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Equilibrium Binding of Proteins to F-Actin

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Summary

This chapter reviews some of the many available methods for measuring the binding of myosin and other proteins to actin. Binding to actin has special considerations because actin is a long lattice and the binding site of many of its binding partners consists of multiple actin protomers. The analysis of binding to a lattice cannot be done by standard methods such as a Scatchard plot. Rational methods of analysis are described.

Key Words: Myosin; S1; actin; G-actin; F-actin; cooperativity; kinetics; binding; binding analysis; equilibrium; association constant; parking problem; ATPase rate; equations; sedimentation; ATP.

1. Introduction

The physical sizes of F-actin and actin-binding proteins place restrictions on the types of assays that can be used for measuring equilibrium binding. This chapter describes variations of sedimentation and fluorescence methods that have broad applications to the study of actin. Sedimentation methods commonly consist of two steps: separation of free ligand from the ligand-actin complex by sedimentation and determination of the free and bound ligand. Fluorescence methods are based on the change in environment of tryptophan residues or covalently attached probes on either actin or the ligand protein. Pyrene on Cys 374 of actin is responsive to binding of myosin subfragment 1 (S1) and other proteins and changes the affinity of binding to S1 by less than a factor of 2 ([1](#)). Fluorescein-labeled S1, 4-(iodoacetamido) salicylic acid (ISAL)-labeled S1, and *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)-amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD)-labeled caldesmon are examples of fluorescent ligand proteins that have been used in actin-binding studies ([2](#)).

Measurement of protein binding to actin has special requirements because of the unique properties of actin. Actin can exist either as a globular monomer (G-actin) or as a very large filamentous polymer (F-actin). Care must be taken to avoid changing the state of actin during the measurement, as the affinities of ligands for actin are normally dependent on the state of actin. The methods described in this chapter are for binding to F-actin. Other physical changes in actin can also affect the measurement of binding. For example, bundling of actin filaments causes increased light scattering that complicates fluorescence measurements.

The affinity of myosin for actin depends on the nucleotide bound to the active site of myosin. **Figure 1** shows the salt dependencies of S1-ATP and S1-ADP binding to F-actin. The large difference in affinity resulting from the substitution of ADP for ATP at the active site of S1 must be considered when measuring binding of S1-ATP to actin. Hydrolysis of a large fraction of ATP during the measurement can lead to grossly inaccurate results. **Figure 1** also illustrates

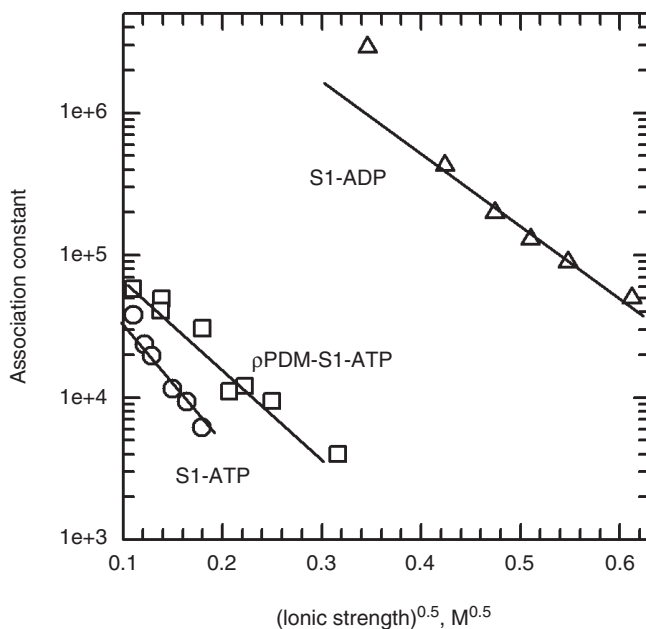


Fig. 1. Dependence of the affinity of myosin S1 to actin on the nucleotide bound to S1 and on ionic strength. Association constants (1/M) obtained at 25°C are shown as a function of the square root of the ionic strength according to the Debye-Hückel relationship (Castaneda-Agullo et al. 1961) (29). [Data from Gafurov et al. 2004 (24); Chalovich et al. 1981 (30), 1983 (28); Chalovich and Eisenberg 1982 (16).]

that the ionic strength can be adjusted to facilitate a particular binding study by bringing the affinity into a region that is most accurately measured.

The affinity between two proteins can be determined by keeping one protein fixed and varying the other. More information can be obtained for long lattices, such as actin, if the experiment is designed so that the occupancy of the lattice is varied. Furthermore, binding measurements are most accurate if the actin concentration is lower than the dissociation concentration of the actin–ligand complex.

The lower limit of actin concentration is set by the sensitivity of the measurement and by depolymerization of F-actin below its critical concentration (3). Phalloidin, a toxin of the angel of death mushroom, binds to actin and lowers its critical concentration by 30-fold (1 : 1 complex) or 90-fold (2 : 1 phalloidin: actin) (4,5). A 1 : 1 complex of phalloidin and actin has been used to determine dissociation constants as low as 10 nM (6).

Ligands vary widely in the stoichiometry of binding to actin. S1 binds to a single actin protomer, fesselin binds to three or four protomers (7), while tropomyosin (8) and caldesmon (9) bind to about seven protomers. One must use a general binding equation that is valid irrespective of the number of protomers that form a binding site. Valid methods of analysis are described below.

Temperature is an important variable for binding studies because it affects protein stability, the rate of reaching equilibrium, and the affinity. The fluorescence intensity may increase at lower temperature because of a decrease in collisional quenching (10). Although the choice of temperature is important, it is most important that the temperature be constant for all measurements.

