured by incubation in a 72°C water bath for 5 min and immediately placed on ice for 20 min.
3. Tissue sections are encircled with a hydrophobic Pap-Pen. A humid chamber consisting of a plastic container with a lid is prepared by layering wet blotting paper at the bottom. Plastic pipets are cut to an appropriate size and laid in parallel to support slides.
4. The solution containing denatured probe is mixed well by pipetting up and down. After placing 10-µL probe on each tissue section, a glass cover slip is placed on top without trapping air bubbles.
5. The slides are placed into the humid chamber and incubated for at least 16 h in a dry incubator at 37°C.

3.3.7. Posthybridization Washes and Immunological Detection

1. Before taking slides from the incubator, a Coplin jar is filled with 2X SSC and equilibrated to 72°C in a water bath.
2. The cover slips are removed carefully from tissue sections by sliding these toward the slide edge. Any excess hybridization solution is carefully wiped from around the tissue sections. The slides are then placed in Coplin jars containing 2X SSC at 72°C and incubated for 5 min without agitation (*see* **Note 8**).

3. Tissue sections are next washed three times for 5 min each in either 1X PBS for peroxidase-based detection or in 1X TBS for alkaline phosphatase-based detection.
4. Enzyme linked anti-DIG solutions are prepared for immunological detection
   For peroxidase detection, dilute peroxidase-conjugated anti-DIG 1:50 in PBS. For alkaline phosphatase detection, dilute alkaline phosphatase-conjugated anti-DIG 1:300 in TBS.
5. Excess PBS or TBS is carefully blotted away from tissue sections and sections are covered completely with 80–100 µL of anti-DIG. The specimens are then incubated in a humid chamber for 1.5 h at 37°C.

3.3.8. Visualization of Hybridization Signals

1. Slides are rinsed in either PBS or TBS three times for 5 min each at room temperature.
2. Substrate solutions are prepared as follows for color development:
   a. For peroxidase detection with diaminobenzidine (DAB+ detection kit), add one drop of liquid DAB chromogen to 1 mL of buffered substrate for DAB+.
   b. For alkaline phosphatase detection with a Sigma Fast Red detection kit, dissolve one tablet of Sigma Fast™ Tris buffer (provided in the kit) in 1 mL of deionized water followed by one tablet of Fast Red Napthol in the solution.
3. Excess PBS or TBS is carefully blotted away from tissue sections and sections are covered with the substrate solution until color development (*see* **Note 9**).
4. After rinsing sections carefully with water, tissues are counterstained with hematoxylin for visualizing nuclei. Mount sections with glycerol or equivalent commercial mounting medium.

The data from an illustrative experiment to identify transplanted human cells in the mouse liver are shown in **Fig. 4**. It is possible to combine *in situ* hybridization with histochemistry or immunostaining to demonstrate gene expression in transplanted cells *(see* **ref.** ***9*** for examples).

4. Notes

1. Negative (mouse) and positive (human) control tissues should be included in each assay. These tissues should be fixed and processed in the same way as tissue samples containing transplanted cells.
2. Tissue preservation is critical and thawing of frozen tissue samples is to be avoided, for instance, during transfer to the cryostat facility. During tissue sampling, the freezing resin is used for adhesion of tissue to the cork plate. Do *not* cover the tissue sample with HistoPrep™ resin because it may affect probe penetration.
3. Air-drying of cryosections for less than 1 h produces poor hybridization signals. Microwaving or baking of tissue sections is not recommended. To reduce hybridization background, avoid contamination of tissue sections with dust. Use of laminar flow hoods for air-drying decreases such contamination.

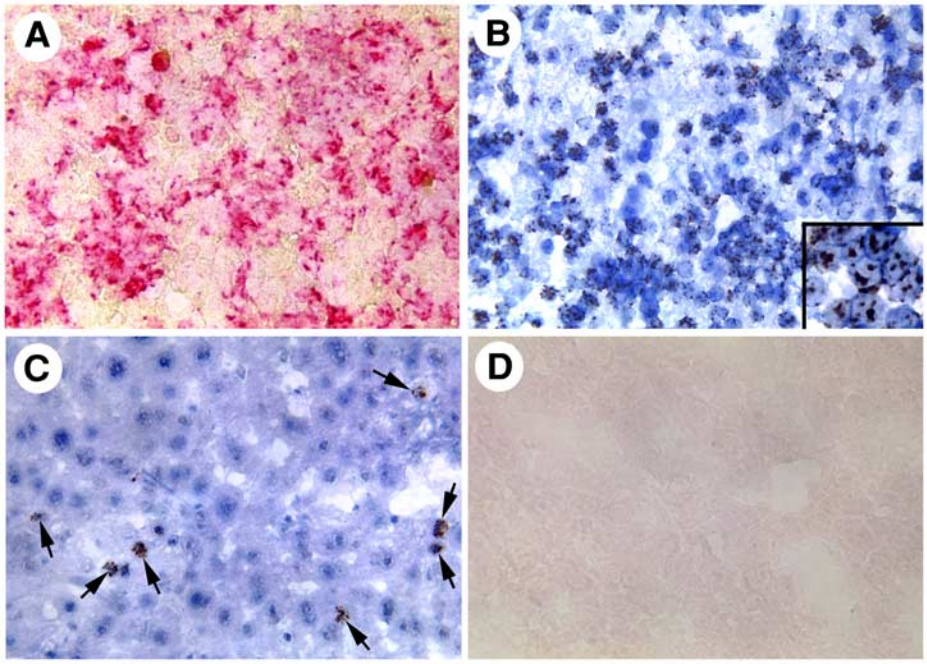

Fig. 4. *In situ* hybridization with digoxigenin-labeled alphoid human DNA probe. **(A)** and **(B)** show hybridization signals in normal human liver with color development using either Fast Red substrate of alkaline phosphatase, producing red spots, or diaminobenzidine substrate of peroxidase, producing brown-black spots. **Inset** in **(B)** shows hybridization signals at higher magnification. Note that the number and intensity of hybridization signals varies due to potential tangential cuts of the nuclei and differences in the cellular content of chromosomes. **(C)** Shows transplanted human hepatocytes in the liver of an immunodeficient NOD-SCID mouse (arrows). **(D)** Shows the negative control mouse liver with the absence of any hybridization signal. Origingal Magnification ×600, except inset in **(B)** ×1000. Tissues in **(B)** and **(C)** were counterstained with hematoxylin.

4. The temperature of solutions *within the Coplin jars* should be verified with a clean thermometer. The temperature should range between 37 ± 1°C for 2X SSC and 72 ± 1°C for denaturation solution. For each procedure, the denaturation solution (70% formamide/2X SSC) should be prepared fresh. Each Coplin jar should contain 35 mL of formamide, 5 mL of 20X SSC, and 10 mL of deionized or distilled water. Not more than six slides should be processed per Coplin jar.
5. The double-fixation method of tissue sections with cold acetone *and* 4% paraformaldehyde is necessary as described.

6. Denaturation of DNA in tissue sections for exactly 2 min is critical.
7. To prevent renaturation of DNA, it is important to transfer slides *immediately* from the denaturation solution into 50% ethanol at –20°C. We obtain best results when tissue slides are kept in 100% ethanol for 5 min rather than just 2 min.
8. Greater stringency of posthybridization washes (e.g., 2X SSC at 72°C with lower salt concentration and greater temperature or wash time will decrease hybridization signals). The wash in 2X SSC should be performed carefully without agitation or shaking.
9. Sections should be observed periodically under a microscope after adding substrate solutions for hybridization signals. Color development usually takes only a few minutes but may require up to 30 min. Brown or bright red spots appear in cell nuclei after peroxidase/DAB+ or alkaline phosphatase/Fast Red detection, respectively (**Fig. 4**). DAB produces sharper signals, whereas Fast Red produces relatively diffuse staining. If hybridization signals are weak, the concentration of DAB+ recommended by the manufacturer should be adjusted by using 0.5 mL of buffered substrate and one drop of chromogen. Use of standard DAB generates weak signals and is not preferred.

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14

In Situ Hybridization to Plant Tissues and Chromosomes

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Summary

In situ hybridization is a basic method in modern plant cell and molecular biology. It is used to locate the chromosomal position of genomic DNA sequences. It is able to determine the patterns of gene transcription in mature tissues and during development. *In situ* hybridization, in combination with other modern methods, has revolutionised our understanding of the structure, function, organization, and evolution of genes and the genome. Here we provide the protocols required for standard RNA and DNA *in situ* hybridization, with enough background information to enable trouble-shooting and data assessment.

Key Words: Plant; fluorescence *in situ* hybridization (FISH); RNA transcripts; high copy DNA sequences.

1. Introduction

1.1. The Location of Specific Sequences on Chromosomes

Plant *in situ* hybridization was first achieved nearly 30 yr ago when a highly repetitious tandem repeat was located, using tritiated cRNA probes, within multiple heterochromatic blocks on the massive chromosomes of *Scilla sibirica* (*1*). Since then, the sensitivity of the technique has been considerably improved.

In situ hybridization on chromosomes using fluochromes for signal detection has a great advantage over *in situ* hybridization using enzymatic detection methods, although the latter predominate in the detection of RNA transcripts. Different DNA probes can be simultaneously detected using different fluochromes, thus allowing their physical order on chromosomes to be determined. Fluorescence *in situ* hybridization (FISH), which initially was developed for mammalian chromosomes, was first applied to plant chromosomes by

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Schwarzacher et al. *(3)* and Yamamoto and Mukai *(4)* and later applied to extended DNA fibres prepared from nuclei *(5)* or chloroplasts *(6)*.

Although nonisotopic detection is widely applied to locate repeated sequences in plants *(7–9)*, the detection of low/single-copy sequences has not yet been routinely established. However, there have been a few reports of some success with single/copy-sequence probes using specialized procedures to strengthen the hybridization signals using tyramide-signal amplification *(10)* or by other means *(11)*. In this chapter, we present a protocol used for the physical mapping of high-copy sequences in a range of different plant species.

1.2. Location of RNA Molecules in Plant Tissues

The functional analysis of a gene requires a detailed characterization of its expression pattern at the tissue and cellular levels. *In situ* hybridization to detect mRNAs is a powerful method for such an analysis. Many techniques and their variations exist, all with specific advantages and disadvantages. Methacrylate embedding can be used if *in situ* hybridization is to be combined with immunolocalization, but the technique is less sensitive than paraffin. Hybridization on frozen sections (cryosectioning) requires relatively expensive equipment and expertise in microscopy. Another approach, the labeling of entire plantlets (whole mount labeling), is a simple technique but can lead to artifacts brought about by tissue accessibility to the probe. Conventional *in situ* protocols that require sectioned material and radiolabeled probes are time-consuming and do not allow the determination of gene expression in single cells. Here, we present one variant of the most widely used method in plant biology, which uses nonradioactive RNA probes on paraffin-embedded material. The protocol is derived from several sources but is largely as described by Guerin et al. *(12)*. Another excellent reference is Jackson *(13)*. It should be noted that, largely because of the complexity of the procedure and the variation between different plant tissues and probe sequences, the method should be viewed as a guideline and may need modification to suit different experiments.

2. Materials

2.1. The Location of Specific Sequences on Chromosomes

2.1.1. Chromosome and Extended DNA Fiber Preparation

1. Cell wall enzyme cocktail: dissolve 2.5% pectolyase Y-23 and 2.5% cellulase "Onozuka R-10" (Serva, Heidelberg, Germany) in 75 m*M* KCl, 7.5 m*M* EDTA, pH 4.0. Store at –20°C in 0.05-mL aliquots.
2. Fixative: 3:1 of ethanol and glacial acetic acid. Make fresh on the day of use.
3. 1% Aceto-carmine: 45% acetic acid and 1% carmine (Merck, Darmstadt, Germany).

4. 45% Acetic acid.
5. 1X PBS: 10 m*M* sodium phosphate, pH 7.0, 140 m*M* NaCl.
6. STE buffer: 0.5% sodium dodecyl sulfate; 100 m*M* Tris-HCl, pH 7.0.
7. 0.5 m*M* Ethylene diamine tetraacetic acid (EDTA).
8. Nucleus isolation buffer: 10 m*M* Tris-HCl, pH 9.5, 10 m*M* EDTA, 100 m*M* KCl, 0.5 *M* sucrose, 4 m*M* spermidine, 1.0 m*M* spermine, and 0.1% (v/v) 2-mercaptoethanol.
9. Triton X-100.
10. Glycerol.
11. Nylon mesh filters (filter size 170, 50, and 20 µm).
12. Petri dish.
13. Mortar and pestle.
14. 0.025% Colchicine.
15. Ethanol (70 and 100%).
16. Dry ice or liquid nitrogen.
17. 37 and 60°C incubator.
18. 10-mL Glass or plastic containers.
19. Slide holders.
20. Slides and cover slips.

2.1.2. Preparation of In Situ *Hybridization Probes*

1. Biotin or digoxigenin (DIG): Nick translation mix (Roche Diagnostics, Penzberg, Germany).
2. 0.5 *M* EDTA, pH 8.0.
3. 15°C water bath.
4. DIG-11-dUTP or biotin-11-dUTP (Roche).
5. dNTPs.
6. 10X PCR buffer.
7. *Taq* DNA polymerase.
8. QIAquick nucleotide removal kit (Qiagen, Valencia, CA).
9. Salmon sperm DNA (100–500 bp fragments obtained by sonication).
10. 3 *M* NaOAc.
11. Ethanol (70 and 100%).
12. Benchtop centrifuge.
13. Thermal cycling machine.

2.1.3. Hybridization

1. 100 µg/mL RNase A (in 2X standard saline citrate [SSC]).
2. Chromosome denaturation solution: 70% formamide in 2X SSC (SSC is 0.15 *M* NaCl, 0.015 *M* NaCitrate, pH 7.5).
3. Deionized formamide.
4. Hybridization mix: 20% dextran sulfate and 4X SSC.
5. Blocking reagent: 5% BSA (fraction V), 4X SSC, 0.2% Tween-20.

6. Ethanol (70 and 100%).
7. 37°C Incubator.
8. Parafilm.
9. Rubber cement.
10. Cover slips.
11. Heating block.
12. Resealable plastic box.

2.1.4. Antibody Detection

1. 4X SSC, 0.1% Tween-20.
2. 2X SSC containing 50% formamide.
3. 2X SSC.
4. FITC-conjugated avidin (Vector Laboratories, Burlinghame, CA).
5. Biotinylated anti-avidin (Vector Laboratories).
6. Rhodamine conjugated anti-DIG antibody raised in sheep (Roche).
7. Rhodamine labeled anti-sheep antibody raised in goat.
8. Antibody reaction buffer: 1% bovine serum albumin (BSA), 4X SSC, and 0.2% Tween-20.
9. Antifade solution: dissolve 10 mg *p*-phenylenediamine in 90% glycerol.
10. 4,6-diamidino-2-phenylindole dihydrochloride (DAPI): 0.2 mg/mL stock solution in water.
11. 42°C water bath shaker.
12. Coplin jars with lids (glass jars that have internal ridges to separate microscope slides).
13. Cover slips.

2.2. The Location of RNA Molecules in Plant Tissues

Note that some solutions appear only in the methods section and are not listed in this section. The entire protocol should be read thoroughly before starting.

2.2.1. Preparation of Plant Tissue Sections

1. Fixative: 4% (w/v) paraformaldehyde (PFA); 0.25% (v/v) glutaraldehyde, in 1X PBS. Prepare on the day of use. To 200 mL of 1X PBS, add 10 *N* NaOH until pH reaches 11.0 (check with pH paper). Heat to 60–70°C in a water bath. Add 8 g of PFA (in fume hood) and stir. PFA should dissolve within a minute or so. Place on ice. When cooled to 4°C, pH to 7.0 with concentrated H_2SO_4. Add glutaraldehyde to 0.25% (v/v; 0.5 mL) and filter through Whatman paper (Brentford, Middlesex, UK) into a sterile screw-cap bottle. Store on ice until use.
2. 10X PBS: 1.3 *M* NaCl, 0.07 *M* Na_2HPO_4, 0.03 *M* NaH_2PO_4.

2.2.2. Preparation of Probe

1. Buffer 1 (spot-test): 0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl.
2. Buffer 2 (spot-test): 0.1 *M* Tris-HCl, pH 9.5, 0.1 *M* NaCl, 0.05 *M* $MgCl_2$.

3. Blocking reagent (spot-test): dissolve 0.025 g of blocking reagent (Roche) in 5 mL of Buffer 1 by heating to 50°C.
4. Staining solution (spot-test): dissolve 1/10 of an NBT/BCIP Tablet (Roche) in 1 mL of Buffer 2, immediately before use.

2.2.3. RNA In Situ *Hybridization of Plant Tissue Sections*

1. 20X SSC: 3 *M* NaCl, 0.3 *M* Na_3-citrate.
2. Proteinase K solution: add 1 mg/mL proteinase K to a solution of 100 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, prewarmed to 37°C.
3. 4% PFA: make as for fixative, but omit glutaraldehyde.
4. 10X *in situ* salts: 3 *M* NaCl, 100 m*M* Tris-HCl, pH 8.0, 100 m*M* Na phosphate buffer, pH 6.8, 50 m*M* EDTA.
5. 50X Denhardts: 1% (w/v) Ficoll, 1% (w/v) PVP, 1% (w/v) gelatin.
6. 1X NTE: 0.5 *M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
7. TST: 100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.3% (v/v) Tween-20.
8. Detection buffer: 0.1 *M* Tris-HCl, pH 9.5, 0.1 *M* NaCl, 0.05 *M* $MgCl_2$.
9. 1X TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.

3. Methods

3.1. Chromosome and Extended DNA Fiber Preparations

It cannot be overstressed that good *in situ* hybridization results begin with good cytological preparations (*see* **Note 1**).

3.1.1. Mitotic Chromosomes

1. Germinate seeds on moist filter paper in a Petri dish.
2. To accumulate metaphases, treat excised roots (2–3 cm long) with one of the metaphase arresting agents. Optimal conditions may differ for different species. Commonly used conditions include placing roots in ice water for 16–24 h or in 0.025% colchicine for 3–6 h at room temperature.
3. Fix the roots in fixative for 2 d at room temperature. If longer storage of the material is required, but less than 7 d, transfer the roots into 70% ethanol at –20°C.
4. Wash roots for 10 min in enzyme buffer or water to remove fixative.
5. Macerate the root-tip meristems in enzyme cocktail at 37°C until the material is soft, usually for up to 25 min.
6. Wash material in 45% acetic acid for at least 10 min.
7. Transfer the material carefully into one drop of 45% acetic acid on a glass microscope slide. Slides with sintered glass labels are best because they identify the surface that carries the biological material. Dissect meristematic tissue (between 0.5 and 3 mm of the root tip, depending on the species and the size of the root) and apply a cover slip. Carefully disperse the material between glass slide and cover slip by tapping the cover slip gently with a needle. Place the slide between one or two layers of folded filter paper and squash the cells using the thumb avoiding any lateral movement. Identify the position of the material on the back of the slide using a diamond marker.

8. Examine the preparation under a phase contrast microscope and keep only those slides that show reasonable numbers of well-separated, well-squashed metaphase chromosome spreads.
9. Place the slide on dry ice or plunge it into liquid nitrogen to freeze the 45% acetic acid. Separate the cover slip almost immediately after removing the slide by inserting a pointed scalpel blade at one corner and carefully flicking off the glass plate, if possible in one piece. Most of the cells should stick to the slide rather than the cover slip.
10. Air-dry and dehydrate the slide in a series of 70 and 100% (v/v) ethanol.
11. The dried slides can be stored desiccated at –20°C or in 100% glycerol at 4°C for up to 1 yr or more.

3.1.2. Meiotic Chromosomes

1. Identify and isolate anthers containing pollen mother cells at the appropriate stage of meiosis and fix the anthers in fixative for 1 d at room temperature. If longer storage of the material is required, but less than 3 d, transfer the roots into 70% ethanol at –20°C (*see* **Note 2**).
2. Wash anthers for 10 min in enzyme buffer or water to remove fixative.
3. Macerate the anther in enzyme cocktail at 37°C until the material is soft, usually for up to 10 min. With some tissues it is possible to gently squeeze the pollen mother cells out of the anther, in others it will be necessary to treat the entire anther in subsequent steps of the procedure.
4. Proceed as described in **step 6** of **Subheading 3.1.1.** for mitotic chromosomes.

3.1.3. Extended DNA Fibers

3.1.3.1. Isolation of Nuclei

1. Grind 2 g of young leaves to a fine powder in a mortar and pestle in liquid nitrogen.
2. Transfer powder to a 50-mL centrifuge tube containing 20 mL of ice-cold nucleus isolation buffer.
3. Incubate and mix for 5 min on ice and filter homogenate through consecutive nylon mesh filters (filter size 170, 50, and 20 µm).
4. Add about 1/20 vol of 10% (v/v) Triton X-100 to the filtrate.
5. Centrifuge at 2000*g* for 10 min at 4°C and resuspend in 200 µL of nucleus isolation buffer at concentration of approx 5×10^5 nuclei per milliliter.
6. Add equal volume of glycerol and store nuclear suspension at –20°C until use.

3.1.3.2. Preparation of Extended DNA Fibers

1. Centrifuge 50 µL of nuclear suspension at room temperature in microfuge at 1200*g* for 5 min.
2. Gently resuspend pellet in 50 µL in 1X PBS and pipet 3 µL of nuclei suspension on to one end of a clean glass slide and allow to air-dry.
3. Disintegrate nuclei structure by adding 20 µL of STE buffer and incubate at room temperature for 3 min.
4. To stretch the DNA fibers drop 200 µL of freshly prepared, ice-cold 3:1 fixative

onto the slide and air-dry. For further stretching of the DNA fibres tilt slowly the glass slide and allow the buffer to float downwards in 4–10 s.
5. Immerse slide in 3:1 fixative for 2 min at room temperature, air-dry and incubate the slides at 60°C for 1 h.
6. The prepared slides should be used for *in situ* hybridization as soon as possible.

The quality of the fibers prepared should be checked after DAPI-staining under the fluorescence microscope.

3.1.4. Probe Labeling

The most widely used approach is to label probes with reporter molecules (haptens). Two typical haptens are biotin and DIG, which can be incorporated as labeled nucleotides by nick-translation or by the polymerase chain reaction (PCR). Fluorochrome nucleotide analogues can be used for direct *in situ* hybridization experiments.

3.1.4.1. Biotin/DIG Incorporation by Nick-Translation

1. Into a microfuge tube place 1 µg of high-quality template DNA in a total volume of 16 µL of sterile, double-distilled water.
2. Add 4 µL of biotin/DIG-nick-translation mix (Roche), mix, and centrifuge briefly.
3. Incubate for 90 min at 15°C.
4. Stop the reaction by adding 1 µL of 0.5 *M* EDTA (pH 8.0) and heating to 65°C for 10 min.
5. Check the size of the products of nick translation by electrophoresis with a 1% agarose minigel using a small sample of the labeled probe. The optimal size of labeled nick-translation fragments is between approx 150 and 250 bp.
6. After labeling, the unincorporated nucleotides are removed using a QIAquick Nucleotide Removal Kit (Qiagen).
7. To precipitate the labeled probe add 1/10 vol 3 *M* sodium acetate and 2.5 vol cold 95% ethanol and leave for at least 30 min at –80°C or overnight at –20°C,
8. Centrifuge at 15,000*g* in a microfuge for 20 min, remove the supernatant, and wash the pellet by carefully adding 0.5 mL of ice-cold 70% ethanol and recentrifuging for 5 min.
9. Discard the supernatant and dry the pellet.
10. Dissolve the labeled DNA in 10–20 µL of water. The labeled probes are stable and can be stored for long periods of time at –20°C.

3.1.4.2. Labeling of DNA With DIG- or Biotin-Labeled Nucleotides by PCR

Where primers are available, a convenient labeling procedure is by PCR.

1. Reaction mixture is given for 100 µL of PCR mix. Add 50 ng of template DNA, 200 n*M* of each primer, 250 µ*M* of each nucleotide (10 µL of 2.5 m*M* dNTP mix containing DIG-11-dUTP [DIG labeling Mix, Roche], or biotin-11-dUTP); 10 µL of 10X PCR buffer, containing 1.5–5 m*M* Mg^{2+}, 2 U of Taq-polymerase, and water to 100 µL.

2. Perform PCR under the same conditions as for the amplification of unlabeled DNA fragments.
3. Stop the PCR, check the size of the PCR fragment by electrophoresis and proceed as described previously (**Subheading 3.1.4.1.**, **step 6**) for nick-translation.

3.1.5. In Situ *Hybridization to Chromosomes*

3.1.5.1. Probe Treatment Before Hybridization

1. Labeled repetitive or genomic DNA probes are denatured in hybridization solution. Per slide dissolve 20–50 ng of labeled probe together with 500 ng of precipitated sonicated salmon sperm DNA by adding 6 µL of deionized 100% formamide and 6 µL of 20% dextran sulfate in 4X SSC.
2. Denature the hybridization mixture at 80°C for 10 min and cool immediately on ice.

3.1.5.2. Pretreatment of Nuclei and Chromosomes on Slides

1. Wash slides three times for 5 min with 2X SSC.
2. Incubate with 100 µL of 100 µg/mL RNase A (in 2X SSC) under a piece of parafilm for 1 h at 37°C. Place in a sealable plastic box to maintain humidity and prevent evaporation.
3. Wash the slides in 2X SSC three times for 5 min in Coplin jars.
4. Dehydrate slides by incubation for 5 min in 70 and 100% ethanol in Coplin jars and allow the slides to air-dry.

3.1.5.3. Slide Denaturation and Hybridization

1. Denature the chromosomal DNA on the slides by immersion in 70% formamide containing 2X SSC at 70°C for 2 min. To prepare 100 mL: 70 mL of 100% formamide plus 10 mL of 20X SSC, plus 20 mL of water.
2. Quickly transfer the slides into a coplin jar with ice-cold 70% ethanol for 5 min and then dehydrate the slides in cold 100% ethanol for 3 min and air-dry.
3. Prewarm the slides to 37°C on a heating block and apply to each slide 12 µL of hybridization mixture containing denatured probe. Cover with a prewarmed cover slip, avoiding air pockets.
4. Seal the edges of the cover slips with rubber cement to minimize evaporation (*see* **Note 3**).
5. Hybridize at 37°C in a humid chamber containing absorbent paper soaked in 2X SSC to maintain humidity. Hybridization overnight is suggested.

3.1.5.4. Detection of Hybridized Probes

1. Remove rubber cement and cover slips using forceps and avoiding lateral movement of the cover slip.
2. Wash the slides in 2X SSC containing 50% formamide three times for 5 min at 42°C with agitation.
3. Further wash the slides 3X with 2X SSC at 42°C for 4 min each with agitation.
4. Apply 50 µL of blocking solution (3% BSA, 0.1% Tween-20, 4X SSC) to each slide. Place a piece of parafilm over the solution and incubate at 37°C for 30 min.

5. Gently peel off parafilm, tilt the slides and allow the blocking solution to drain.
6. Biotin- and DIG-labeled probes are detected with FITC-conjugated avidin (Vector Laboratories) and rhodamine conjugated anti-digoxigenin Fab fragment (Roche), respectively. Apply 50 µL of rhodamine conjugated anti-DIG antibody raised in sheep and/or of FITC-conjugated avidin (diluted in 1% BSA, 4X SSC, 0.2% Tween-20) to each slide and cover with parafilm. Incubate the slides in darkness for 1 h at 37°C in a humid chamber.
7. Remove the parafilm and wash the slides three times with 4X SSC, 0.1 % Tween-20 at 42°C for 5 min each with agitation. It is important to minimize exposure to light.
8. Apply 50 µL of rhodamine-labeled goat anti-sheep antibody for amplification of DIG signals and/or apply biotinylated anti-avidin for amplification of biotin-signals (diluted in 1%BSA, 4X SSC, 0.2% Tween-20) to each slide and cover with parafilm. Incubate the slides in darkness for 1 h at 37°C in a humid chamber. For amplification of biotin-signals an additional antibody step is necessary using FITC-conjugated avidin (diluted in 1% BSA, 4X SSC, 0.2% Tween-20).
9. Remove the parafilm and wash the slides three times with 4X SSC, 0.1% Tween-20, at 42°C for 5 min each with agitation.
10. Mount each slide in 50 µL of antifade solution containing 1 mg/mL DAPI and cover with a cover slip. DAPI is used for counterstaining. Apply gentle downward pressure to reduce the amount of solution between the cover slip and the slide before microscopic examination.
11. The specimens are now ready to be viewed under an epifluorescence microscope using appropriate filters. Use the DAPI filter (excitation wavelength 359 nm, emission wavelength 461 nm) to identify DAPI-stained chromatin first, and then switch to the FITC (excitation wavelength 490 nm, emission wavelength 525 nm) or rhodamine (excitation wavelength 540–560 nm, emission wavelength 580 nm) or dual filter to localize the FISH signals. Specialized equipment such as cooled sensitive charged-couple device (CCD) camera is recommend for fiber FISH. If a black and white CCD camera is used, microscope filters should be distinct for each fluorochrome and aligned to ensure an exact merging of digitized images

3.2. The Location of RNA Molecules in Plant Tissues

Standard practices for working with RNA should be observed until the posthybridization steps (**Subheading 3.2.3.3.**) in the protocol (*see* **Note 4**).

3.2.1. Preparation of Plant Tissue Sections

3.2.1.1. Day 1: Fixing of Plant Material

1. Collect plant tissue of interest into freshly prepared ice-cold fixative. Work as quickly as possible, keep tissue pieces small and collect into 25-mL fixative in 50 mL tubes, on ice. Apply vacuum (–75 kPa) to samples until PFA fixative starts to bubble. Hold vacuum for 15 min and release slowly. Repeat if necessary until tissue begins to sink (some tissue, such as flowers, will never sink using this method because of air spaces in the tissue).
2. Replace fixative with fresh fixative and shake gently overnight at 4°C.

3.2.1.2. Day 2: Dehydration

All the following steps are at 4°C or on ice with gentle shaking.

1. Rinse tissues twice for 5 min each in 1X PBS.
2. Dehydrate with an ethanol series of 30, 50, and 70% ethanol for 30 min each treatment. The tissue can now be stored under 70% ethanol for several days at 4°C or several months at –20°C.
3. At this stage, we transferred the tissue to embedding cassettes (Tissue-Tek®, Sakura Finetechnical, Tokyo, Japan) and loaded the cassettes into a Shandon Duplex tissue processor for automated paraffin embedding. Otherwise, continue dehydration in 85 and 95% ethanol at room temperature for 1 h each and store tissue in 0.1% (v/v) Eosin Y (Sigma; to visualize the tissue), 95% ethanol overnight at 4°C.

3.2.1.3. Days 3–8: Paraffin Infiltration

All the following steps are at room temperature with gentle shaking.

1. Replace the Eosin Y solution with 100% ethanol and wash for 1 h. Repeat the ethanol wash for 30 min and again if needed to remove all the Eosin from the solution.
2. Pass the tissue through 25% Histo-ClearII (National Diagnostics, Atlanta, GA), 75% ethanol for 30 min, 50% Histo-ClearII, 50% ethanol for 30 min, 75% Histo-ClearII, 25% ethanol for 30 min, and 100% Histo-ClearII twice for 1 h each.
3. Change the Histo-ClearII solution once more and transfer the solution and tissue to a 50-mL tube containing approx 1/5 of the volume of paraffin wax chips (Paraplast Plus, Tyco Healthcare, Mansfield, MA), leave to melt at 58°C overnight (no shaking). Place another bottle of Paraplast chips to melt overnight.
4. The next day, replace the wax/Histo-ClearII with freshly melted wax and incubate at 60°C overnight. Change the wax twice a day, with several hours between changes, for 3–5 d.
5. Warm molds (plastic or metal) in an oven, place them on a warm plate (50°C) and add a small amount of recently melted wax. Working quickly, use warm forceps to transfer the tissue pieces into the centre of the mold so that they stick to the partially set wax in the base. Fill with molten wax to the top and place the molds on a bench to solidify. Once the wax is about half solid, float the molds on an ice-water bath and dunk them when the wax has completely solidified.
6. Wrap and label the molds and store at 4°C until use.

3.2.1.4. Day 9: Sectioning

1. Cut sections 7- to 8-µm thick using a microtome. The sections should emerge as a continuous “ribbon” which can be collected onto a piece of black paper and individual sections separated if necessary.
2. Float the sections onto a 40°C water bath (diethylpyrocarbonate [DEPC]-treated water) using a wet paintbrush and allow to sit for several minutes, during which time the ribbon should flatten out.

3. Catch the sections onto a slide (*see* **Note 5**) by moving a submerged slide near the section and withdrawing slowly, the section should move onto the slide.
   Drain excess water from the slides on paper towel and leave on a slide warmer or in an oven for 42°C overnight. Store in a clean, dry box at room temperature until use (slides keep indefinitely).

3.2.2. Preparation of Probe

This method uses the DIG labeling system (Roche), with the label detected indirectly using immunocytochemistry. The normal precautions for working with RNA (*see* **Note 4**) should be observed.

3.2.2.1. In Vitro Transcription

1. Linearize clean plasmid template DNA at a site downstream of the cloned insert in a vector that includes T7 and SP6 promoter sequences to allow sense and antisense transcripts by the appropriate polymerases. Do not use restriction enzymes which generate 3' overhangs. Check that the digest is complete by agarose gel electrophoresis of a small sample, purify restricted plasmid DNA using standard phenol/chloroform techniques, precipitate with ethanol and dissolve to give a final concentration of 0.5 µg/µL.
2. Set up the following reactions on ice, using components of the DIG RNA labeling kit (SP6/T7) (Roche). There should be one sense and one antisense reaction per experiment. The sense probe is a negative control.
   a. 2 µL of purified template (1 µg)
   b. 2 µL of dNTP labeling mixture
   c. 2 µL of transcription buffer
   d. 1 µL of RNase inhibitor
   e. 11 µL of DEPC-treated H_2O
   f. 2 µL of RNA polymerase

   for a total of 20 µL. Incubate at 37°C for 2 h.
3. Add 2 µL of RNase-free DNase and incubate at 37°C for 15 min to remove DNA template. Add 2 µL of 0.2 *M* EDTA to stop the reaction.
4. Remove 0.5 µL of probe for a spot test to check the labeling reaction, and precipitate the remainder by the addition of 1 µL of transfer RNA (Sigma; 10 mg/mL), 2.5 µL of 4 *M* LiCl, and 75 µL of 100% ethanol. Incubate at –80°C for 30 min or –20°C for 2 h.
5. Centrifuge at 15,000*g* in a microfuge for 15 min at 4°C, remove the supernatant and wash the pellet by carefully adding 0.5 mL of ice-cold 70% ethanol and recentrifuging for 5 min. Discard the supernatant and dry the pellet under vacuum. Dissolve the labeled RNA in 100 µL of DEPC water. Remove a 50-µL sample for hydrolysis and store the remainder at –80°C.