2. Materials (see Note 1)

1. Actin depolymerization buffer: 0.5 mM ATP (Cat. #A7699, Sigma, St. Louis, MO), 2 mM tris-(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.8, 0.1 mM CaCl_2 .
2. Preparative ultracentrifuge with type 50 Ti and type 50.2 Ti rotors (Beckman Coulter, Fullerton, CA).
3. Column chromatography equipment.
4. Water bath sonicator.
5. Gel filtration resins such as Sephadex G100 or Superdex 200 resins (Pharmacia-LKB, Uppsala, Sweden) and Sephacryl S-300 HR (Sigma, St. Louis, MO).
6. Actin modification buffer: 100 mM Tris-HCl, pH 8.0, 1 M KCl, 20 mM MgCl_2 , 1 mM CaCl_2 , 0.1% NaN_3 . Add 10 mL 1 M Tris-HCl pH 8, 50 mL 2 M KCl, 2 mL 1 M MgCl_2 , 5 mL 20 mM CaCl_2 , and 10 mL 1% NaN_3 to a 100-mL graduated cylinder. Add water to 100 mL. This stock can be stored in the refrigerator for several months.
7. Fluorescence probes such as *N*-(1-pyrene)iodoacetamide (Molecular Probes, Eugene, OR).

8. Dithiothreitol (Invitrogen, Carlsbad, CA).
9. UV spectrophotometer Bio-Spec-1601 (Shimadzu, Kyoto, Japan).
10. Phalloidin (Sigma, St. Louis, MO).
11. Beckman Optima TL ultracentrifuge with a TLA 120.1 rotor or a Beckman Airfuge (Beckman Coulter, Fullerton, CA).
12. Teflon beakers for mixing proteins, 3–5 mL capacity.
13. Highly purified bovine serum albumin or Tween 20.
14. 2X protein loading buffer: 0.5 M Tris-HCl, pH 6.8, 4.4% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and bromophenol blue in distilled/deionized water.
15. Circulating water bath model RTE-110 (Thermo-Neslab, Newington, NH).
16. Fluorescence spectrophotometer (Thermo Electron, Madison, WI).
17. Reducing buffer: 100 mM KCl, 10 mM phosphate buffer pH 6.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol.
18. ATP stock: Dissolve 0.12 g ATP (Cat. #A7699, Sigma, St. Louis, MO) in 2 mL water and adjust the pH to 7.0. Dilute 0.05 mL ATP solution to 50 mL using a volumetric flask. Measure the absorbance at 259 nm. The concentration of the undiluted ATP stock in mM units is then $64.9 \times A_{259}$ (see **Note 2**).
19. ATP-Tris solution: To 3 mL 200 mM Tris-HCl (pH 8.0) add about 100 mg solid ATP (see **18**, above). Adjust the pH to 8.0 with Tris base (NaOH or KOH should be avoided because the metal ions interfere with the subsequent assay). Dilute a 0.05-mL aliquot of the ATP to 50 mL in a volumetric flask. Measure the O.D. at 259 nm and calculate the ATP concentration as described above. Add enough of the ATP stock to make 2 mL 35 mM ATP. Add 0.2 mL ^{32}P -ATP and bring the total volume to 2.0 mL by the addition of 200 mM Tris-HCl pH 8.
20. ^{32}P -ATP stock: To 1 mCi ATP [γ - ^{32}P] (Perkin Elmer-NEN, Boston, MA), add sufficient unlabeled ATP stock solution to make 4 mL 1 mM stock (final specific activity, about 250 mCi/mmol). Store frozen in 1-mL aliquots in a container rated for storage of ^{32}P such as an acrylic box (see **Note 3**). ^{32}P -ATP should be as fresh as possible and disposed of after 3–4 weeks because decreases in observed ATPase activity may occur.
21. Tris base, 2 M stock: Dissolve 24.2 g Tris base (F.W. 121.14) in water. Bring to 100 mL final volume.
22. Tris buffer, 200 mM, pH 8.0: Dissolve 4.8 g Tris base in 150 mL room temperature water. Adjust pH to 8.0 with HCl. Bring final volume to 200 mL with water.
23. Ammonium-EDTA reagent: Add 4 g NH_4Cl (F.W. 53.49) and 2.5 g EDTA disodium salt (372.24) to about 75 mL water. Adjust pH to 8.0 with Tris base. Adjust volume to 100 mL.
24. 1 M MOPS buffer: Dissolve 20.9 g MOPS [3-(*N*-morpholino)-propanesulfonic acid] (F.W. 209.26) in about 80 mL water. Adjust pH to 7.0 and volume to 100 mL.
25. ATPase quench solution: Add 64 mL 12 N HCl to 300 mL water (see **Note 4**). Add 0.11 g NaH_2PO_4 (F.W. 137.99). Adjust to 500 mL and store in a repipet dispenser bottle set to deliver 0.4-mL aliquots.