3.2.2.2. Carbonate Hydrolysis

1. Calculate the optimal time for carbonate hydrolysis to generate probe lengths of about 150 bp, using:

$$\text{time (min)} = (Li - Lf)/(K \times Li \times Lf)$$

where Li = initial length of probe (kb, e.g., 1.0 kb); Lf = final length of probe (0.15 kb); and K = rate constant (0.11 kb/min). For the aforementionede example: $(1.0 - 0.15)/(0.11 \times 1.0 \times 0.15) = 51.5$ min.

2. To 50 µL of labeled probe RNA, add 30 µL of 0.2 *M* Na_2CO_3 and 20 µL of 0.2 *M* $NaHCO_3$ (both freshly made) and incubate for the calculated time at 60°C.
3. Stop the reaction by placing on ice and adding 3 µL of 3 *M* NaAc (pH 6.0) and 5 µL of 10% glacial acetic acid.
4. Add 8 µL of 3 *M* NaAc (pH 6.0), 1 µL of tRNA (10 mg/mL), 1.2 µL of 1 *M* $MgCl_2$, 250 µL of 100% ethanol, and precipitate the probe as per **Subheading 3.2.2.1.**, **step 5**. Resuspend the probe to give a final concentration of 100 ng/µL and store in small aliquots at –80°C.

3.2.2.3. Spot-Test to Check Probe Labeling

1. Spot 0.5 µL of DIG-labeled probe onto a small piece of nylon membrane (such as Hybond-N+; Amersham, Little Chalfont, Buckinghamshire, UK) and allow to dry. Include appropriate controls provided in DIG RNA labeling kit. The following steps are all carried out in small Petri dishes, with shaking.
2. Wash the membrane for 5 min in Buffer 1.
3. Wash for 30 min in freshly made blocking reagent, and then again in Buffer 1 twice for 5 min each.
4. Incubate in antibody conjugate (Anti-DIG-AP Fab fragments; Roche), diluted 1:2000 in Buffer 1, for 30 min.
5. Wash filter in Buffer 2 for 10 min.
6. Incubate (in the dark) in staining solution for 10–20 min, not shaking. Check frequently for staining and when developed, wash filter for 1 min in water and allow to air-dry. Use the intensity of the spots, compared to the positive control, to obtain a crude estimate of the amount of probe synthesized.

3.2.3. RNA In Situ *Hybridization of Plant Tissue Sections*

3.2.3.1. Prehybridization Treatments

Slides are placed in racks (we processed a rack of 20 slides at a time) and then into dishes (200-mL capacity) for the following treatments.

1. Wash twice for 10 min each in Histo-ClearII to remove paraffin wax.
2. Hydrate with an ethanol series of 100% (twice), 95, 90, 80, 60, 30% ethanol and water for 2 min each. Keep these solutions and dishes for later if possible.
3. Wash for 15 min in 2X SSC.
4. Incubate for 30 min at 37°C in proteinase K solution.
5. Incubate for 2 min in 2 mg/mL glycine (make up fresh in PBS).
6. Wash twice for 2 min each in PBS.
7. Incubate for 10 min in freshly made 4% PFA (in a fumehood).
8. Wash twice for 5 min each in PBS.
9. Incubate for 10 min in 0.1 *M* triethanolamine and acetic anhydride. This treatment neutralizes positive charges and aids in reducing background. Elevate slide

rack in container of freshly made 0.1 *M* triethanolamine with a stir bar. Just before adding the slides, dispense acetic anhydride (1.25 mL per 250 mL) into the triethanolamine, stir hard to mix and immediately add the slides (the half-life of acetic anhydride in aqueous solution is less than 1 min).

10. Wash twice for 5 min each in PBS.
11. Dehydrate with an ethanol series in 30, 60, 80, 90, 95, and 100% ethanol (twice) for 30 s each. Slides can be stored for a short time in a rack at 4°C with a little 100% ethanol in the bottom.

3.2.3.2. *In Situ* Hybridization

1. Air-dry the slides, separate them in clean, RNase-free racks and dry in the laminar flow until completely dry.
2. Make up an appropriate amount of hybridization solution, allowing 100 µL per slide. For eight slides:
   a. 100 µL of 10X *in situ* salts.
   b. 400 µL of deionized formamide.
   c. 200 µL of 50% dextran sulfate (warmed to aid pipetting).
   d. 20 µL of 50X Denhardts.
   e. 10 µL of transfer RNA (10 mg/mL).
   f. 70 µL of DEPC H_2O.
3. Warm the solution to 65°C and mix gently without forming bubbles.
4. Calculate the amount of probe required. It will probably be necessary to optimize the probe amount for each experiment, but begin with a concentration of 0.5 ng/µL/kb probe complexity. For example, for a 0.5-kb probe, need $0.5 \times 100 \times 0.5 =$ 25 ng probe/slide. The probe concentration may need to be varied up to 5 times higher or lower for optimal results.
5. Dilute the probe to 25 µL in 50% deionized formamide and denature at 80°C for 2 min. Spin down briefly and store on ice.
6. Mix the appropriate amount of diluted probe with the warmed hybridization solution, again without forming air bubbles, and apply to the slide, taking care not to scrape the tissue.
7. Cover with a siliconized cover slip or a piece of Parafilm and place slides in a sealed box containing absorbent paper soaked in 4X SSC to maintain humidity. Elevate the slides (we use pairs of glass pipets).
8. Incubate overnight at 50–55°C.

3.2.3.3. Posthybridization Treatments

All wash solutions should be prewarmed to the appropriate temperature before use.

1. Remove the cover slips by dipping the slides into a beaker containing 2X SSC at 55°C and letting them fall off.
2. Place the slides into a rack and into a dish of 0.2X SSC and agitate gently at 55°C for 45 min.

3. Repeat twice more with fresh 0.2X SSC.
4. Wash twice in NTE for 5 min each at 37°C with gentle agitation.
5. Wash in NTE containing 20 mg/mL of RNaseA for 30 min at 37°C with gentle agitation.
6. Wash twice in NTE for 5 min each at 37°C with gentle agitation.
7. Wash in 0.2X SSC containing 0.3% (v/v) Triton X-100 for 1 h at 55°C. At this point slides can be stored under PBS at 4°C overnight if desired.

3.2.3.4. Blocking and Detection

All subsequent steps are conducted at room temperature.

1. Place slides flat on an elevated platform in a humid box (as above) and add 200 µL of filtered 1% (w/v) blocking reagent (made according to Roche instructions) per slide. Leave for 30 min.
2. Drain off first block and add 200 µL of filtered 1% (w/v) BSA in TST. Leave for 30 min.
3. Drain off second block and add Anti-DIG-AP Fab fragments (Roche), diluted 1:100 in filtered 1% BSA (w/v) in TST, incubate in humid box 1 h.
4. Place the slides into a rack and into a dish of TST and wash with agitation, three times for 10 min each.
5. Wash slides for 10 min in detection buffer.
6. Wash slides for 10 min in detection buffer containing 1 m*M* levamisole (to inhibit endogenous AP activity).

3.2.3.5. Color Reaction

1. Place slides flat and add 500 µL (or enough to cover the slide) per slide of Western Blue (Promega) AP substrate, seal slides in a box and leave in the dark for color to develop.
2. Check color development periodically by draining the majority of the substrate solution and adding a cover slip to a test slide and examining the tissues under a microscope. Remove the cover slip, replace the solution and place the slide in the humid chamber if the reaction is incomplete. Color development should be completed within 16 h but could take up to 3 d, although an unsatisfactorily high background may result.
3. Stop the reaction by draining the slides and rinsing in 1X TE, then in water.

3.2.3.6. Dehydration and Mounting

1. Rapidly dehydrate the slides in an ethanol series of 30, 60, 85, 95, and 100% ethanol (twice). Keep the time in ethanol to a minimum (5–10 s), as the color product is slightly soluble in ethanol.
2. Let slides air-dry in a dust-free environment and mount in Entellen mounting medium (Merck). View under the light microscope and compare sense (control) and antisense (hybridizing) treatments for RNA expression.

4. Notes

1. Well-spread chromosome preparations with low amounts of cytoplasm give the best hybridization signals. Most FISH analyses have been made on mitotic root tip preparations and pollen mother cells at meiosis.
2. It may be convenient to fix/store whole flower buds at various stages of meiosis, then remove and identify anthers containing the required meiotic stages before the next stage (**Subheading 3.1.2.**, **step 2**). A single young anther, or often, all the anthers of a single flower bud, normally contains pollen mother cells at the same (synchronous) stage of meiosis. The size and color of the bud, although often difficult to determine accurately, may be the only clues to the progress of meiosis. Some plants, such as many of the grasses and cereals, have flowering spikes with a series of florets that exhibit a temporal sequence of maturity, and each floret, therefore, differs slightly in meiotic stage, which can be very useful in finding the best chromosomes for analysis. In addition, grasses often contain three meiotically synchronized anthers so the examination of one anther indicated the appropriateness of the other two for the *in situ* procedure. Variants of this approach may be useful in preparing meiotic chromosomes from a variety of different species, but only experience within a particular species will yield reliable results.
3. Some rubber cements work better than others.
4. All plasticware and glassware should be dedicated to RNA work, with plasticware new and glassware treated by dry heat sterilization or preferably by treatment with "RNaseZAP®" (Ambion, Austin, TX) and rinsing in DEPC-treated water. All solutions are treated with DEPC (0.05% v/v) and if not possible (e.g., compounds containing amine groups), made up with DEPC-treated water in RNase-free bottles. Gloves are to be worn and changed frequently, and the work conducted in a dedicated RNase-free area if possible.
5. We use Menzel SuperFrost Plus (Microm International, Walldorf, Germany), which are precleaned and charged.

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15

Histochemical Localization of Cell Proliferation Using *In Situ* Hybridization for Histone mRNA

Tim D. Hewitson, Kristen J. Kelynack, and Ian A. Darby

Summary

Monoclonal antibodies to proliferation associated antigens have long been used to histologically localize mitogenesis. However, techniques that distinguish cells in the synthetic or S phase have tended to rely on the in vivo incorporation of tritiated thymidine or thymidine analogs such as bromodeoxyuridine. The necessity to pulse with these labels before retrieving tissue means that they cannot be used in humans and are not available retrospectively. Measuring expression of histones serves as a useful adjunct to these techniques. As expression of histone proteins (H2A, H2B, H3, H4) are restricted to the synthetic phase of the cell cycle, hybridization for histone mRNA precisely distinguishes those cells in the S phase. Measuring their expression can easily be applied to the histological localization of proliferation, and can be used both prospectively and with archived tissue specimens. Several histone *in situ* hybridization probes and nonradioactive detection systems are now available commercially. A generalized protocol for their use in measuring *in situ* proliferation is provided in this chapter.

Key Words: Proliferation; mitogenesis; histone; cell cycle; *in situ* hybridization.

1. Introduction

Studies of cell kinetics are an important part of understanding clinical and experimental disease, with it now increasingly recognized that we must account for multiple processes, including cell proliferation, differentiation, and death. In this chapter, we present a protocol for using *in situ* hybridization to localize local proliferation. This method complements the *in situ* technique for assessing cell apoptosis provided elsewhere in this book.

For a number of years, monoclonal antibodies to nuclear proliferation associated antigens have been used successfully to localize cell proliferation (**Table 1**, reviewed in **ref. *1***). These antigens are cell cycle-associated proteins

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Table 1
Specificity of the Various Histochemical Markers Used to Determine Proliferation

Histochemical marker	*Specificity*
BrdU	S phase
Ki-67	G_1, S, G_2, M
PCNA	All phases: not cycle specific
Histones (H2A, H2B, H3, H4)	S phase

Histones, bromodeoxyuridine (BrdU), Ki67, and PCNA have different specificities and therefore measure different aspects of the cell cycle.

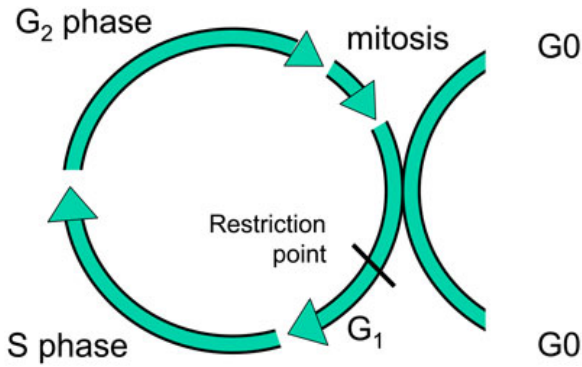

Fig 1. Schematic representation of the cell cycle and its various phases. Quiescent cells are in G_0 phase and re-enter the cell cycle at G_1. After passing the restriction point in late G_1, cells are committed to enter the S phase, where DNA replication occurs. In G_2, cells prepare for mitosis (M phase) where cells divide (derived from **ref. *1***).

and include proliferating cell nuclear antigen (PCNA) and Ki-67, both of which detect proliferating cells in G_1, S, G_2, and M phases (**Fig. 1** ***[1]***). However, techniques that distinguish cells in the synthetic or S phase have tended to rely on the in vivo incorporation of tritiated thymidine, or thymidine analogues such as bromodeoxyuridine ***(2)***. The necessity to pulse with these labels before retrieving tissue means that they cannot be used in humans and are not available retrospectively.

Histones are a class of highly abundant nuclear proteins that play a fundamental role in the packing and expression of the eukaryotic genome and are rapidly degraded when the S phase is completed (**Fig. 1**). Histones play a crucial part in the packing of very long DNA molecules into the limited space within the cell nucleus. Their role in DNA folding appears to be of great importance given the observation that not all DNA is packed in the same way.

Measuring expression of histones serves as a useful adjunct to other techniques measuring proliferation *(3–5)*. Because the expression of histone proteins (H2A, H2B, H3, H4) is restricted to the synthetic phase of the cell cycle, *in situ* hybridization for histone mRNA precisely distinguishes those cells in the S phase *(4,6)*, which are useful, for instance in evaluating G_1/S phase transition *(4)*. Furthermore, the use of *in situ* hybridization to localize mRNA expression means that we are detecting cytoplasmic labeling *(5)* (**Fig. 2A**,**B**). As other measures of cell proliferation use nuclear expression (**Fig. 2C**), *in situ* hybridization for histone H3 provides a unique opportunity to double label with other nuclear markers. Finally, the high degree of conservation among histone sequences between species allows these probes to be used for any mammalian tissue.

Several histone in situ hybridization probes and nonradioactive detection systems are now available commercially. These can easily be applied for localization of proliferation, and can be used both prospectively and with archived tissue specimens.

2. Materials

2.1. Tissue Pretreatment

1. Silanized slides.
2. Staining jars.
3. Pepsin (cat. no. P7000; Sigma, St. Louis, MO).
4. Hydrochloric acid, 0.2 *N*.
5. Heated water bath.
6. Distilled water.

2.2. Buffers

1. Tris-buffered Saline (TBS): 8.77 g of NaCl, 6.06 g of Tris (hydroxymethyl aminomethane) per liter of distilled water. Adjust pH to 7.4 before use.

2.3. Probes

A variety of probes for histones exist commercially. Perhaps the best known of these are a 550-base, fluorescein-labeled, single stranded cDNA sequence for Histone H3 (DAKO Cytomation, Glostrup, Denmark) and an oligonucleotide cocktail for detection of H2B, H3, and H4 mRNA sequences (Novocastra, Newcastle, UK). In both cases, the probes have been applied to the detection of histones in tissue sections or cell suspensions. Although these probes represent an example of two fundamentally different approaches in probe design, the incorporation of a fluorescein label provides for a common generic detection method.

To confirm specific hybridization, the use of parallel application of control probes is recommended. For example, an anti-sense Beta-actin cDNA probe

similar in length and labelling density is a suitable positive control, confirming the presence of hybridizable mRNA in the tissue section. Likewise, a random oligonucleotide is a suitable negative control to be run alongside oligonucleotides complementary for histone mRNA and is useful to determine the specificity of hybridization

2.4. Hybridization and Washes

1. Lint-free tissues.
2. Acid-washed siliconized or plastic cover slips.
3. Incubator.
4. Coplin jar.
5. Humidified incubation chamber.
6. Stringent wash solution (*see* **Note 1**).

2.5. Immunodetection (see *Note 2)*

1. Alkaline phosphatase conjugated Rabbit F(ab') anti-fluorescein isothiocyanate (FITC; Vector Laboratories, Burlingame, CA).
2. Antibody diluent (cat. no. S3022; DAKO).
3. Sigma Fast™ BCIP/NBT tablets (cat. no. B5655; Sigma).
4. Counterstain (e.g., methyl green, nuclear Fast Red).
5. Aquamount (BDH, Poole, UK) or equivalent aqueous mounting media.

3. Methods

The technique described here is applicable to the detection of FITC-labeled probes described previously. In both cases an alkaline phosphatase-conjugated antibody fragment is used to detect the fluorescein label incorporated in probe synthesis. The site of hybridization is then visualized by colorimetric reaction of the enzyme conjugate with its substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and the concomitant reduction of nitro blue tetrazolium (NBT). This reaction results in an insoluble blue-purple product at the site of hybridization (**Fig. 2A,B**).

3.1. Tissue Preparation (see *Note 3)*

3.1.1. Dewaxing Paraffin-Embedded Tissue

1. Collect paraformaldehyde (4% w/v) fixed, 2- to 5-µm thick tissue sections onto silanized glass slides and dry them in slide racks overnight at 40°C.
2. Dewax paraffin-embedded sections by placing them into staining jars containing histoclear (two changes for 5 min each), and then into 100, 75, and 50% alcohol for 2 min each.
3. Rinse slides in distilled water for 5 min.