26. Silicotungstic acid solution: Add 20 mL 36 *N* H₂SO₄ to 300 mL water (*see Note 4*). Add 22 g silicotungstic acid (Cat. #A289, Fisher, Fair Lawn, NJ) and stir until dissolved. Bring volume to 500 mL. Store in a repipet dispenser bottle set to deliver 0.2-mL aliquots. The final solution is 1.4 *N* H₂SO₄, 4.4% silicotungstic acid.
27. Isobutanol:benzene (1:1): Add 250 mL isobutanol to 250 mL benzene and store in a repipet dispenser bottle set to deliver 1-mL aliquots. Use a hood and other normal safety precautions when using benzene (*see Note 5*).
28. Ammonium molybdate solution (5%): Dissolve 5 g ammonium molybdate (Cat. #A674, Fisher, Fair Lawn, NJ) in water to make 100 mL solution. Stir overnight or until completely dissolved. Filter. Store in a repipet dispenser bottle set to 0.2 mL. This solution should be discarded after 3–4 weeks or when a white precipitate is visible.
29. *N*-Pyrenyliodoacetamide stock: Weigh out between 1.4 and 2 mg pyrenyl iodoacetamide (Molecular Probes, Eugene, OR). Add dimethylformamide (Sigma, St. Louis, MO) to bring to 14 mg/mL. Vortex vigorously or sonicate in a water bath sonicator to dissolve the solute. Use immediately after preparation.
30. Hexokinase stock: To 2500 IU yeast hexokinase (Cat. #376811, Calbiochem, La Jolla, CA) add 2 mL 50 mM Tris-HCl, 5 mM MgCl₂. This stock solution is stable for 6 months at 4°C.
31. ADP stock: Dissolve 0.012 g ADP (Cat. #117105, Calbiochem, La Jolla, CA) in 2 mL water. Adjust to pH 7 with 5 *N* KOH. Dilute 0.05 mL of the ADP solution to 50 mL using a volumetric flask. Measure the absorbance at 259 nm. The concentration of the stock ADP (in mM) is then $64.9 \times A_{259}$.
32. ADP buffer for fluorescence measurements: This buffer contains ADP and ATP scavengers ([11](#)). To 1.66 mL 12 mM ADP, add 0.025 mL 2 *M* glucose, 0.14 mL hexokinase stock, 0.125 mg diadenosine pentaphosphate (Cat. #D4022, Sigma, St. Louis, MO) (0.031 mL 4 mg/mL diadenosine pentaphosphate stock), 1 mg bovine serum albumin (0.052 mL 38 mg/mL stock), and 0.59 mL water. This solution should be made on the day of the assay.
33. Ca²⁺ or ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) buffer for fluorescence measurements: Other components of the final reaction mixture are present in this buffer. We normally prepare this buffer at 1.54X the concentration that is required in the final fluorescence assay mixture (1.3 mL of this buffer is present in each 2 mL of the final solution). A typical buffer is 30.7 mM MOPS pH 7, 7.6 mM MgCl₂, 209 mM KCl (adjust this to the desired ionic strength), 1.54 mM EGTA (or 0.75 mM CaCl₂), and 1.54 mM dithiothreitol.
34. Actin-pyrene labeled ([12,13](#)): To 20 mg highly purified F-actin in 4 mM imidazole, 2 mM MgCl₂, add 1 mL actin modification buffer and water to 10 mL. Stir until the actin is homogeneous. Add 0.1 mL of 14 mg/mL solution of *N*-(1-pyrene)iodoacetamide in dimethylformamide with rapid stirring. Incubate 15 h at 10°C in the dark with slow stirring. Stop the reaction by adding a small flake of dithiothreitol. Centrifuge the actin in a Beckman 50 Ti rotor for 20 min at 60,000g. Save the supernatant containing the pyrenyl actin. Centrifuge the supernatant in a 50Ti rotor at 134,000g for 1 h. Remove the supernatant. Add 5 mL 4 mM

- imidazole, 2 mM MgCl₂ to the pellet, and allow the tube to stand on ice in the dark for at least 1 h. Homogenize the softened pellet and dialyze against two changes of buffer of choice. Determine the concentration of actin by the Lowry assay and the concentration of bound pyrene by the absorbance at 344 nm using an extinction coefficient of $2.2 \times 10^4 M^{-1} \text{cm}^{-1}$.
35. Phalloidin-F-actin: To 0.67 mL 100 mM NaCl, 10 mM MOPS pH 7.0, add 0.03 mL 1 mM phalloidin (Cat. #P2141, Sigma, St. Louis, MO) made in methanol and mix. Add 0.3 mL 100 μ M actin or pyrene-labeled actin and mix gently.
 36. Actin-binding proteins of interest: The protein should be pure and homogeneous. The protein should be in a buffer that will not destabilize the protein and which will not interfere with subsequent analysis. Proteins used for co-sedimentation with actin should be centrifuged at 106,000g in a Beckman 50Ti rotor (or equivalent) for 1 h to remove aggregates.
 37. Myosin S1: S1 prepared by enzymatic digestion should be purified by gel filtration chromatography on Sephacryl S-300 HR or an equivalent resin. Skeletal muscle S1 has 2 isoforms that bind differently to actin at low ionic strength (14). These isoforms may be resolved by ion-exchange chromatography on DEAE resin (15).
 38. Ligand proteins modified with ¹⁴C or fluorescent probes: This procedure may be optimized by adjusting the molar ratio of reagent to protein and the reaction time. Dialyze the protein against reducing buffer. After dialysis, add sufficient phosphate buffer, pH 6.0, to bring the final buffer concentration to 50 mM. Add dithiothreitol to bring the final concentration to 10 mM and incubate for at least 30 min at 37°C. Dialyze twice against a large excess of 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol, or run over a desalting column equilibrated with the same buffer. Adjust the protein concentration to about 2 mg/mL. Add the fluorescent reagent at a concentration equal to 0.1 mM plus three times that of the concentration of the protein. Incubate in the dark at 18°C for 4 h; stop the reaction with a small flake of dithiothreitol. Clarify the modified protein by centrifugation and remove unreacted probe by extensive dialysis or gel filtration chromatography. If the thiol groups of the protein are not particularly labile, the 0.1 mM dithiothreitol may be omitted from the dialysis buffer or column buffer. In that case, the amount of fluorescent reagent should be simply three times that of the concentration of the protein. Note that the amount of fluorescent reagent should be adjusted to obtain the desired extent of labeling for the particular protein studied.

3. Methods

3.1. Binding Methods Based on Pelleting Actin-Ligand Complexes

Protein mixtures are centrifuged. The actin-ligand complexes in the pellets and the supernatants are analyzed to determine the bound and free ligand, respectively. Measurement of both the ligand protein and actin may be done by several methods including polyacrylamide electrophoresis, capillary electro-

phoresis, HPLC, and enzymatic assays of the ligand protein. Enzymatic assays are useful when the ligand concentration is very low relative to the actin concentration, as in the case of S1 binding to actin during steady-state ATP hydrolysis.