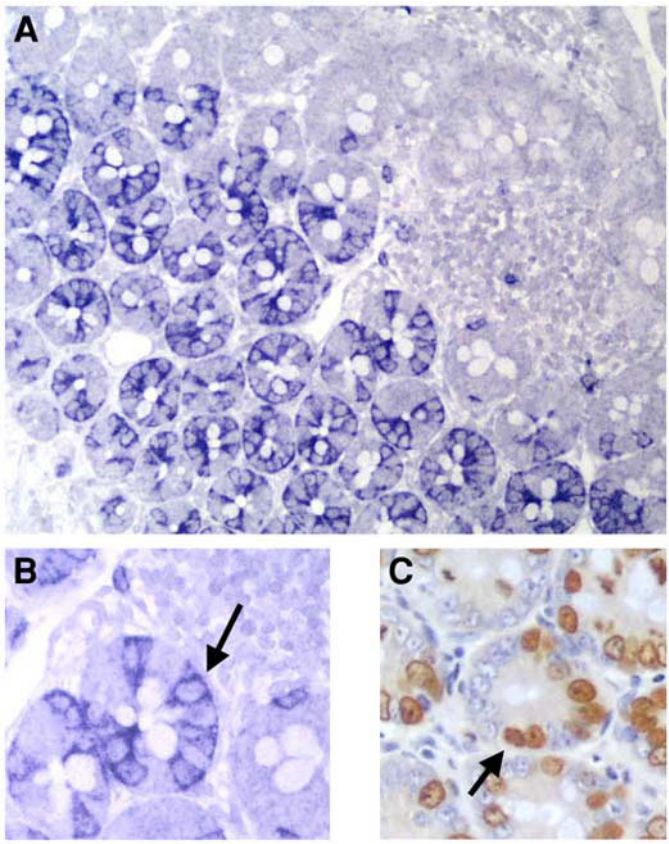

Fig. 2. **(A)** *In situ* hybridization for Histone H3 mRNA identifies rapidly proliferating cells in the crypt of rat intestine. **(B)** At higher magnification, the cytoplasmic localization of histone H3 mRNA expression (arrow) can clearly be seen. In each case *in situ* hybridization was performed with a FITC-labeled H3 histone cDNA that was detected immunohistochemically with an alkaline phosphate-labeled anti-FITC conjugate. **(C)** Nuclear immunohistochemical staining (arrow) for the thymidine analog BrdU. After injection of BrdU 4 h before sacrificing the animal, incorporation of BrdU was detected immunohistochemically with anti-BrdU, avidin biotin complex, and diaminobenzidine chromogen.

3.1.2. Pretreatment of Paraffin-Embedded Sections

1. Pretreat slides by incubating them in a prewarmed solution of 0.8% pepsin (Sigma) in 0.2 *N* hydrochloric acid at 37°C for 10 min.
2. Wash slides in two changes of distilled water for 5 min each.

3.1.3. Cryostat Sections and Cell Preparations

1. Cryostat sections from frozen tissue or cell preparations may also be used if collected on silanized slides and fixed in 100% cold acetone for 20 min.
2. Air-dry sections before proceeding (*see* **Note 4**).

3.2. Hybridization

1. Remove each slide from distilled water and wipe excess water from around the section using a clean tissue.
2. Add approx 20 to 50 µL of prediluted probe in hybridization solution to each specimen, and place a cover slip over the sample (*see* **Notes 5** and **6**).
3. Transfer slides to a prewarmed, humid chamber and incubate at 55°C for 2 h.

3.3. Posthybridization Wash (see Note 7)

1. Remove slides from humid chamber and gently wash them in a Coplin jar containing TBS for 5–10 min. Soak slides until cover slips are removed (*see* **Note 8**).
2. When cover slips have been removed, place slides in a staining rack and wash in fresh TBS for 2 min.
3. Immerse slides into prewarmed stringent wash solution (1X SSC) and incubate at 55°C for 30 min.
4. Remove slides and wipe excess liquid from around the sections.

3.4. Detection (see Note 2)

1. Place slides in a humidified staining chamber, apply anti-fluorescein- alkaline phosphatase conjugated ant-FITC reagent diluted in antibody diluent (Dako), and incubate at room temperature for 20 min (*see* **Note 9**).
2. Dissolve a single Sigma Fast™ BCIP/NBT tablet (Sigma) in 10 mL of distilled/deionized water. Vortex if necessary and use within 1 h.
3. Wash slides in fresh TBS for 5 min.
4. Remove slides from TBS, wipe excess liquid from around sections, and place them into staining chamber.
5. Add enough BCIP/NBT substrate (and incubate at room temperature for 90 min.
6. Wash slides in water for 5 min.
7. Counterstain nuclei using aqueous Methyl Green (or alternative) for 2–10 min at room temperature.
8. Wash slides in water for 5 min and mount sections using aqueous mounting medium.

3.5. Interpretation

Cells containing histone mRNA will show intense blue/black labeling of the cell cytoplasm under microscopic examination (**Fig. 2A,B**).

4. Notes

1. Stringent wash solution can consist of either a proprietary wash solution provided with commercial probes, or a 1/20 dilution of 20X SSC (3 *M* NaCl, 0.3 *M* Sodium citrate, pH 7.0).
2. Commercially available immunodetection kits may be substituted.
3. Always wear gloves and protective clothing when conducting the procedure. As RNase contamination is a primary concern, use autoclaved or decontaminated glassware whenever possible. Plastic equipment may be treated with 70% ethanol for 5 min and rinse with sterile water before use.
4. Cryostat sections generally do not require any pretreatment before commencing the hybridization procedure.
5. The exact amount of probe applied will depend on the size of the cover slip. Take care not to introduce bubbles under cover slips when applying them to the sections. This interferes with probe-target binding. It is important that sections remain hydrated throughout the hybridization procedure, as high background labeling is associated with sections that dry out.
6. In each case, the various histone probes available commercially are provided prediluted and ready to use.
7. A posthybridization wash is not part of the Novacastra protocol for *in situ* hybridization using the histone oligonucleotide cocktail.
8. When soaking slides in TBS to remove cover slips, do not use slide racks. Placing cover slipped slides in slide racks makes it more difficult to displace cover slips without damaging tissue sections.
9. Dilution will need to be empirically determined but a useful starting range is 1:100 to 1:1000. When applying detection reagents, use enough alkaline phosphatase anti-FITC to completely cover each tissue section.

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16

Histochemical Localization of Apoptosis With *In Situ* Labeling of Fragmented DNA

Tim D. Hewitson, Teresa Bisucci, and Ian A. Darby

Summary

Cell death by apoptosis is now recognized widely as an important constituent of cell turnover and disease pathology. Characterized by the cleavage of DNA into oligonucleosome-sized DNA fragments, end-labeling of fragmented DNA often is used as an *in situ* histological marker of apoptosis. The judicious and appropriate use of this technique therefore provides us with an important tool for assessing cell kinetics. Protocols for both terminal transferase-mediated UTP nick end-labeling, so-called TUNEL, and the combination of TUNEL with immunohistochemical staining are presented here, along with a discussion of its significance and interpretation.

Key Words: Apoptosis; TUNEL; fragmented DNA; cell death.

1. Introduction

Apoptosis is a morphologically distinct form of programmed cell death. It has a role in such processes as embryogenesis, immune regulation, and defense against viruses and also can be induced by a variety of physical and chemical stimuli *(1)*. Importantly, apoptosis leads to the safe removal of cells by phagocytosis, whereas in contrast, necrosis provokes tissue injury and inflammation. Apoptotic cell death is associated with a number of biochemical and morphological changes, including *de novo* gene expression, condensation of chromatin, and DNA degradation. Many attempts have been made to use these changes in the identification of apoptosis, which have met with varying degrees of success. Although a number of specific gene products are now known to be associated with apoptosis *(2)*, histochemical studies still rely on the specific morphological changes that occur during this process. These changes have been defined as the sequential condensation of nuclear chromatin, the formation of

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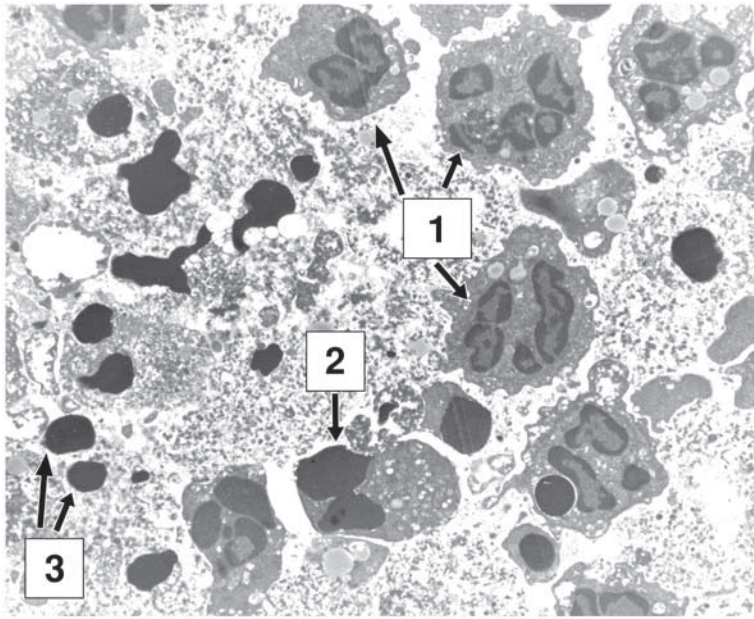

Fig. 1. Electron micrograph of polymorphonuclear granulocyte apoptosis in a rat model of experimental renal infection. Resolution of tubulointerstitial nephritis occurs through apoptosis of inflammatory cells and drainage of apoptotic cells through the tubular lumen. The various stages of polymorph apoptosis are labeled 1–3. Several normal polymorphs are visible with irregular outlines and granular appearance of nuclear chromatin **(1)**. The increased electron density of chromatin can be seen in the early stages of budding **(2)**, whereas numerous apoptotic bodies **(3)** are distributed throughout the lumen.

membrane-bound cell fragments termed apoptotic bodies and, finally, the engulfment by professional and recruited phagocytes. Although these criteria are easily recognized in vivo by electron microscopy (**Fig. 1**), the infrequence of its occurrence makes its quantitation by this means problematic. Activation of endonuclease activity during the process of cell death ***(3)*** causes DNA fragmentation in apoptotic cells, which produces a characteristic ladder of oligonucleosome-sized DNA fragments on agarose gel electrophoresis. Although applicable to pure cell populations, gel electrophoresis is difficult to apply to *in situ* studies with mixed cell populations.

However, the presence of DNA fragmentation has now been adapted to the in vivo identification of apoptosis. Apoptotic cells may be localized by the *in situ* labeling of this fragmented DNA using terminal transferase-mediated UTP nick end-labeling (TUNEL ***[4]***). In this technique, labeled dUTP is attached to

the 3' end of these breaks by terminal transferase (TdT) and is then detected using immunohistochemical techniques. TUNEL has been used successfully to study apoptosis in a diverse range of biological systems including, among others, developmental biology ***(5)***, immunoselection ***(6)***, wound healing ***(7)***, and glomerulonephritis ***(8)***.

However, it is important to remember that DNA fragmentation is not confined to apoptosis exclusively. Fragmentation of DNA also is found in the late stages of necrosis, although the nuclear floculation and diffuse labeling pattern results in differences in histological appearance. Several in vitro studies have, however, confirmed that end-labeling of fragmented DNA correlates with the incidence of apoptosis, as measured by other parameters ***(9)***. Furthermore, some data exist to suggest that single-strand DNA breaks, as occur in necrosis, are labeled less easily that the double stranded fragmentation in apoptosis ***(10)***. In summary, despite the limitations of the TUNEL method, the judicious use of this technique may aid considerably in the identification and quantitation of apoptosis *in situ*.

2. Materials

2.1. TUNEL on Paraffin-Embedded Tissue

1. Paraffin-embedded sections of 10% neutral-buffered formalin or 4% paraformaldehyde fixed tissue collected onto 3'-aminopropyltriethoxysilane (Sigma, St. Louis, MO)-coated slides (*see* **Notes 1** and **2**). A positive control tissue section should be included to verify that the TUNEL reaction has worked (*see* **Note 3**).
2. Proteinase K (Sigma): stock solution of 20 mg/mL made up in distilled water and stored at –20°C.
3. Biotin-labeled dUTP (Roche Diagnostics, Penzberg, Germany).
4. Terminal deoxynucleotidyl transferase (TdT; 25 U/µL; Roche).
5. Buffer 1: sodium chloride. (30 mL of 5 *M* stock), sodium citrate (15 mL of 1 *M* stock), and distilled water added to a total volume of 500 mL (final composition 0.3 *M* sodium chloride, 0.03 *M* sodium citrate).
6. Tris/ethylene diamine tetraacetic acid (EDTA) (TE) buffer: Tris-HCl, pH 7.4 (0.5 mL of 2 *M* stock); EDTA, pH 8.0 (20 µL of 0.5 *M* stock); and distilled water to 100 mL (final composition 0.01 *M* Tris, 0.0001 *M* EDTA).
7. TdT buffer: 15.0 g of sodium cacodylate, 0. 119 g of cobalt chloride, 15 mL of 1 *M* stock Tris-HCl, and distilled water added to a total volume of 500 mL (final composition: 140 m*M* cacodylate, 1 m*M* cobalt chloride, 30 m*M* Tris).
8. Phosphate-buffered saline (PBS), pH 7.2.
9. Vector ABC Elite staining kit (Vector laboratories, Burlingame, CA).
10. Chromogen substrate: 4 mL of 3.3'-diaminobenzidine tetrahydrochloride (DAB; DAKO) mixed with 3 µL of 30% H_2O_2. An equivalent commercial preparation (e.g., metal-enhanced diaminobenzidine [DAB], Pierce, Rockford, IL) may be substituted.

11. Humidified staining tray.
12. Microscope.
13. Staining racks and baths.
14. Wax pen (DAKO or equivalent).
15. Harris hematoxylin.
16. Scott's tap water: 20 g of $MgSO_4 \cdot 7H_2O$, 3.5 g of $NaHCO_3$, made up to 1 L with distilled water (final concentrations: 0.081 *M* $MgSO_4$, 0.041 *M* $NaHCO_3$).
17. DNase I (Sigma).
18. Absolute ethanol.
19. Xylene.
20. 0.45-µm Syringe filter.
21. Depex (BDH, Poole, UK) or other nonaqueous mounting media.

2.2. Additional Materials for Combined Immunohistochemistry and TUNEL

1. Tris-buffered saline (TBS), pH 7.4: 25 m*M* Tris base, 0.9% NaCl, adjust pH to 7.4 with HCI.
2. Primary cell specific antisera (e.g., leukocyte marker).
3. Alkaline phosphatase anti-alkaline phosphatase complex (DAKO).
4. Alkaline phosphatase conjugated anti-IgG serum (species specific for primary antiserum; DAKO).
5. Fast red substrate (Sigma).
6. Aquamount (BDH) or other aqueous mounting media.

3. Methods

3.1. TUNEL Protocol

1. Heat-paraffin-embedded tissue sections in a 60°C oven for 10 min.
2. Dewax tissue sections by transferring slides immediately to a xylene bath for 5 min.
3. Rehydate tissue in graded (100, 75, 50%) alcohols and wash in distilled water for 5 min.
4. Digest sections by treatment with proteinase K (1 µL of 20 mg/mL stock in 1000 µL of TE buffer) for 15 min at room temperature (*see* **Note 4**).
5. Wash tissue sections in TdT buffer for 5 min.
6. Outline the section with a wax pen to create a hydrophobic barrier around the tissue section, thereby reducing the volume of reagents required.
7. Incubate sections at 37°C with 1 µL of biotin-labeled dUTP and 1 µL of TdT in 100 µL of TdT buffer for 30 min in a humid atmosphere.
8. Wash in buffer I to terminate the reaction.
9. Detect *in situ* incorporation of biotinylated dUTP by incubating sections with biotin horseradish peroxidase complex (ABC Elite kit) for 15 min or according to the manufacturer's instructions.
10. Wash in PBS twice for 5 min.

11. Develop reaction product by staining with DAB/H_2O_2 substrate for 3–7 min at room temperature. Most DAB solutions made from DAB in powder or tablet form should be filtered through a 0.45-µm syringe filter prior to use. Monitor DAB/H_2O_2 on wet slides using a microscope with a low-power (×10) objective.
12. Terminate the staining by rinsing in distilled water when the ratio of positive staining of apoptotic nuclei to background staining is maximal.
13. Wash in distilled water (5 min).
14. Counterstain sections with Harris' hematoxylin (1–2 min depending on strength of hematoxylin), rinse in tap water, dip briefly in Scott's tap water (three times) until the sections are visibly "blued," and wash in tap water for 5 min.
15. Finally, dehydrate in graded (50, 75, 100%) alcohols, rinse in xylene, mount with Depex (BDH), and examine by light microscope (*see* **Notes 6** and **7**).

3.2. Combined Immunohistochemistry and TUNEL Protocol

The TUNEL technique can be combined with immunohistochemistry for cytoplasmic or surface markers to identify apoptotic cell phenotype ***(6)*** and/or phagocytosis of apoptotic bodies ***(11)***.

1. Follow the TUNEL protocol in **Subheading 3.1.** for **steps 1–11** inclusive.
2. Wash in TBS (*see* **Note 5**) twice for 3 min (*see* **Note 8**).
3. Incubate with primary antiserum for 1 h at room temperature in a humidified container (determine appropriate dilution empirically).
4. Wash sections in TBS twice for 3 min.
5. Incubate for 20 min with alkaline phosphatase conjugated anti-IgG serum (species specific for primary antiserum; 1:50 dilution in TBS).
6. Wash in TBS twice for 3 min.
7. Incubate for 20 min with alkaline phosphatase anti-alkaline phosphatase complex (1:50 dilution in TBS).
8. Wash in TBS (3 min, twice).
9. Detect alkaline phosphatase reaction product by incubation with fast red substrate for 10–20 min at room temperature. Monitor substrate reaction microscopically, terminating the reaction by washing in distilled water for 5 min.
10. Counterstain sections as described previously.
11. Mount sections in Aquamount (BDH; *see* **Note 9**), and examine by light microscope.

4. Notes

1. Fixatives: 4% paraformaldehyde or paraformaldehyde-lysine-periodate may be substituted for neutral-buffered formalin. Fixatives that denature DNA (e.g., mercuric formalin) may result in labeling of nuclei and are therefore unsuitable for TUNEL. False-positive labeling is an artifact associated with any delay between retrieval of tissue and fixation.
2. 3'-Aninopropyltriethoxysilane (APES) coating of slides: pretreatment of microscope slides with APES prevents sections falling off during protease digestion.

Importantly, it also avoids having to "bake" sections in a hot oven, as is often routinely used in histology laboratories to ensure adherence of the tissue. APES-coated slides can be prepared by sequentially washing microscope slides in: (1) dilute laboratory detergent overnight, (2) running tap water for 3 h, (3) distilled water twice for 5 min, and (4) 95% alcohol twice for 5 min. Slides are then air dried before being dipped in a freshly prepared 2% solution of APES (Sigma) in 100% acetone (BDH) for 10 s. Slides are then washed in: (1) acetone twice for 5 min and (2) distilled water twice for 5 min and air dried at 40°C in a hot air oven for 12 h. Slides may be stored at room temperature in a dust free container until use.

3. Controls: positive controls consist of TUNEL labeling of rat ovary sections and of tissue sections pretreated with DNase I at a concentration of 1 µg/mL TBS for 5 min. TUNEL labeling of tissue sections in the absence of TdT is used as a negative control. DNase digestion results in positive labeling of all nuclei *(4)*, whereas the TUNEL reaction in the absence of TdT leaves all nuclei unlabeled. Sections of small intestine may not be a suitable positive control. Despite Gavrieli et al. *(4)* describing labeling of the intestinal villus in their original description of the TUNEL method, doubts have been raised about the specificity of this reaction *(9)*.
4. Digestion: optimal concentration and time of protease digestion may have to be established empirically for individual tissues. Microwave treatment has been substituted for protease digestion by some investigators *(12)*. Overdigestion using proteinase K may result in false-positive results; therefore, digestion times need to be established for each tissue.
5. Tris-buffered saline is used in all washes in the double labeling procedure as phosphate-buffered saline is incompatible with subsequent alkaline phosphatase detection.
6. Interpretation: the definition of apoptosis is still essentially based on morphological criteria. The presence of apoptosis may therefore need to be substantiated by electron microscopy (**Fig. 1**) or the identification of morphological features in TUNEL-positive cells. Apoptotic cells are often recognizable on light microscopy as condensed TUNEL positive chromatin lying within a "halo" of what was the cell cytoplasm (**Fig. 2**). Such a phenomenon is presumably attributable to the contraction of cell cytoplasm in combination with the rapid kinetics of apoptosis.
7. Quantitation: Apoptotic cells are usually expressed as a proportion (%) of total cell number or as the number of apoptotic cells per unit area (e.g., 10×0.25-µm fields). Kinetic studies have suggested that apoptotic cells are histologically recognizable for 0.5–2.0 h before clearance *(8)*. Identification of a low rate of apoptosis, therefore, still indicates the removal of a significant proportion of the cell population. For example, if apoptotic bodies are visible for 2 h, a 1% incidence of visible apoptosis may represent clearance of up to 12% of cells within 24 h. This study highlights that for statistical validity it is necessary to examine sufficient nuclei (at least 1000) when enumerating the incidence of apoptosis.
8. *In situ* end-labeling, as described by Wijsman et al. *(13)*, is a variant of the above

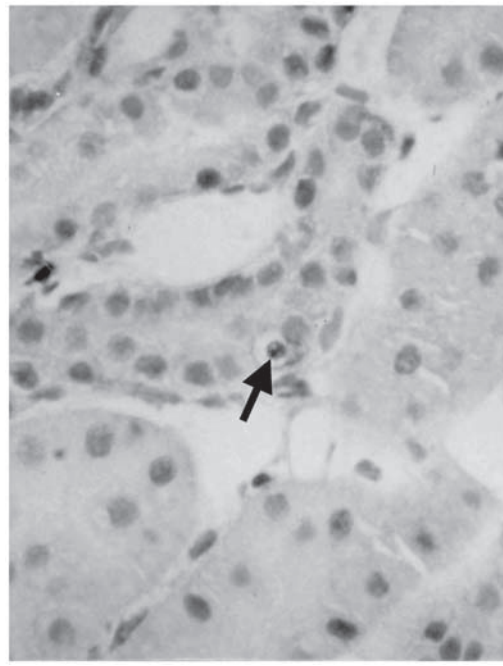

Fig. 2. End-labeling of fragmented DNA in a rat tubular epithelial cell. Condensation of nuclear chromatin (arrow) is easily observed. The rapid kinetics of apoptosis frequently result in the formation of "halos" around apoptotic cells.

technique where the enzyme Klenow DNA polymerase I is substituted for TdT. There is some evidence that TdT is preferable to DNA polymerase because more favourable kinetics are reflected in shorter incubation times ***(14)***.

9. Avoid section dehydration because it will remove fast red reaction product.

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17

The Use of Combined Immunohistochemical Labeling and *In Situ* Hybridization to Colocalize mRNA and Protein in Tissue Sections

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Summary

This chapter explores the combination of a nonradioactive *in situ* hybridization technique to detect mRNA with an imunnohistochemical labeling technique for use in formalin-fixed, paraffin-embedded, or frozen tissue sections. This technique allows the combination of detecting mRNA by *in situ* hybridization with immunohistochemical detection of a protein product or a cell surface marker without using any radioactive procedures. This technique is ideal for use on tissue sections when the aim is to identify which cells are producing a secreted protein product, such as a cytokine.

Key Words: mRNA; secreted protein; cytokine; cell adhesion molecules; cell surface markers; frozen tissue sections; formalin-fixed; paraffin-embedded tissue sections.

1. Introduction

The development of sensitive techniques to detect biologically relevant proteins at the mRNA and protein levels has been a major research tool in basic and applied biomedical research *(1–6)*. The combination of these two techniques has been particularly valuable when the biological protein being studied is a secreted cell product that has the ability to bind to components of the extracellular matrix or to receptors on target cells within the same tissue *(4,5,7–9)*. The best example of this is the study of cytokine production in human tissue sections, in which standard immunohistochemical labeling techniques will detect cytokine production, secreted cytokine and cytokine bound to receptors on target cells *(4,5,9)*. If it is important to determine which of the cellular components of the tissue under study is producing the biological protein, the combination of *in situ* hybridization to detect mRNA and immunohis-

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tochemical labeling to detect the protein offers the ability to distinguish between the cells responsible for production of and the target cells for the biological protein.

We describe here a method that combines *in situ* hybridization and immunohistochemical labeling of tissue sections *(8)*. The technique uses nonradioactive methods, which avoids the use of radioactivity, allows a permanent recording of mRNA detection with colorimetric techniques, a more precise localization of mRNA within cells than radioactive methods, and greater speed and efficiency compared with radioactive methods *(10,11)*. The use of riboprobes with the higher affinity and thermal stability of RNA–RNA hybrids compared with RNA–DNA hybrids avoids the problems of competitive hybridization to the complementary strand of nick-translated cDNA probes and permits higher stringency washing conditions, resulting in reduced nonspecific background hybridization signals *(8)*. This technique therefore offers several advantages over previous techniques, including the ability to use archival, paraffin-embedded sections, the use of a nonradioactive label for *in situ* hybridization, the inclusion of an amplification step to enhance the sensitivity of *in situ* hybridization, and the ability to vary the color end products in the immunohistochemical detection steps as determined the particular requirements of the tissue sections and the research plan.

2. Materials

2.1. Immunoperoxidase Technique

1. 1 *M* Tris-HCl Buffer (BDH chemical, Poole, UK); pH to 8.0 and sterilize before use.
2. 5X phosphate-buffered saline (PBS) buffer: 45 g of NaCl, 15.2 g of sodium hydrogen phosphate Tris (BDH), and 3.93 g of sodium dihydrogen phosphate Tris (BDH). Dissolve in 1 L of distilled water.
3. 0.05 *M* Tris-1X PBS buffer, pH 7.5: 50 mL of 1 *M* Tris-HCl buffer, pH 8.0; 200 mL of 5X PBS buffer. Make up to 800 mL with distilled water and pH to 7.5 using HCl. Add distilled water to 1 L. Autoclave before use.
4. Endogenous Peroxidase Block: 3% (v/v) hydrogen peroxide in methanol.
5. Proteinase K solution: dilute proteinase K (Roche Diagnostics, Penzberg, Germany) stock (5 mg/mL) to the desired concentration using proteinase K Buffer (*see* **Note 1**).
6. Proteinase K Buffer: dissolve 0.185 g of ethylene diamine tetraacetic acid (EDTA) in 100 mL of 1X PBS. Autoclave before use.
7. Normal serum: 20% normal donkey serum (Jackson Immuno Research, Avondale, PA) in 0.05 *M* Tris–1X PBS buffer, pH 7.5.
8. Primary antibody: dilute primary antibody in 0.05 *M* Tris–1X PBS buffer, pH 7.5. The dilution must include 10% normal serum and 1/200 of 1X PBS–0.1% thimerosal (Sigma, St. Louis, MO).

9. Secondary antibody (linking antibody): biotinylated donkey anti-mouse antibody (Jackson Immuno Research): use 1/200 dilution in 0.05 *M* Tris–1X PBS buffer, pH 7.5, plus 5% normal serum in solution.
10. ABC complex (Standard Elite ABC Kit;Vector Laboratories, Burlingame, CA): it must be made 30 min before use. 10 µL of Bottle A (Avidin), 10 µL of Bottle B (biotinylated horseradish peroxidase), and 1000 µL of 0.05 *M* Tris–1X PBS buffer, pH 7.5.
11. 3,3'-diaminobenzidine tetrachloride (DAB, Sigma) Solution: Add 500 µL of DAB (10 mg/mL) to 4.5 mL of 0.05 *M* Tris–1X PBS buffer, pH 7.5, and add 2 µL of hydrogen peroxide just before use (*see* **Note 2**).

2.2. APAAP Technique

1. Tris-buffered saline (TBS): 0.02 *M* Tris-HCl, 0.15 *M* NaCl, pH 7.6.
2. 20X TBS: 90 g of NaCl, 200 mL of 1 *M* Tris-HCl, pH 8.0, distilled water to 500 mL, and pH to 7.6. Autoclave before use.
3. 1 *M* Tris HCl buffer, pH 8.0: 60.57 g of Tris in 500 mL of distilled water. Adjust to pH 8.0. Autoclave before use.
4. Normal serum: 10% (v/v) normal sheep serum (Jackson Immuno Research) in TBS.
5. Linking antibody (Ab): 1/200 dilution sheep anti-mouse in TBS plus 10% normal sheep serum. The linking Ab can be stored in 4°C in 1/10 dilution with 1X PBS + 0.1% thimerosal.
6. APAAP Complex (Roche Diagnostics): use 1/50 dilution in TBS.
7. Fast Red Substrate: dissolve 5 mg of naphthol AS-MX phosphate (Sigma) in 200 µL dimethyl formamide (BDH). Add 10 mL of 0.2 *M* Tris-HCl buffer, pH 8.0, and 2 mg of levamisole (Sigma), and finally add 10 mg of Fast Red TR Salt (Sigma). Filter substrate for immunohistochemistry (*see* **Note 3**).

2.3. In Situ *Hybridization*

1. 0.1 *M* Tris-HCl–0.2 *M* glycine solution pH 7.2: 1.2114 g of Tris, 1.5014 g of glycine. Dissolve in 100 mL of distilled water and adjust the pH to 7.2 with concentrated HCl. Autoclave and store at 4°C.
2. 20X standard saline citrate (SSC) solution: 175 g of NaCl, 88 g of Na citrate dihydrate. Make up to 1000 mL in distilled water and pH to 7.0.
3. Prehybridization solution: 10% dextran sulfate (500,000), 0.05% polyvinylpyrrolidone (PVP), 20X SSC, 50% deionized formamide, 0.05% Triton X-100, 500 µg/mL Herring Sperm DNA (Roche Diagnostics). Dissolve the dry ingredients in 20X SSC, with manual stirring, then add formamide and Triton X-100. Boil the herring sperm DNA for 10 min and chill on ice before adding to the solution. Store the solution in –20°C.
4. Nail polish with enough acetone added to allow free flow from a Pasteur pipet.
5. Acid cleaned slides and coverslips: place slides or cover slips in Petri dish with 2.0 *N* HCl for 20–30 min. Pick up slides or cover slips with clean forceps and place in 70% alcohol for a few minutes before drying on paper towel.

2.4. Digoxigenin Detection

1. Hybridization Buffer I solution: 0.1 *M* Tris-HCl, 0.15 *M* NaCl, pH 7.5.
2. Protein block: 10% normal sheep serum in Hybridization Buffer I.
3. Anti-digoxigenin (DIG)-Alkaline phosphatase (anti-DIG-AP, Roche Diagnostics), sheep antibody, used in 1:500 dilution with 10% normal sheep serum and hybridization Buffer I as diluent.
4. Hybridization Buffer III: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 0.05 *M* $MgCl_2$, pH 9.5.
5. Nitro blue tetrazolium (NBT, Roche Diagnostics) substrate: 80 µL of dimethylformamide, 135 µL of NBT solution (7.5 mg NBT in 300 µL of distilled water), 105 µL of X-P (5-bromo-5chloro-3-indolyl-phosphate, Roche Diagnostics) solution (5.0 mg X-P in 300 µL of distilled water), 2.4 mg of levamisole in 10 mL of Hybridization Buffer III (*see* **Note 4**).

3. Methods

3.1. Preparation of Tissue Sections

3.1.1. Frozen Sections

1. Cut 4- to 6-µ thick sections on a cryostat.
2. Place sections on acid cleaned slides coated with 2% 3-amino propyl triethoxy silane (APTS, Sigma; *see* **Note 5**).
3. Fix in acetone for 10 min at 4°C, air-dry, and use immediately or store at –20°C, individually wrapped in aluminium foil.
4. Wash sections in Tris-PBS before use (*see* **Note 6**).

3.1.2. Formalin-Fixed, Paraffin-Embedded Sections

1. Cut 4-µ thick sections on a microtome and place onto acid cleaned APTS-coated slides.
2. De-wax sections in xylene and rehydrate in 100, 90, 80, and 70% alcohol.
3. Place sections in Tris-PBS before use (*see* **Note 6**).

3.2. Generation of Riboprobe

1. Restrict cDNAs to a size of equal to or less than 2000 bp within the coding sequence, using appropriate restriction endonucleases (*see* **Note 7**).
2. Insert cDNA into an appropriate vector, e.g., SP72, pGEM 3, pGEM 7z- (Promega, Madison, WI).
3. Transform an appropriate strain of *Escherichia coli* using the calcium chloride procedure.
4. Select colonies, grow up bacteria, and make plasmid DNA minipreparations.
5. Check the suitability of selected clones by restriction endonuclease digests of plasmid DNA to confirm the presence of the correct sized insert.
6. Make large scale preparation of plasmid DNA using a Maxiprep kit (Qiagen, Chatsworth, CA).
7. Recheck cDNA by restriction endonuclease digests to confirm the presence of the correct insert.

8. cDNA fragments cloned into the plasmid vector were linearized using appropriate restriction endonucleases in such a manner that the cDNA insert could be transcribed into an RNA copy from either the SP6 or T7 RNA polymerase promoters without including any vector sequence.
9. Generate riboprobes using a commercial riboprobe generation kit containing either biotin-labeled UTP (Gibco-BRL, Glen Waverley, Victoria, Australia) or DIG-labeled UTP (Roche Diagnostics) as part of the nucleotide mix. This resulted in biotin- or digoxigenin-labeled riboprobes which were a copy of either the sense or antisense strand of the cDNA (*see* **Note 8**).
10. Remove template DNA with an RNase free DNase (Roche Diagnostics) and unincorporated nucleotides by passage through affinity columns (Qiagen).
11. Precipitate riboprobes in 2 *M* $LiCl_2$ in 100% ethanol and resuspend in diethylpyrocarbonate (DEPC)-treated water.
12. Confirm labeling with biotin or DIG by dot-blot hybridization and appropriate size by Northern gel analysis (*see* **Note 9**).