3.1.1. Binding of Ligand to Actin

1. For moderate affinity complexes prepare 0.6-mL solutions containing various concentrations of the ligand protein and $0.5\mu\text{M}$ phalloidin-F-actin. It is best to use small Teflon beakers to stir the mixtures slowly at 5°C for 30 min or until the mixtures are homogeneous.
2. Transfer 0.5 mL of each mixture to a centrifuge tube and incubate for 5 min at the desired temperature.
3. Centrifuge the samples at the desired temperature for a sufficient time to produce a firm actin pellet (see below).
4. Remove all the supernatant and place into a small clean tube.
5. Carefully wash the pellet with 0.5 mL buffer to remove residual supernatant. Discard the wash.
6. Analyze the actin pellet to determine the ratio of bound ligand to actin monomer.
7. Analyze the supernatant to determine the amount of free ligand. It is also possible to determine the bound ligand from the free and vice versa using the conservation of mass equation: $[\text{Ligand}]_{\text{Bound}} = [\text{Ligand}]_{\text{Total}} - [\text{Ligand}]_{\text{Free}}$. Analyze both the supernatants and pellets whenever feasible.

3.1.2. Sedimentation of Actin–Ligand Complexes

Several combinations of centrifuges and rotors can be used for these assays. Sedimentation can be done in a Beckman Airfuge with rotor A-95 at 134,000 *g* for 20 min (16) with a TLA 120.1 rotor in a Beckman Optima TL ultracentrifuge at 43,500 *g* for 30 min, or with in a 50 Ti rotor in a preparative ultracentrifuge at 134,000 *g* for 30 min. The manufacturer of the rotor should be consulted for optimal volumes to be used in the respective centrifuge tubes. The rotor and centrifuge must be at the desired temperature before starting the binding reaction.

3.1.3. Controls for Sedimentation Assay

3.1.3.1. SEDIMENTATION IN THE ABSENCE OF ACTIN

The most important control to run is the ligand in the absence of actin. Partial sedimentation of the ligand protein alone is normal. Ligand proteins may also be lost by adsorption to the walls of the vessel. The value of the amount of ligand in the control pellet should be added to the experimental supernatant and subtracted from the experimental pellet value prior to calculating binding constants. This correction can be minimized by using Teflon beakers and by adding

a carrier protein or a detergent to the assay mixture. Highly purified bovine serum albumin at 0.1 mg/mL or 0.05% Tween 20 works well with a number of actin-binding proteins.

3.1.3.2. DETERMINATION OF THE OPTIMUM ADDITIVE CONCENTRATION

The optimum additive concentration maximizes the amount of ligand in the supernatant in the absence of actin and does not inhibit ligand binding to actin.

1. Prepare several centrifuge tubes each containing buffer and the pure ligand.
2. Prepare a duplicate set of tubes that also contain actin.
3. To each tube add a different concentration of a detergent or carrier protein.
4. Centrifuge all tubes and analyze the supernatants and pellets as in a normal binding assay. The optimum concentration of additive gives the maximum amount of bound ligand after correction for sedimentation in the absence of actin.

3.1.3.3. CORRECTIONS FOR INACTIVE ACTIN OR LIGAND

Other corrections that may be significant are for “dead” actin and ligand. The fraction of actin that is functional may be equated to that fraction that binds to S1 in the absence of nucleotide at moderate ionic strength. It is difficult to correct binding studies for the fraction of inactive actin unless the ligand binds to a single actin protomer. The best way to handle inactive actin is to eliminate it by further purification such as by going through another cycle of polymerization.

One can test the quality of the ligand protein by conducting a binding experiment at a low and constant ligand concentration with increasing actin concentrations. All of the ligand should be bound in the limit of infinite actin concentration. A Lineweaver–Burke plot ($1/\text{initial velocity}$ vs. $1/[\text{actin}]$) can be used to estimate the fraction of ligand that does not bind to actin. If the fraction of damaged ligand determined by this method is relatively small, it is possible to correct the total ligand concentration by multiplying the total concentration by $(1 - \text{fraction dead ligand})$.

3.1.3.4. REMOVAL OF NONFUNCTIONAL LIGAND

Nonfunctional ligand can often be removed by employing a method of purification that selects for functional ligand. In the case of S1, it is possible to take advantage of the effect of ATP on actin affinity.

1. Prepare a buffer solution containing about 200mM NaCl, 5 μ M S1, and 5 μ M actin.
2. Centrifuge for a sufficient length of time to produce a tight actin pellet and discard the supernatant.

3. Allow the pellet to swell in a small volume of the same buffer.
4. Homogenize pellet gently in a glass–Teflon homogenizer in a buffer containing 2mM ATP to release the bound S1. This step must be done quickly so that the ATP does not become depleted.
5. Rapidly centrifuge again to form another actin pellet. The supernatant is enriched in functional S1.
6. Remove residual actin and ATP by column chromatography. A modification of this procedure can be used for other actin-binding ligands by employing a combination of conditions that alternatively favor binding and that promote dissociation.

3.1.4. Analysis of Pellets and Supernatants

3.1.4.1. GEL ELECTROPHORESIS

Supernatants from sedimentation experiments are readily analyzed by adding an SDS-containing sample buffer directly to the samples. Analysis of the pellets requires a little care.

1. Soften pellets by incubating on ice for 1 h after the addition of water equal to half of the initial reaction volume.
2. Add a volume of a 2X concentrated sample buffer equal to the volume of water added in step 1 and suspend the pellets using a bath sonicator.
3. Place supernatants and pellet solutions in a boiling water bath for 2 min to denature all proteins.
4. Following electrophoresis, scan the stained gels to determine the density of the actin and ligand bands. Densities can be converted into masses by the use of standard curves.
5. Prepare standard curves over a wide range of protein masses to ensure linearity over the range actually used in the assay. Run at least two concentrations of standards (a mixture of known amounts of actin and the ligand proteins) on each gel to correct for gel-to-gel differences in staining intensity.

3.1.4.2. HPLC

Reverse-phase HPLC is useful because the organic solvent keeps the actin in a monomeric state so that it can run readily on the column.