3.3. In Situ *Hybridization*

1. Place slides in 0.2 *N* HCl for 20 min at room temperature. Rinse sections in DEPC-treated water for 5 min in two changes (*see* **Note 6**).
2. Digest slides with 50 µg/mL proteinase K (Roche Diagnostics) for 30 min at 37°C. Rinse sections in DEPC-treated water for 5 min in two changes (*see* **Note 1**).
3. Incubate sections with 0.1 *M* Tris-HCl 0.2 *M* glycine solution (pH 7.2) for 10 min at room temperature followed by rinsing in DEPC-treated water.
4. Rinse sections in 2X SSC solution for 5 min (two changes).
5. Cover sections in prehybridization buffer and incubate for 90–120 min at 50°C.
6. Remove the prehybridization solution and replace with the desired concentration of riboprobe in prehybridization buffer.
7. Cover each section with an acid-cleaned cover slip, seal with nail polish and incubate overnight at 50°C (*see* **Note 10**).
8. Remove cover slips with a scalpel blade. Wash slides twice in 2X SSC solution for 10 min, 1X SSC solution for 10 min, and 0.1X SSC solution for 15 min at 45°C, in 0.1X SSC for 5 min at room temperature, and finally in water for 5 min at room temperature. The sections were then ready for the detection of biotin or DIG.

3.4. Detection of In Situ *Hybridization*

3.4.1. Biotin Detection System

1. Place sections in endogenous peroxidase blocking solution for 10 min at room temperature, washed in water followed by Tris–PBS buffer wash for 5 min.
2. Remove sections from Tris–PBS, drain, and incubate with 20% normal serum (30 min, room temperature) to block nonspecific protein binding.
3. Incubate sections with anti-biotin antibody (Roche Diagnostics) 1:100 dilution containing 10% normal donkey serum (Jackson Immuno Research) for 45 min at room temperature (*see* **Note 11**).

4. Wash sections in Tris–PBS then incubate with a biotin-labeled antimouse immunoglobulin (Roche Diagnostics) 1:200 dilution containing 10% normal donkey serum for 30 min.
5. Wash sections in Tris–PBS then incubate with ABC complex for 30–60 min.
6. Wash sections in Tris–PBS, then incubate with a diaminobenzidine solution for 5 min.
7. Wash sections in Tris–PBS (*see* **Note 12**).

3.4.2. DIG Detection System

1. Wash slides in Hybridization Buffer I solution for 5 min.
2. Block nonspecific binding by incubating the slides in 10% normal sheep serum (Jackson Immuno Research) diluted in Hybridization Buffer I for 45 min.
3. Incubate sections with anti-DIG alkaline phosphatase-labeled antibody (anti-DIG AP, Roche Diagnostics) in 1:500 dilution in hybridization buffer containing 10% normal sheep serum.
4. Wash sections with Hybridization Buffer I and equilibrate in Hybridization Buffer Solution III for 2 min.
5. Incubate sections with the color substrate, NBT (Roche Diagnostics) in the dark for 1–4 h. Stop color development by washing slides in water (*see* **Note 13**).

3.5. Immunohistochemical Labeling for Protein

1. Cover with normal sheep serum (Jackson Immuno Research) for 2 h to block nonspecific binding then wash in TBS buffer.
2. Incubate sections overnight at room temperature with the primary antibody (mouse IgG).
3. Wash sections in TBS buffer the incubate with secondary sheep anti-mouse antibody (Roche Diagnostics, Castle Hill, NSW) for 60 min.
4. Wash sections in TBS buffer, then incubate with alkaline phosphatase anti-alkaline phosphatase complex (APAAP complex; Roche Diagnostics, Castle Hill, NSW) for 60 min at room temperature.
5. Wash sections in TBS buffer wash then apply a chromogenic substrate, e.g., Fast Red (Sigma) to the sections for 15 min.
6. Stop the color development was stopped by washing in water.
7. Counter-stain the sections with dilute hematoxylin and mount in Aquamount (BDH, Poole, UK; *see* **Note 14**).

4. Notes

1. Proteinase K is necessary to expose epitopes in formalin-fixed tissues and to permeabilize tissue to allow access to the riboprobes with the *in situ* hybridization step. Alternatives include Pepsin digestion and microwave retrieval steps which, in our hands, are less reliable than the use of proteinase K digestion.
2. Although silver enhancement of the DAB reagent can be used to produce a darker stain, it adds little to the procedure.

3. Other color substrates can be used to produce a blue, green, or yellow color product, but the combination of a brown *in situ* hybridization product with a red immunohistochemical reaction product gave the best color combination when both procedures were combined on the same tissue section.
4. It is possible to vary the color end product of the DIG-labeling system, including using an anti-digoxigenin antibody conjugated to horseradish peroxidase to convert to a DAB end product.
5. Coating of slides with APTS ***(12)***, rather than poly-L-Lysine, is necessary to avoid loss of part or all of the tissue section, especially when a combination of *in situ* hybridization and an immunohistochemical reaction is performed on the same tissue section.
6. Although DEPC treatment is not necessary in most steps, because of the inactivation of RNAse by the fixation step as well as other steps in the procedure, it may give added security when first establishing this procedure. Autoclaving all solutions is a necessary procedure to prevent bacterial contamination of solutions and the use of DEPC-treated solutions and the wearing of gloves is essential for the *in situ* hybridization steps in **Subheading 3.3.**
7. Where possible, restriction endonucleases were chosen so that the cDNA fragments generated had different restriction enzyme sites at the 5' and 3' ends of the cDNA, allowing insertion in a known orientation within the polycloning site of an appropriate plasmid (SP72, pGEM 3, pGEM 7z-; Promega) in such a way that the cDNA fragment was flanked by the SP6 and T7 RNA polymerase promoters.
8. The commercial riboprobe generation kits will generate a labeled copy of the antisense (positive probe) and the sense strand (negative probe), which gives an inbuilt control for each probe. The use of riboprobes also increases the strength of hybridization of an RNA probe to a mRNA, allowing greater stringency of washing conditions, which will reduce nonspecific hybridization.
9. Aliquots were stored at –20°C for up to 12 mo. Aliquots of riboprobes were thawed as needed and diluted 100- to 500-fold in hybridization buffer, in which they were stable at 4°C for up to 1 mo. Probe concentration were measured using QuantaGene DNA/RNA Calculator (The Australian Chromatography Company, Pharmacia, Cambridge, UK) and the same concentration of sense and antisense probes were used for *in situ* hybridization.
10. Ensure that there are no bubbles under the cover slip, otherwise the labeled riboprobe will not be in contact with all parts of the tissue section. Alternatives such as parafilm on top of the sections was rarely adequate to prevent drying of the sections in the overnight hybridization step.
11. This amplification step with anti-biotin antibody greatly enhanced the sensitivity of the technique.
12. We have attempted several combinations of *in situ* hybridization with immunohistochemistry, including performing either the *in situ* hybridization or immunohistochemical labeling step first and either developing the color reaction immediately or at the end of the combined procedure. Best results were achieved by performing the *in situ* hybridization step first including developing the color

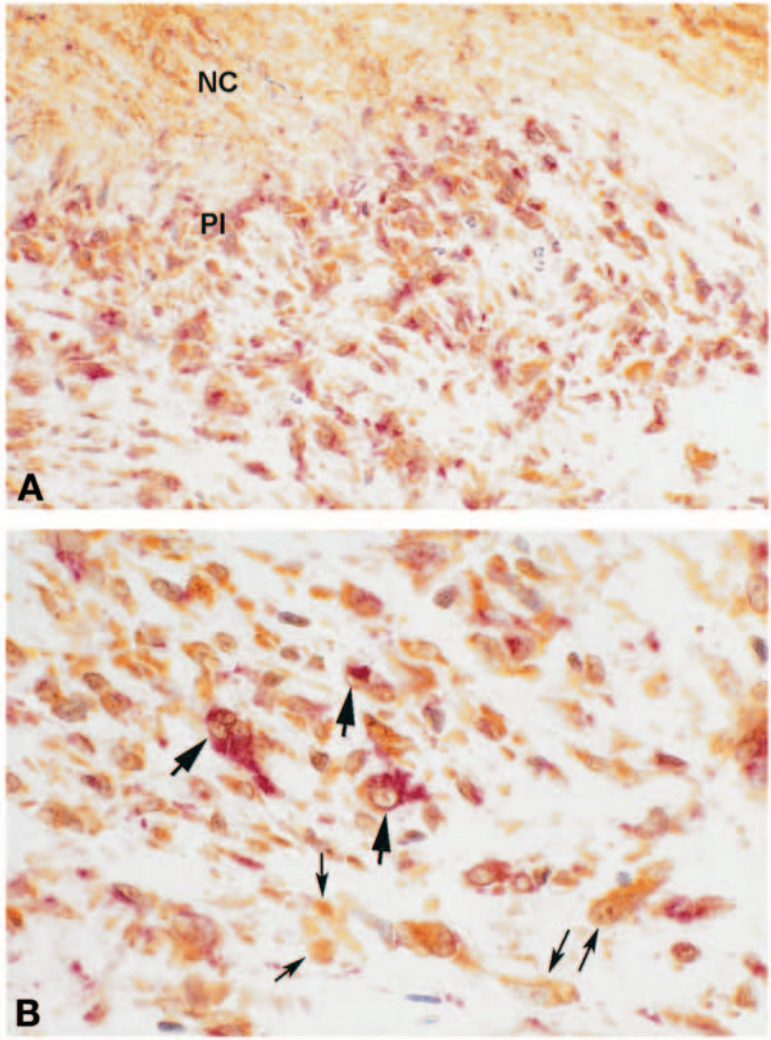

Fig. 1. Low (**A**, ×66) and high (**B**, ×132) power views of combined *in situ* hybridization for IL-1β mRNA (brown) and macrophage lineage marker CD68 (red) in a tissue section from a rheumatoid nodule. Large arrows show macrophages containing IL-1β mRNA (red and brown) whereas small arrows show fibroblasts containing IL-1β mRNA (brown only). NC, necrotic center, Pl, palisade layer, St, stromal region.

reaction, followed by the immunohistochemical reaction *(8)*. **Figures 1** and **2** demonstrate examples of combining in situ hybridization for cytokine mRNA with immunohistochemical detection of either cell lineage markers (**Fig. 1**) or cytokine (**Fig. 2**).

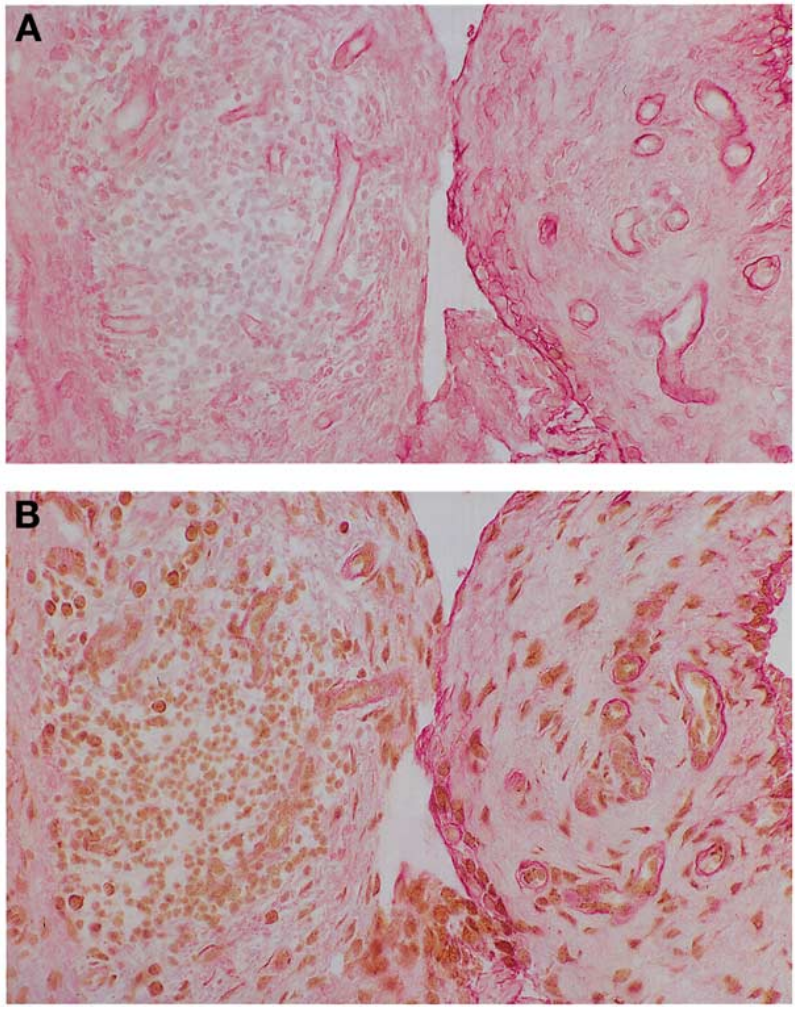

Fig. 2. Low-power (×66) view of combined *in situ* hybridization for tumor necrosis factor (TNF)-α mRNA (brown) and immunohistochemistry for TNF-α (red) on a synovial membrane biopsy from a patient with rheumatoid arthritis. **(A)** Sense riboprobe and anti-TNF-α antibody. **(B)** Antisense riboprobe and anti-TNF-α antibody.

13. If excess background is a problem with a biotin based system as a result of endogenous biotin in the tissue sections that is not adequately blocked, a DIG system will overcome this and can be converted to a DAB color end-product by using an anti-DIGantibody linked to horseradish peroxidase.
14. The color end product from an immunoalkaline phosphatase reaction is not stable in xylene or alcohol; therefore, an aqueous mounting medium must be used.

15. Quantification of *in situ* hybridization and immunohistochemical labeling can be performed using computer-assisted image analysis techniques, as previously described ***(13–16)***. The reproducibility of this method of quantitation is excellent, with an intra- and inter-observer variability of <10%, mainly to the result of operator variability in field selection ***(16)***.

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18

Semiquantitative *In Situ* Hybridization Using Radioactive Probes to Study Gene Expression in Motoneuron Populations

Paul D. Storer and Tracey DeLucia

Summary

In situ hybridization is a powerful technique for examining changes in mRNA levels. Its primary advantage over the Northern blot and reverse transcription polymerase chain reaction is the ability to localize specific species of mRNA to a particular cell population in a heterocellular system. This consideration is important when studying gene expression, especially in areas of high cellular heterogeneity, such as the central nervous system, where the differentiation between neuronal and glial gene expression is critical in evaluating the effects of physiological or pathological stimuli. We have used radioactive *in situ* hybridization to study changes of mRNA levels in several motoneuron populations following axonal injury. Considering that many neuronal populations have multiple targets of innervation, we used *in situ* hybridization in a manner that focused on the examination of changes in gene expression at the single cell level. This chapter describes the *in situ* hybridization protocol that our laboratory has used to demonstrate alterations in gene expression in specific motoneurons whose cell bodies are localized to the central nervous system.

Key Words: mRNA; motor neuron; *in situ* hybridization; protocols.

1. Introduction

Quantitative *in situ* hybridization is a powerful technique for examining changes in gene expression. Its primary advantage over the northern blot and reverse transcription polymerase chain reaction (RT-PCR) is the ability to localize changes in gene expression to a particular cell population in a heterocellular system. This consideration is important when studying gene expression, especially in areas of high cellular heterogeneity, such as the central nervous system, where the differentiation between neuronal and glial gene

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expression is critical in evaluating the effects of physiological or pathological stimuli.

We have used radioactive *in situ* hybridization extensively to study changes in gene expression in several motoneuron populations after peripheral or central nerve injury ***(1–8)***. Specifically, we have investigated the effects of axotomy alone or in combination with the administration of gonadal steroids on the expression of numerous regeneration-associated genes in facial, rubrospinal, and sciatic motoneuron cell bodies of the adult hamster and rat. Considering that these nuclei have multiple targets of innervation, we were required to use *in situ* hybridization in a manner that focused on the examination of changes in gene expression at the single cell level. This chapter describes the *in situ* hybridization protocol that our laboratory has used to demonstrate changes in gene expression in motoneurons of the central nervous system.

2. Materials

1. Cryostat sections of fresh-frozen brain or spinal cord tissue cut at 12 µm and mounted onto Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA).
2. Nuclease-free pipet tips and microfuge tubes.
3. Nuclease-free Pyrex glassware baked at 220°C for at least 4 h.
4. Slide racks.
5. Glass loaf pans.
6. Water baths.
7. Nalgene trays with covers (Nalge Nunc, Rochester, NY).
8. Whatman no. 2 filter paper (Whatman, Brentford, Middlesex, UK).
9. ^{33}P[dATP].
10. Double-stranded cDNA probe template.
11. Nick-translation kit.
12. Diethylpyrocarbonate (DEPC; *see* **Note 1**).
13. 100% Ethanol.
14. 2X Pre-hybridization/Hybridization buffer (*see* **Note 2**): 1.2 *M* sodium chloride, 20 m*M* Tris-HCl, pH 7.6, 2 m*M* ethylene diamine tetraacetic acid (EDTA), 0.2 mg/mL bovine serum albumin, 0.5 mg/mL sodium pyrophosphate, 0.4 mg/mL Ficoll, 0.4 mg/mL polyvinyl pyrrolidone, 100 µg/mL yeast transfer ribonucleic acid (RNA), 100 µg/mL yeast total RNA, 0.5 mg/mL salmon sperm deoxyribonucleic acid (DNA), 20 µg/mL dithiothreitol (DTT), and 20% dextran sulfate (hybridization buffer only), diluted 1:1 with deionized (DI) formamide.
15. Photographic emulsion (Kodak NTB-2, Kodak-Eastman, Rochester, NY).
16. Developer and fixative (Kodak D-19 and Kodak Rapid Fix).
17. Thionin, pH 3.5.
18. Microscope.
19. Computer-based image analysis system.
20. Statistical analysis program.