1. Mix protein pellets with 0.05 mL 20% acetonitrile, 0.1% trifluoroacetic acid in water.
2. Incubate the pellets in this solvent for a minimum of 1–2 h and then homogenize them mechanically or by using a water bath sonicator. It is critical that the proteins be totally solubilized.
3. Load the samples onto a C18 column (i.e., μ Bondapak; Waters Inc.) equilibrated with 20% acetonitrile and 0.1% trifluoroacetic acid.
4. Wash with the same buffer for 5 min at 1 mL/min.
5. Elute the proteins with a gradient to 70% acetonitrile over 25–30 min.

6. Monitor the protein peaks at 214 nm and analyze the peak areas either by weighing the cut-out peaks from chart paper or by utilizing a software package (i.e., Chrom Perfect Spirit Tiger II; Justice Laboratory).
7. Determine the amount of each protein in each mixture by comparing the areas of all peaks in the mixture with standards of the respective proteins. For an example of this procedure, see Fredricksen et al. (2003) (9).

3.1.4.3. RADIOACTIVELY LABELED LIGAND

Ligand proteins containing thiol groups may often be readily labeled with ^{14}C -iodoacetamide. The amount of ligand bound may be determined by dissolving the actin pellet after centrifugation and determining the radioactivity in a scintillation counter. One can also measure the loss of radioactivity from the supernatant as a measure of binding (17,18).

Radioactive probes simplify quantitation of binding and allow a wide range of ligand concentrations to be analyzed. However, the investigator must determine the extent to which modification has altered the affinity of the ligand protein for actin. Competitive binding studies are useful for determining the relative functionality of a modified ligand. The labeling procedure has not altered the affinity if binding of the radioactive ligand is reduced to 50% when the concentrations of unlabeled and labeled ligands are equal.

It is also important to confirm that the label (whether radioactive or fluorescent) is on the desired ligand.

3.1.4.4. SPECIFIC LIGAND ASSAYS: ATPASE ACTIVITY

The concentration of free and bound ligand can be determined by an assay specific for the ligand. A common example of this approach is the use of ATPase rates to determine free myosin S1 concentrations when examining binding to actin during steady-state ATP hydrolysis (*see Subsection 3.1.3.3.*). Low S1 concentrations must be used to ensure that the ATP has not been exhausted during the mixing and sedimentation steps. (*See Note 6.*) Binding may be measured by varying the actin concentration at a constant low S1 concentration (typically $0.1\ \mu\text{M}$ for assays at 25°C). A good way to ensure that the binding is valid is to show that the same fraction bound is obtained when the S1 concentration is reduced.

The rate of ATP hydrolysis in the presence of ammonium ions may exceed the physiologically relevant actin activated rate and is well suited for determining the concentration of myosin in sedimentation assays. Metal ions interfere with this assay so Tris base should be used for adjusting the pH of stock solutions. F-actin also inhibits this ATPase reaction, so F-actin must be eliminated or depolymerized. F-actin is depolymerized in

the buffer described below by the combined effects of EDTA and high ionic strength.

1. The binding mixture consists of 1 mL of a solution containing 0.1–0.3 μM S1, a variable amount of F-actin, 2 mM MgATP, 2 mM MgCl_2 , 10 mM imidazole pH 7.0, 1 mM dithiothreitol, and ammonium chloride to obtain the desired ionic strength. Add all components, except the S1, into small Teflon beakers and stir on ice for 15 min.
2. Initiate binding reactions by adding S1 from a 2 μM stock. Mix for 1 additional min.
3. Transfer known amounts of the mixtures to chilled centrifuge tubes. Incubate in a water bath for approximately 5 min to reach the desired temperature.
4. Centrifuge at 134,000 g for 25 min in a 50Ti rotor.
5. Remove the entire supernatant and place into ice-cold glass tubes.
6. Determine the S1 content of each supernatant using the ammonium-EDTA ATPase assay (see below).
7. Add 0.35 mL of each supernatant to a tube or Teflon beaker containing 0.5 mL ammonium-EDTA reagent. When measuring low levels of S1, it is helpful to add 1 mg/mL tropomyosin or bovine serum albumin as a carrier.
8. Incubate the samples in a water bath at 25°C. Start the reaction by adding 0.15 mL 35 mM γ - ^{32}P ATP-Tris.
9. Remove 0.2 mL reaction mixture at 2, 5, 10, and 20 min after initiating the reaction for determination of released phosphate. Place each 0.2-mL aliquot into a disposable tube (about 1 cm $\times$ 9 cm) containing 0.4 mL ATPase quench solution. Vortex briefly, and extract the ^{32}P i by the modification of a standard method (19) described below.
10. To each tube add sequentially 0.2 mL silicotungstic acid solution, 1.0 mL 1:1 isobutanol: benzene (use in a hood), and 0.2 mL 5% ammonium molybdate. Vortex rapidly for exactly 30 s.
11. Allow the tube to rest for several minutes until the bright yellow organic phase separates from the lower clear aqueous phase. The upper phase contains the ^{32}P i, which can be analyzed by scintillation counting. If the phases fail to separate well (i.e., when high protein concentrations are used), centrifuge the tubes in a hood for 10 s at low speed.
12. Remove 0.25 mL of upper yellow phase and add to a 10-mL scintillation vial. Add 8 mL scintillation fluid and count for sufficient time to collect at least 10^4 counts. (See **Note 7**.)

The ATPase rate of the no-actin control ($v_{A=0}$) is equivalent to 0% bound or 100% free S1. The fraction of bound S1 (FB) bound at a given actin concentration can be determined directly by Equation 1 where $v_{A=C}$ is the rate at some fixed actin concentration.

$$(1) \quad \text{FB} = 1 - (v_{A=C}/v_{A=0})$$

3.2. Fluorescence-Based Actin Binding

Fluorescence assays can be used to measure binding when there is a change in the environment of a probe on either actin or the actin-binding protein. Environmentally sensitive probes are most easily utilized when placed on the lattice protein, actin. Changes in the fluorescence of a pyrene probe placed on Cys 374 of actin form the basis for several assays.

The following paragraph describes the measurement of binding of S1-ADP to pyrene-labeled actin. Variations of this method may apply to other actin-binding proteins.

3.2.1. Fluorescence Method

1. Into a 1 × 1 cm fluorescence cuvette, add 0.2 mL 3 mM phalloidin pyrene-actin, 1.3 mL EGTA buffer, and 0.5 mL 8 mM ADP buffer.
2. Incubate in the fluorimeter until the solution is at the desired temperature.
3. Set the excitation and emission wavelengths for 364 and 384 nm, respectively.
4. Record the initial fluorescence. Add S1 in a stepwise manner using a concentrated stock to minimize dilution. Subsequent additions should not be made until the fluorescence amplitude is stable; it may take several minutes for equilibrium to be reached. Begin by adding 1-μL aliquots of 50 μM myosin S1. When the fluorescence changes become small, add 3- or 5-μL aliquots of S1. Finally, add 5-μL fractions of 200 μM S1.
5. Continue the titration until there is no fluorescence change on addition of ligand. The experiment should be planned so that the plateau is reached when the added ligand volume is less than 0.2 mL so that the correction for the volume change will be small.

3.2.2. Fluorescence Data Corrections

Correct the data for dilution of the fluorescent probe and for changes in concentrations of the proteins. Corrections are imperfect, so it is preferable to avoid changes in volume greater than 10%. Spreadsheet programs are convenient for making these corrections. (See **Note 8**.)

3.3. Analysis of Actin-Binding Data

3.3.1. Noncooperative Binding with $n = 1$

A simple hyperbola can be fitted to the data to obtain the dissociation constant, K_{diss} , when there is no cooperativity when the binding site consists of one actin protomer ($n = 1$). Let θ be the ratio of the ligand-actin complex to total actin and let L_F be the free ligand concentration. θ is given by Equation 2.

$$(2) \quad \theta = (n \times L_F) / (K_{\text{diss}} + L_F)$$

A poor fit of a hyperbola to data may occur if the binding is cooperative, if the endpoint had not been determined accurately, or if there is nonspecific binding.

The dissociation constant can also be determined by an equation expressed in terms of total actin, $[A_T]$, and total ligand, $[L_T]$, concentrations (20):

$$(3) \quad \theta = \{([L_T] + K_{\text{diss}} + [A_T]) - \sqrt{([L_T] + K_{\text{diss}} + [A_T])^2 - 4[L_T][A_T]}\} / 2[A_T]$$

3.3.2. General Model

In cases where a ligand interacts with several actin protomers of the actin lattice, the relationship between theta and the free ligand concentration depends on additional factors. **Figure 2** illustrates the binding of a ligand (solid bars) to three actin protomers (open circles) within a long actin filament. The actin filament shown is partially decorated with ligand. Consider the possible fates of another ligand as it binds to actin. If the ligand were to bind to the region of actin designated as “A,” the binding would be described with an association constant K . If that same ligand were to bind instead to region “B,” the affinity would be equal to $K\omega$. If $\omega > 1$, the binding would be stronger than to an isolated site and there would be positive cooperativity. If $0 < \omega < 1$, the binding would be weaker than to an isolated site, and there would be negative cooperativity. Binding to a doubly tandem site as in “C” would be described by $K\omega^2$. Binding is impossible if the gap between two bound ligands is less than the size of the binding site, as in “D.” This last case illustrates a parking problem.

Saturation of the actin lattice with ligand is difficult when there is a parking problem, as the individual ligands must rearrange themselves to eliminate these gaps. An experimental way to minimize the parking problem is to mix the ligand with G-actin and initiate polymerization (*see* Fredricksen et al. 2003) (9). In that way the ligand can bind to the growing filament so that gap formation is unlikely. Obtaining full saturation and thus identifying the true number of protomers in a binding site, n , may be experimentally difficult when the value of n is large.

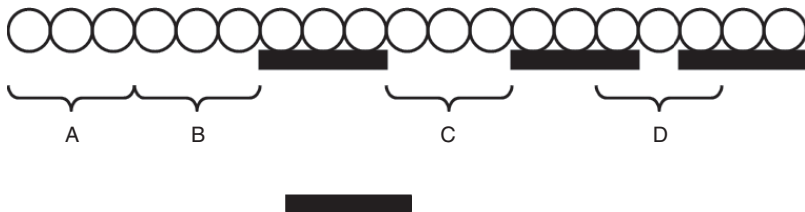


Fig. 2. Binding of a ligand (solid bar) where the binding site consists of three actin protomers: (A) binding to an isolated site; (B) binding to a singly contiguous site; (C) binding to a doubly contiguous site; (D) a gap too small for binding to occur.

Binding to an actin-like lattice of sites is correctly described by Equation 4 below (21). (See Notes 9 and 10.)

$$(4) \quad \frac{\theta}{L_F} = K \cdot (1-n\theta) \cdot \left(\frac{(2\omega-1)(1-n\theta) + \theta - R}{2(\omega-1)(1-n\theta)} \right)^{n-1} \cdot \left(\frac{1-(n+1)\theta + R}{2(1-n\theta)} \right)^2$$

Relationships of the binding curves to the parameters n and ω are shown in Fig. 3. Note that the ordinate of Fig. 3 is $\theta \cdot n$ (rather than θ) so that all curves reach a maximum of 1.0 for ease of comparison. The log of the concentration is shown so that the full binding profile can be examined. The curve with $n = 1$ is a simple S-shaped binding curve on the log plot and a hyperbola in the direct plot (see inset). Saturation of the actin filament becomes progressively more difficult as the value of n increases as a result of the parking problem. When $n = 7$, saturation is not reached even when the free ligand concentration is 10^4 times the dissociation constant.