3. Methods

This protocol is for detecting mRNA in sectioned brain or spinal cord tissue on a single cell level using ^{33}P-labeled cDNA probes and emulsion autoradiographic techniques. Using emulsion-based *in situ* hybridization provides information on the number of cells expressing the mRNA of interest and the relative level of expression with individual cells, and is a more sensitive measure than X-ray film. Strict adherence to a nuclease-free environment is critical to the success of this protocol, including the use of DEPC-treated water and solutions, baked glassware and spatulas, and the wearing of gloves throughout the procedure (s*ee* **Note 1**). It is a good idea to have an inventory of glassware and chemicals that are used exclusively for *in situ* hybridization; it will prevent accidental contamination with RNase from other sources in the laboratory. Minor variations of this protocol can be done in order to perform *in situ* hybridization with either RNA probes or ribosomal DNA probes as well.

3.1. Tissue Preparation

1. After experimental manipulation, rats are anesthetized and killed by decapitation. Brains or spinal cords are quickly extracted, frozen on dry ice, and stored at –80°C.
2. Prior to sectioning, brains are thawed to –20°C and blocked coronally. Coronal sections are cut at 12 µm and thaw-mounted onto Superfrost Plus slides perpendicular to the axis of the slide.
3. Finally, slide-mounted sections are fixed for 15 min with 4% paraformaldehyde/0.1 *M* PBS, rinsed in PBS buffer, dehydrated in a graded ethanol series (50, 75, 90, and 100%), and stored at –80°C (*see* **Note 3**).

3.2. Probe Labeling

cDNA probes are labeled using a nick-translation kit and ^{33}P-labeled dATP. After the reaction, labeled probe is purified using spin columns. ^{33}P emits β particles, which have a wavelength that is greater than the thickness of the emulsion, so standardizing procedures for dipping, drying, and development of emulsion-coated slides are essential. However, probes labeled with ^{33}P result in emulsion autoradiographs with high cellular resolution. Remember that the nick-translation reaction will have to be optimized for different kits and different cDNA probes to result in an adequate incorporation of the isotope.

3.3. In Situ *Hybridization*

3.3.1. Prehybridization Treatment

1. Thaw slides to room temperature and arrange on Whatman filter paper in Nalgene boxes.
2. Heat 2X prehybridization solution at 100°C for 10 min.
3. Snap cool in dry ice/ethanol bath.

4. Immediately add 1 vol of DI formamide and vortex.
5. Add DTT to a final concentration of 160 µ*M* and vortex.
6. Pipet 20 µL of prehybridization solution onto each section.
7. Wet filter paper underneath slides with 2X SSC:DI formamide to keep the slides humidified throughout the incubation period. Cover Nalgene trays.
8. Incubate slides at 37°C for 2 h.

3.3.2. Hybridization

1. Add labeled cDNA probe to 2X hybridization solution at a concentration of 100,000 cpms per brain section.
2. Heat hybridization solution/probe at 100°C for 10 min.
3. Snap cool in dry ice/ethanol bath.
4. Immediately add 1 vol of DI formamide and vortex.
5. Add DTT to a final concentration of 160 µ*M* and vortex.
6. Aspirate prehybridization solution using a vacuum apparatus. Do not let sections dry!
7. Replace prehybridization solution with 20 µL of hybridization solution/probe.
8. Humidify boxes with 2X SSC/DI formamide and cover Nalgene trays.
9. Incubate slides at 37°C for 16 h.

3.3.3. Posthybridization Treatment

1. After incubation, remove slides from Nalgene boxes and place into slide racks that will fit into glass loaf pans containing wash buffers.
2. Place the slides in 4X SSC + β-mercaptoethanol at room temperature for 10 min.
3. Do not agitate the slides during these washes.
4. Wash in 2X SSC + β-mercaptoethanol at 45°C for 30 min.
5. Wash in 1X SSC + β-mercaptoethanol at 45°C for 90 min.
6. Wash in 0.5X SSC + β-mercaptoethanol at 45°C for 30 min.
7. Dehydrate in graded series of ethanol/300 m*M* ammonium acetate and air-dry.

3.4. Autoradiography

After posthybridization procedures, the slides are dipped into 43°C liquid emulsion (Kodak NTB-2) under a safelight. A separate group of hybridized brain sections are used as "test" slides, which should be developed periodically over the next couple of weeks to ascertain exposure times. Assessing test slides before development of the experimental slides prevents saturation of the emulsion over the most heavily labeled cells, making visualization and analysis of these cells more accurate. After all slides have been dipped in emulsion, let air-dry in a light-tight box for approx 3 h and then place into light tight slide boxes containing desiccant. Store slides at 4°C until ready for development. Slides are ready to be developed when the signal to noise ratio of hybridization signal is adequate for quantitation.

3.5. Developing Exposed Slides

1. Develop all test slides and subsequent experimental slides in Kodak D-19 developer at 19°C for 2 min.
2. Wash in water for 30 s.
3. Wash twice in Kodak Rapid Fix for 5 min. Do not use hardener in the fixative. Wash in cold running water for 10 min.
4. Lightly counterstain sections using thionin (pH 3.5), dehydrate through a graded series of ethanol (50, 75, 90, and 100%) and clear overnight in HemoDe or acetone. Light counterstaining is critical to the analysis of *in situ* hybridization because silver grains must be clearly distinguished from the underlying tissue.
5. Cover slip using Permount.

3.6. Quantitation of Hybridization Signal and Statistics

To measure the relative changes in gene expression that occur in motoneurons after experimental manipulation, the relative number of silver grains that overlay individual neuronal cell bodies is quantitated using a 40× or 60× objective and a computer-assisted image analysis system. Our laboratory utilizes a Zeiss Axioskop microscope (Carl Zeiss, Gottingen, Germany) outfitted with a Photometrics CoolSnap charge-coupled device (CCD) camera (Princeton instruments, Trenton, NJ), which provides visualization on a computer monitor in association with MetaView v.4.5r2 image analysis software. This software allows us to measure the number of silver grains overlaying neuronal cell bodies and to measure the area of the neuron that was measured. Additionally, this system allows various image enhancement functions, which aid in increasing the contrast between silver grains and the underlying stained tissue. Data are presented as the average number of grains per neuron or as the average density of neuronal labeling. Changes in gene expression can then be presented as the ratio of the average labeling density over injured neurons to the average labeling density over uninjured neurons.

3.6.1. Image Analysis

1. Before measuring silver grain densities, all slides must be coded such that the data are collected under "blind" conditions.
2. To not duplicate neurons that are counted from section to section, a systematic sampling strategy is used for section selection, that is, at least every other section is used for quantitation.
3. In collecting data from motoneurons that are sectioned through different levels of the cell body, specific criteria concerning neuronal selection must be used, including the presence of (1) a clear somal boundary and (2) a visible nucleus **(Fig. 1A)**.

   Using the computer-assisted image analysis system, threshold gray levels are set to define and tag silver grains overlying the cells. Set the threshold to a level that

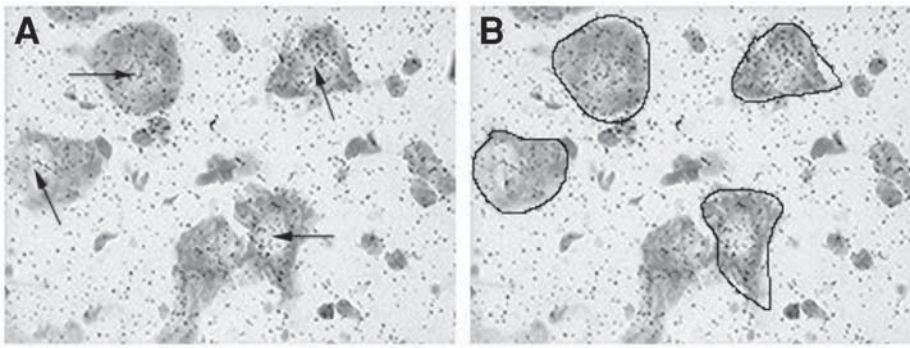

Fig. 1. Measurement of silver grain densities from montoneurons after *in situ* hybridization with ^{33}P-labeled cDNA probes. (**A**) Identify motoneurons to be used for quantification byt he presence of a clear somal boundqary and nucleus. (**B**) Delineate those cells and measure the somal size and number of silver grains using image analysis software.

is high enough to distinguish labeled from non-labeled cells, but also to a level that can accurately reflect the number of grains counted manually over a range of grain densities. The average size of a grain is calculated to convert numbers of pixels (as measured by the computer) to numbers of grains.

Because axonal injury produces changes in the somal size, grain density is a more reliable measure of RNA abundance than number of grains alone. Thus, the average number of grains per neuron, somal area of individual cells, and grain density (average number of grains/somal area) are recorded from at least 100 neurons from both the control and experimental nuclei for each animal and time point.

a. Obtain image and perform image-enhancement techniques to optimize the contrast between grains and tissue.
b. Set threshold to accurately tag silver grains. To compensate for lighting variations and differences in staining intensity between cells or sampling fields, adjust the threshold as needed to maintain the accurate tagging of silver grains.
c. Define the boundary of neurons to be counted using the geometric tools provided by the image analysis software **(Fig. 1B)**.
d. Instruct the image analysis software to automatically count the number of pixels/grains that are above the threshold from the user-defined areas in the field of view.

4. Background labeling is determined by counting grains overlying non-neuronal areas within each section, obtaining an average number of grains/area of background and subtracting that from the values for neuronal labeling. True background levels of silver grains can be determined using appropriate controls run simultaneously with the experimental slides (*see* **Note 4**).

3.6.2. Statistics

The values obtained from individual neurons of both control and experimental nuclei are averaged separately (mean ± SEM) and statistically compared using a multi-factorial analysis of variance (one-way or two-way analysis of varianec) followed by the Newman–Keuls *post-hoc* comparison. Ultimately, average grain densities from each nucleus are plotted as the percentage change observed in the injured nucleus compared with the control nucleus.

4. Notes

1. DEPC should be added to all solutions used in this procedure. We add 20 µL of DEPC into 1 L of solution, stir overnight, and then autoclave for 90 min to deactivate the DEPC. DEPC reacts with histidine residues of proteins and is specifically used to inactivate RNase activity. However, DEPC can also interact with RNA, so heat is needed to break down the DEPC into CO_2 and ethanol. Tris-containing solutions will also react with DEPC, so only add Tris to DEPC-treated water after autoclaving.
2. Components of the prehybridization and hybridization solutions are important in inhibiting nonspecific labeling by decreasing the interaction of the probe with polysaccharides, proteins and other nucleic acids contained within the tissue. The addition of dextran sulfate to the hybridization solution increases the rate and signal amplification of hybridization by an excluded volume effect. Additionally, dextran sulfate promotes probe networking (the formation of probe aggregates) by overlapping sequences between probe molecules. The addition of DI formamide aids in disrupting hydrogen bonds and destabilizes nucleic acid duplexes resulting in decreased melting temperature. DTT is effective in preventing end ligation of DNA strands.
3. Fixation of tissue with paraformaldehyde minimizes background labeling by acetylation of basic proteins resulting in the prevention of electrostatic interactions between the probe and endogenous proteins.
4. To assess the specificity of hybridization, we include sections that have been hybridized with labeled probe, but in the presence of excess (10-fold) nonlabeled probe. The competition for the target mRNA by the "cold" probe should result in the appearance of a background level of silver grains. To control for the autoradiographic technique itself, we include sections that are taken through the procedure but that are not hybridized with probe. These slides allow us to assess the contribution of any other source of excitation other than the labeled probe that would result in false-positive grain accumulation.

Acknowledgments

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19

Quantitative *In Situ* Hybridization of Tissue Microarrays

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Summary

Tissue microarrays enable the rapid histological localization of gene expression in hundreds of archival samples by *in situ* hybridization. However, the scoring of tissue microarray data may be influenced by intra- and inter-observer variations, and categorizing continuous variables risks discarding potentially meaningful information. Quantitation imposes a greater degree of objectivity, is more reproducible than subjective discriminations, and facilitates the communication and clarity of definitions. Phosphorimaging has been successfully used to quantitate the hybridization signal intensity from arrayed tissues. The process is rapid and has a wide dynamic range, surpassing the densitometric analysis of autoradiograms. This paper presents a detailed method for quantitative isotopic *in situ* hybridization on formalin-fixed paraffin-embedded tissue microarrays. In addition, the method includes a protocol for the development of synthetic agarose cores to control for the specificity and sensitivity of hybridization.

Key Words: *In situ* hybridization; quantitative evaluation; storage phosphor screen; tissue microarrays.

1. Introduction

Tissue microarrays (TMAs) enable hundreds of archival samples to be examined concurrently for gene expression by *in situ* hybridization (ISH ***[1]***). The technology allows high-throughput molecular profiling, permits rapid correlations to be made between tumor genotypes and clinicopathologic traits, and ensures the preservation of archival tissue for future research or clinical needs ***(1,2)***. The morphological information offered by *in situ* studies often surpasses the utility of biochemical methods, which give a composite result without due consideration of the heterogeneity of cells ***(2)***. Moreover, the use

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of TMAs ensures that all specimens are processed under near-identical conditions, optimizing preanalytical and analytical standardization *(3)*.

However, the value of TMA studies is limited because they typically are scored by pathologists on a semiquantitative scale. The data generated may be influenced by intra- and interobserver variations and categorizing continuous variables risks discarding potentially meaningful information *(4,5)*. Quantitation by phosphorimaging imposes a greater degree of objectivity, is more reproducible than subjective discriminations, and facilitates the communication and clarity of definitions *(4)*. Phosphorimage analysis is very sensitive, can be completed within hours, and has a wide linear dynamic range (across five orders of magnitude), permitting a rapid and accurate comparison of gene expression levels between samples *(2,6)*. Phosphor image analysis offers significant advantages over the densitometric analysis of autoradiograms, which rapidly saturate at high signal intensities. Furthermore, phosphor imaging is not affected by emulsion-based artifacts, including negative chemography, variations in emulsion thickness, variations in development conditions, and potential loss of data from accidental exposure.

We detail here our method for quantitative isotopic ISH on TMAs, which has been peer-reviewed for the quantitation of angiogenic signal transductants *(7,8)*. In addition, the method includes a protocol for the development of synthetic agarose cores to control for the specificity and sensitivity of hybridization and to orientate the TMA *(2,7,8)*.

2. Materials

Appropriate caution should be exercised when handling and disposing of radioactive materials.

2.1. Probe Preparation

1. Marathon-ready complementary (c)DNA (BD Clontech, Palo Alto, CA).
2. RNA Stat-60 (Tel-Test, Friendswood, TX).
3. Prostar Ultra High Fidelity reverse transcription polymerase chain reaction (RT-PCR) System (Stratagene, La Jolla, CA).
4. Advantage cDNA Polymerase Mix (BD Clontech).
5. QIAquick PCR Purification Kit (Qiagen, Valencia, CA).
6. [α-^{33}P]UTP (MP Biomedicals, Irvine, CA). Ordered fresh each week and stored at –70°C.
7. Transcription Optimized 5X buffer (Promega, Madison, WI).
8. 100 m*M* Dithiothreitol (Promega).
9. 10 m*M* each rATP, rCTP, and rGTP (Promega).
10. rRNasin Ribonuclease Inhibitor 20–40 U/µL (Promega).
11. T3 and T7 RNA Polymerase10–20 U/µL (Promega).
12. RQ1 RNase-free DNase 1 U/µL (Promega).

13. DE81 Ion Exchange Paper (Whatman, Clifton, NJ).
14. RNeasy Mini Kit (Qiagen).
15. BioSafeII Scintillation Fluid (Research Products International, Mt. Prospect, IL).
16. 6% polyacrylamide TBE/Urea gel (Invitrogen, Carlsbad, CA).
17. 2X TBE/Urea Sample Buffer (Invitrogen).
18. RNA Molecular Weight Marker III (Roche Diagnostics Indianapolis, IN).
19. Biomax MS Film (Eastman-Kodak, Rochester, NY). Light sensitive.

2.2. Synthetic Controls

1. Microgranular Cellulose (Sigma-Aldrich, St. Louis, MO).
2. T7 and T3 RNA Megascript Kits (Ambion, Austin, TX).
3. NuSieve 3:1 Agarose (FMC Bioproducts, Rockland, ME).
4. 15 × 15-mm diSPO Base Molds (Baxter, Deersfield, IL).

2.3. Hybridization

1. RNase A 100 Kunitz U/mg (Sigma-Aldrich, St. Louis, MO).
2. Proteinase K (Roche Diagnostics).
3. Hybridization Buffer: 10% dextran sulfate (Bioworld, Dublin, OH), 2X SSC, and 50% fluka formamide (Sigma-Aldrich). Vortex well to mix and store on ice. Extra hybridization buffer can be stored and reused by wrapping the tube in foil and storing at –80°C. Restrict use to a fume-hood.
4. Yeast transfer RNA (Sigma-Aldrich).
5. Box Buffer: 4X SSC with 50% formamide. Make fresh as required and restrict use to a fume-hood.
6. RNase Buffer: 10 m*M* Tris-HCl, pH 8.0, 0.5 *M* NaCl. Make fresh as required.
7. Autoradiography NTB-2 Emulsion (Eastman-Kodak). Light sensitive.
8. D-19 Developer (Eastman-Kodak). Light sensitive.
9. GBX Fixer (Eastman-Kodak). Light sensitive.
10. Biomax MR-1 Film (Eastman-Kodak). Light sensitive.

2.4. Phosphorimaging and Analysis

1. Low-energy phosphorscreen (light sensitive) and Typhoon 8600 Phosphorimager (Amersham Biosciences, Piscataway, NJ).
2. Phoretix Array v.3.0.1 (Non-linear Dynamics, Newcastle-upon-Tyne, UK).