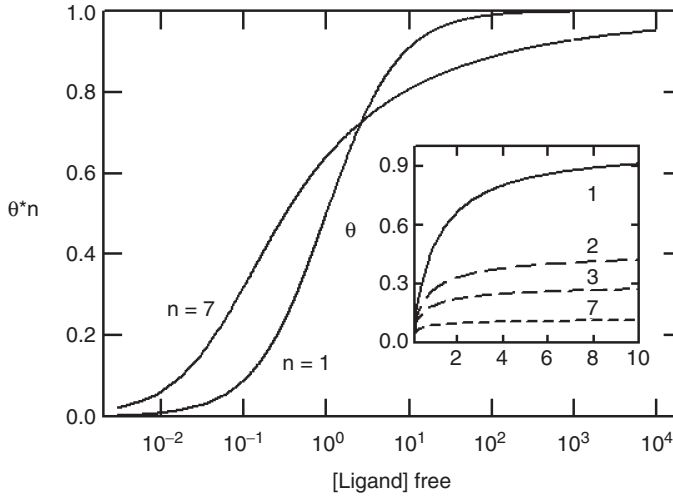


Fig. 3. Relationship of the binding curve to the number of actin protomers comprising a binding site. The ordinate is the product of theta (θ) and n so that both curves reach the same maximum value. Simulations used the McGhee and von Hippel equation with values of $K = 1$ and $\omega = 1$. Ligand concentration is on a log scale. Inset shows the low concentration region of binding. Data are plotted as theta (not $\theta \cdot n$) against the concentration (linear scale). Note that values of $n > 1$ give the appearance of negative cooperativity so that saturation is not reached even when the ligand concentration is 10^4 that of the binding constant, K .

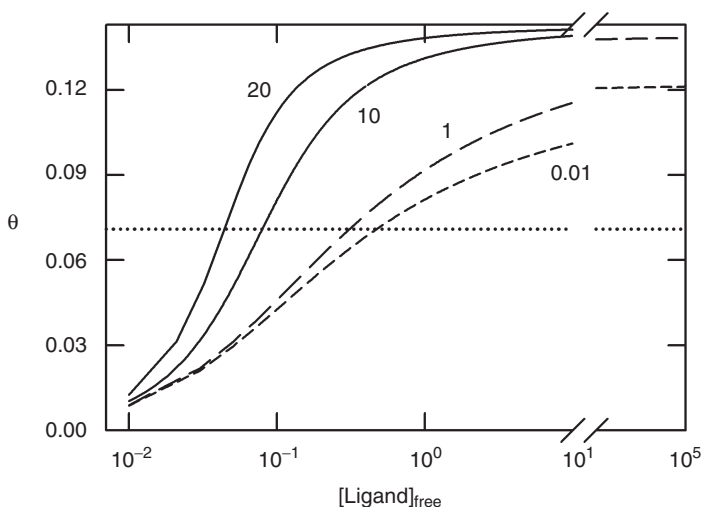


Fig. 4. Effect of the cooperativity parameter, ω , on the binding of a hypothetical ligand to actin having $n = 7$ and $K = 1$. Simulations used Equation 5. As in Fig. 5, the value of $n = 7$ caused saturation with the ligand to be difficult to achieve even at extreme ligand concentrations. However, the presence of positive cooperativity ($\omega > 1$) permits saturation to be achieved.

Positive cooperativity (negative free energy of interaction among adjacent ligands) can compensate for the parking problem. Figure 4 shows a series of binding curves on a log plot each having $K = 1$ and $n = 7$ but with different values of the cooperativity parameter, ω . Values of $\omega < 1$ exaggerate the effect of the parking problem. However, when the value of ω is greater than 1, and particularly when $\omega > n$, the apparent negative cooperativity disappears.

An important point from Figs. 3 and 4 is that it may be experimentally impossible to reach saturation with ligands having $n > 1$. This creates an ambiguity in the analysis because values of n and ω can compensate for each other and a unique fit may not be obtained. Another complication that can occur is that there may be different interactions of each part of a long ligand protein with each actin protomer. Some analytical approaches to this problem have been published earlier (9,22). Experimental approaches other than binding isotherms explored here are usually required in these cases.

3.3.3. Specific Model for Cooperative Binding of Myosin S1 to Actin-Tropomyosin

In the presence of tropomyosin or troponin-tropomyosin, the binding of S1-ADP or rigor S1 to actin exhibits apparent positive cooperativity. Plots of theta versus $S1_{\text{free}}$ are sigmoidal in the absence of Ca^{2+} (see Greene and Eisenberg, 1980) (17). The attachment of S1-ADP to actin-tropomyosin is thought to stabilize a conformation that binds more tightly to S1 (23). Two models that are based on somewhat different assumptions are commonly used to evaluate this special case. The Hill model describes the binding in terms of four parameters. K_1 is the affinity of S1 to the inactive state of actin-tropomyosin. K_2 is the affinity of S1 to the active state of actin-tropomyosin. Y is a cooperativity parameter. In the case of negative cooperativity $1 > Y > 0$, whereas for positive cooperativity $Y > 1$. The final parameter, L' , describes the tendency of an entire actin filament to exist in the inactive state in the absence of bound S1. The dependence of theta on the free ligand concentration, c , is given by the Hill relationship shown in Equation 5 below. Additional details about that model can be found elsewhere (24). (See **Note 11.**)

$$(5) \quad \begin{cases} \theta = p_1 \frac{K_1 c}{1 + K_1 c} + p_2 \frac{K_2 c}{1 + K_2 c} \\ p_1 = \frac{2aY^{-1}}{\delta(a-1+\delta)} \quad p_2 = \frac{2aY^{-1}}{\delta(1-a+\delta)} \\ \delta = \left[(1-a)^2 + 4aY^{-1} \right]^{1/2} \\ a = (1 + K_2 c)^7 / L' (1 + K_1 c)^7 \end{cases}$$

Figure 5 shows the effect of changes in L' and Y on the simulated binding of a ligand (such as S1) to actin-tropomyosin-troponin. The plots are shown both on a direct scale and on a log scale for comparison to the other figures. A large value of L' means that the inactive state is stabilized. **Figure 5** shows that as L' increases, the free S1 concentration required to stabilize the active state increases (compare curves a, b, and c). Increasing the value of Y increases the sensitivity of the switch so that the transition from the inactive state to the active state occurs over a narrower range of free S1 concentrations. Curves c and d have the same value of L' but d has a larger value of Y . Note that curve d rises more sharply than curve c.