3. Methods

3.1. Probe Synthesis

Private and public expression databases are screened to identify tissue types and pathologies that contain the transcript of interest. Examples of databases frequently used include the Gene Logic® (Gaithersburg, MD) probearray database and the National Center for Biotechnology Information (NCBI, Bethesda, MD) serial analysis of gene expression (SAGE) database [http://www.ncbi.nlm.nih.gov/SAGE/]). cDNA libraries representing the tissue(s) of

interest are purchased where available (Marathon Ready cDNA). Alternatively, RNA is extracted from frozen tissues (RNA STAT-60) and reverse transcribed (Prostar Ultra High Fidelity RT-PCR system) to provide a cDNA template (following the manufacturer's instructions). Oligonucleotide primers are designed and synthesized to span a region approx 500–700 bp in length with a guanine-cytosine (GC) content of approx 50–55%, using Oligo software (v.6.2, Molecular Biology Insights, Cascade, CO). Specificity for the transcript of interest is confirmed with a BLASTn (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) search. T7 and T3 polymerase recognition sequences (respectively 5'-GGATTCTAATACGACTCACTATAGGGC-3' and 5'-CTATGAAATTA-ACCCTCACTAAAGGGA-3') are applied 5' to the sense and anti-sense primers respectively. ISH probe templates are synthesized by polymerase chain reaction (PCR) using the Advantage cDNA polymerase mix (according to the manufacturer's instructions). Note, for GC-rich sequences Advantage-GC cDNA polymerase mix can be used.

For example, an anti-sense human β-actin probe spanning nucleotides 796 to 1086 in the Genbank sequence (accession NM'001101) can be synthesized with the following primers: Sense, 5'-GGATTCTAATACGACTCACTATAG-GGCGCTGCCTGACGGCCAGGTC-3'; Anti-sense, 5'-CTATGAAATTAA-CCCTCACTAAAGGGAGAGTACTTGCGCTCAGAGGAG-3'. The PCR mix is prepared according to the manufacturer's instructions and the PCR conditions are as follows: 94°C for 1 min, 25 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min, with a final extension of 68°C for 5 min.

Amplified products are purified with the QIAquick PCR Purification kit, their size is checked by agarose gel electrophoresis and their concentration calculated using a spectrophotometer (*see* **Note 1**).

3.2. Transcription

1. A 12-µL aliquot of [α-^{33}P]UTP (125 µCi) is speed-vacuumed until dry.
   To each tube with dried [α-^{33}P]UTP, the following reagents are added in order:
   a. 2 µL of transcription optimized 5X buffer.
   b. 1 µL of distilled H_2O.
   c. 1 µL of 100 m*M* dithiothreitol.
   d. 2 µL of rNTP mix (2.5 m*M* each rATP, rCTP, and rGTP).
   e. 1 µL (20–40 U) of rRNasin ribonuclease inhibitor.
   f. 1 µg of DNA template (in 2 µL).
   g. 1 µL (10–20 U) of RNA polymerase (T3 RNA polymerase for an anti-sense probe or T7 RNA polymerase for a sense probe).
2. The solution is then incubated for 1 h at 37°C.

3.3. Digestion and Resuspension of Template

1. To the aforementioned mixture, add 1 µL (1U) of RQ1 RNase-free DNase and return to 37°C for 15 min, before adding 90 µL of TE buffer.

3.4. Removal of Unincorporated Nucleotides

1. Pipet 1 µL of probe onto a 0.5-cm^2 piece of DE81 ion exchange paper and place in a scintillation vial labeled "pre-filtered."

   Remove unincorporated nucleotides and the cDNA template from the remaining probe solution using the RNeasy mini kit. In brief,
2. Add 350 µL of buffer RLT, mix, and then add 250 µL of ethanol.
3. Apply the sample to a spin-column, centrifuge for 15 s at 8000*g*, and discard the flow-through.
4. Pipet 500 µL of buffer RPE onto the column centrifuge for 15 s at 8000*g*, discard the flow-through, and repeat.
5. Centrifuge at 8000*g* for 1 min to dry the membrane and place the column in a new microcentrifuge tube.
6. Apply 30 µL of RNase-free H_2O to the column, wait 3 min, and centrifuge for 1 min at 8000*g*.
7. Add 100 µL of TE to the elutate, pipet 1 µL of probe onto DE81 paper, and place in a scintillation vial labeled "filtered."
8. Check the efficiency of probe-labeling by measuring the scintillation of the prefiltered and filtered samples in 6 mL of BioSafe II using the LS-6500TD scintillation counter (Beckman Coulter, Fullerton, CA). Counts from filtered samples should be no less than 50% of the counts from prefiltered samples.

3.5. Checking of Probe Size

1. Add 3 µL of probe or 5 µL of RNA ladder to an equal volume of 2X TBE/Urea sample buffer.
2. Denature for 3 min at 95°C and quench for 3 min on ice (*see* **Note 2**).
3. Load samples into 6% polyacrylamide TBE/urea gels and run at 180–250 V for 45 min.
4. Remove the gel casing, cover in Saran Wrap and expose to Biomax MS film for 1 h at –70°C.
5. Develop the film and mark on it the gel position.
6. Agitate the gel in 0.5 µg/mL ethidium bromide for 15 min and view under ultraviolet light to visualize the ladder (be sure to wear an acrylic face shield).
6. Mark the positions of the bands on the film to check the size of the labeled probe.
8. The probe may then be stored at –80°C overnight or used immediately.

3.6. Preparation of Synthetic Control Blocks

1. Sense and anti-sense riboprobes are transcribed from the cDNA PCR template using T7 and T3 RNA Megascript kits exactly according to the manufacturer's instructions.
2. An 8% aqueous solution of Nusieve 3:1 agarose is prepared in 250 µL of aliquots and incubated at 95°C.
3. Serial dilutions of each riboprobe are then prepared and mixed thoroughly with the agarose to give final concentrations of 5 µg/mL, 1 µg/mL and 0.5 µg/mL in a total volume of 1 mL (2% agarose).
4. The mixture is then incubated for a further 10 min at 95°C.

5. After mixing thoroughly, each control is pipetted into a 15 × 15-mm diSPO base mold and allowed to set for 2 h at 4°C.
6. Remove the blocks from the molds using a clean razor blade, then fix the blocks overnight in 10% neutral-buffered formalin before embedding them in paraffin.
7. Blank 2% agarose control blocks are prepared in an identical manner.
8. Cellulose binds strongly to radioactive RNA probes and, when incorporated into TMAs, can be used for orientating slides on a phosphorimager. To synthesize a block containing cellulose, 1 g of microgranular cellulose is added to 3 mL of water and mixed to form a suspension. Finally, 750 µL of the cellulose suspension is added to 250 µL of 8% agarose and processed as described previously.

3.7. TMA Construction

For this purpose, TMAs are constructed using a Beecher Instruments Microarrayer (Silver Springs, MD) as previously described *(1)*. In brief, cores (600 µm in diameter) are punch-biopsied from representative regions of the donor blocks and brought into recipient paraffin blocks. Sampling of formalin-fixed, paraffin-embedded tissues is undertaken in triplicate to provide representative data on the parent block; synthetic controls are sampled in duplicate. In addition, cores taken from pelleted, paraffin-embedded cell lines or transfected cells can be incorporated into the blocks as additional positive and/or negative controls. Cellulose orientation markers can be placed asymmetrically in three corners. Sections, 3 µm thick, are cut from the recipient blocks and mounted on Superfrost-plus glass slides.

3.8. Hybridization

On the day of hybridization:

1. TMA slides are heated in a 65°C oven for 20 min.
2. They are then deparaffinized in a Leica Autostainer XL (Deerfield, IL) through a series of three xylenes (5 min each) and a gradient of ethanols (100, 95, 75% aqueous for 3 min each), and are finally moved into RNase-free water.
3. Slides are then washed twice for 5 min in 2X SSC at room temperature (RT).
4. Deproteination is carried out for 15 min at 37°C in 20 µg/mL proteinase K (made up in 250 mL prewarmed RNase-free RNase buffer).
5. A further wash in 0.5X SSC for 10 min at RT is followed by dehydration in a gradient of ethanols (75, 90, 100% aqueous) for 2 min each.
6. Slides are air-dried at RT for 1 h.
7. For each slide, 2×10^6 cpm of probe is mixed with 2 µL of 100 mg/mL tRNA and denatured for 3 min at 95°C, before quenching on ice.
8. Hybridization buffer is added to this solution and mixed to give a final volume of 200 µL per slide.
9. Hybridization is performed by laying the slides in a plastic Tupperware box, lined with Whatman 3MM filter paper that is saturated with box buffer. Hybridization solution is applied to each TMA, the boxes tightly sealed and the slides incubated overnight at 55°C (*see* **Notes 3** and **4**).

3.9. Washes

All washes are conducted with agitation (*see* **Note 5**).

1. Slides are washed twice in 2X SSC for 10 min at RT.
2. Unhybridized probe is digested with 20 mg/mL RNase A (in RNase buffer) for 30 min at 37°C (*see* **Note 6**).
3. Wash twice in 2X SSC with 1 m*M* ethylene diamine tetraacetic acid (EDTA) for 10 min at RT.
4. Wash for 2 h at 55°C in 0.1X SSC with 1 m*M* EDTA.
5. Finally, the slides are washed twice in 0.5X SSC for 10 min at RT.
6. Slides are dehydrated in a gradient of ethanols (50, 70, 100% aqueous) and left to air-dry for 2 h at RT.

3.10. Phosphorimaging

1. Slides are typically placed face-up in two columns in a hypercassette with the slide labels oriented towards the middle and are carefully taped down along the labeled edge.
2. The TMAs are exposed to a low energy phosphorscreen for 16 h at RT (*see* **Note 7**).
3. The phosphorscreen is scanned immediately on a Typhoon 8600 in phosphorimaging mode at 50-µm resolution.
4. The resulting ".gel" file is imported into Phoretix Array software.
5. A measurement grid (with a six-unit core radius) is aligned over the cores of each TMA and background correction performed using the image rectangle function (drawn alongside each TMA; *see* **Note 8**).
6. The volume measurement of each core above the background provides a quantitative value for the hybridization signal, which can be imported into Excel (Microsoft, Redmond, WA) for manipulation (*see* **Fig. 1**).

   For comparison with an autoradiographic image, the slides may then be exposed for 3 d at RT to Biomax MR-1 film.

3.11. Developing the Slides

1. Under safe-light illumination, slides are dipped in emulsion (1:2 dilution with water) at 42°C, dried overnight at RT, and exposed for 4–6 wk at 4°C (*see* **Note 9**).
2. Each TMA is then immersed in D-19 developer (1:1 dilution with water) for 3 min, washed in water for 30 s, immersed in GBX fixer for 3 min, and washed in water three times for 5 min each at RT.
4. Slides are stained with hematoxylin and eosin, and cover slipped.
5. Silver-grain deposition (indicating hybridization of the probe) is reviewed by bright- and dark-field microscopy.

4. Notes

1. Frequently, it is necessary to nest the PCR when the primers with T3 and T7 sequences cannot specifically amplify the sequence of interest. The transcript may also be cloned into a plasmid with T7 and T3 primer sequences around the

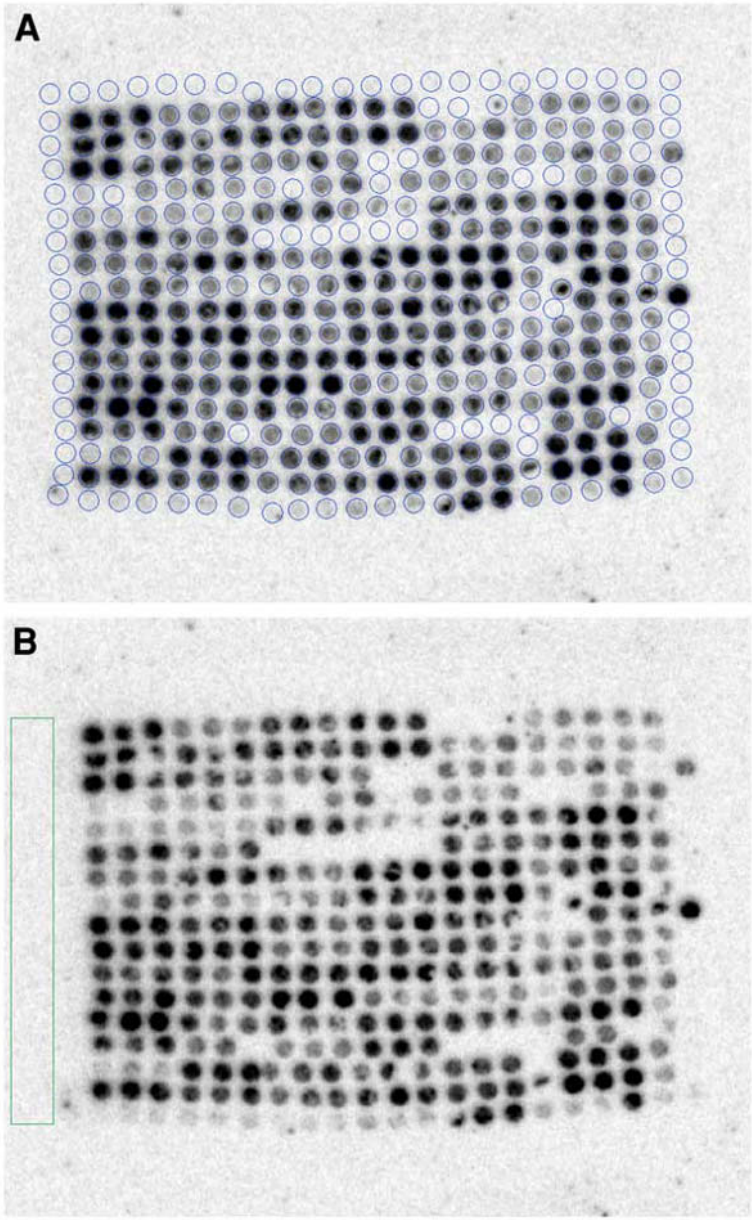
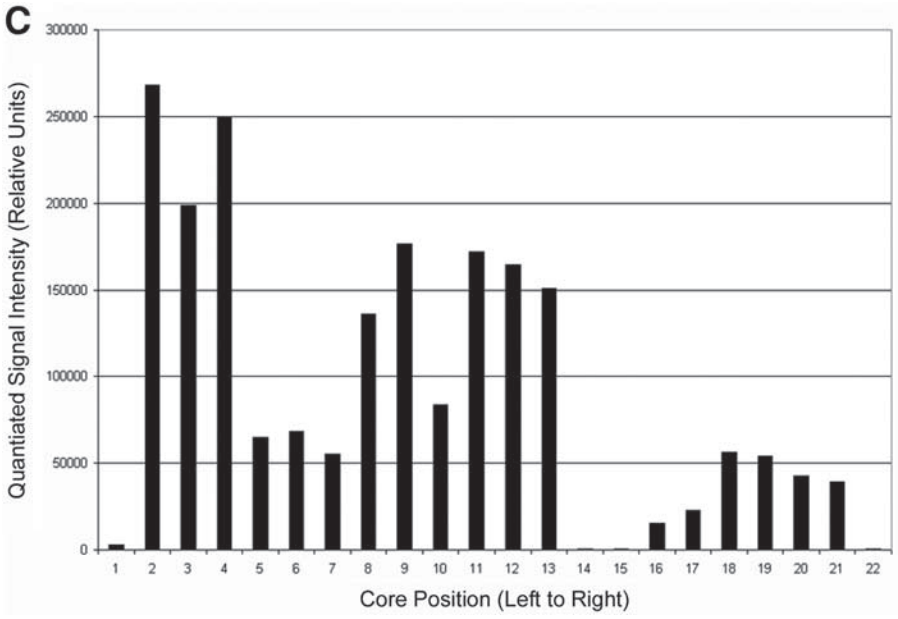


Fig. 1 Phosphor images of a human tissue microarray (TMA) hybridized with an anti-sense probe against β-actin. The darker the pixel, the more intense the hybridization signal. **(A)** A gridded array, with each grid circle positioned around a TMA core. **(B)** The image rectangle (left) used to measure the background signal intensity. **(C)** A bar chart of the quantitated signal intensity from the second row down, left to right.



insert. Using restriction enzymes, the vector can then be linearized to provide a DNA template for in vitro transcription and sequencing. Most problems of sensitivity and specificity (for instance, high sense background or binding of probes to intestinal goblet cells) can be resolved by changing the region of the transcript to which the probe is designed; it is rarely necessary to change other aspects of the protocol.

2. Be sure the lids of the tubes are secured to prevent the lids from popping open when heating, causing probe loss and potential radioactive contamination.
3. Plastic coverslips (Apoptag; Intergen, Burlington, MA) may be used to ensure complete coverage of all TMA elements during hybridization.
4. For probes with a high guanine-cytosine content, the hybridization temperature is increased to 65°C.
5. Dispose of all wash solutions up to the first 2X SSC, after the RNase A treatment, as liquid radioactive waste.
6. It is essential to ensure that all solutions are RNase-free up until this point. Separate containers and slide racks should be used during and after RNase treatment and care must be taken not to contaminate RNase-free equipment.
7. Ensure the phosphorscreen is completely "blanked" on a light box before use.
8. Grids are custom designed to the dimensions of the array (i.e., numbers of rows and columns, and the spot radii) and can then be manually aligned to the phosphorimage spots. If the signal is relatively weak, it may be advantageous to take an autofluorescence image of the array to help line up the grid. Grid layouts can be saved as analysis templates for future use.

9. It is essential to ensure that there are no bubbles in the emulsion and that there is an even coverage over the entire slide. The emulsion will cause the hematoxylin and eosin to streak over the slide; however, it will not affect the appearance of the tissue or the silver grains.

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