An alternative description of the binding of S1 to actin-tropomyosin has been proposed by Geeves and co-workers. Details of this model have been described elsewhere (24,25).

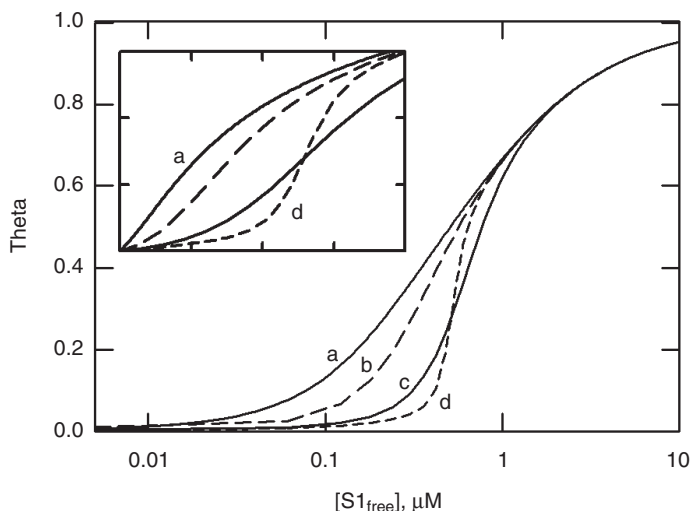


Fig. 5. Simulations of the Hill model for binding of S1 to actin. Each S1 binds to a single actin protomer. Values of theta are shown as a function of the free S1 concentration shown on a log scale. Inset shows the initial part of the curves plotted on a direct scale. The abscissa of the inset is from 0 to $0.8\mu\text{M}$ in steps of $0.2\mu\text{M}$. The ordinate of the inset has values of theta from 0 to 0.6. Curves a through d are simulations with $K1 = 0.1$ and $K2 = 2\mu\text{M}^{-1}$. Values of L' and Y (L' , Y) varied: a (1,1), b (10,1), c (100,1), d (100,10).

4. Notes

1. All chemicals must be A.C.S certified reagent grade or better. Water must be distilled or otherwise purified. Proteins must be pure and the concentrations must be accurate. Most proteins are determined by absorbance measurements corrected for light scattering. Protein concentrations may also be measured by the Lowry assay with a bovine serum albumin standard. The Lowry assay is somewhat dependent on the amino acid composition of the protein and does not necessarily give the absolute protein concentration. Several methods are available for determining the absolute protein concentration directly. We have had success with the *o*-phthalaldehyde method (26). One must hydrolyze the protein and compare the fluorescence of the product formed by reaction with *o*-phthalaldehyde with a standard curve made using alanine *o*-phthalaldehyde complex fluorescence as a standard.
2. The extinction coefficient for adenosine, AMP, ADP, and ATP is $15,400\text{ M}^{-1}\text{ cm}^{-1}$.
3. For added shielding, we slide the individual vials into blocks of acrylic plastic with dimensions $2 \times 2 \times 4\text{ cm}$ containing a hole of appropriate size to accommodate

the vial. For common cryogenic tubes we use holes 12–23 mm in diameter and 3.5 cm deep.

4. Use normal safety precautions when diluting strong acids.
5. Ethyl acetate or isobutanol alone are not as effective as isobutanol:benzene but may be used when safe handling conditions for benzene are not available.
6. An alternative method to study S1-ATP-“like” complexes is to modify S1 with ρ -phenylenedimaleimide to reduce the rate of ATP hydrolysis (27,28).
7. The HCl in the quench stops the reaction while the excess Pi acts as a carrier for the ^{32}Pi . The silicotungstic acid solution precipitates the proteins. Molybdate forms a complex with Pi that is soluble in isobutanol:benzene. It is essential to extract the same fraction of ^{32}Pi in each assay.
8. Theta and free ligand concentrations can be determined from fluorescence measurements by the use of a spreadsheet as shown below. Let the initial volume of solution before addition of ligand be v_i .
 col(1): cumulative volume of ligand added.
 col(2): concentration of the ligand stock.
 col(3): total volume equal to $v_i + \text{col}(1)$.
 col(4): total concentration of ligand = $[\text{col}(1) \times \text{col}(2)]/\text{col}(3)$.
 col(5): insert the raw fluorescence readings for each point of col(2).
 col(6): corrected fluorescence = $\text{col}(5) \times [\text{col}(3)/v_i]$.
 At this point determine the minimum (min) and maximum (max) values of col(6).
 col(7): normalized fluorescence = $[\text{col}(6) - \text{min}]/[\text{max} - \text{min}]$.
 col(8): theta, moles of ligand bound/moles of actin total = $\text{col}(7) \times (1/\text{number of actin protomers in a ligand-binding site})$. Note that if either the initial point or the endpoint is inaccurate the values of theta will be inaccurate. The assumption here is that the fluorescence reaches its maximum when the actin is saturated with ligand. It is a good idea to confirm that assumption with another type of measurement.
 col(9): bound ligand concentration = $\text{col}(8) \times c_A$ where c_A is the total actin concentration.
 col(10): free ligand concentration = $\text{col}(4) - \text{col}(9)$.
 Now produce a plot of theta versus free ligand concentration or col(8) versus col(10). This is the curve that must be analyzed to obtain equilibrium binding parameters.
9. Equation 15 in the original article contains an error; the term $(2\omega + 1)$ should be $(2\omega - 1)$.
10. A sample program for analysis of Equation 4 is given here using the modeling program MLAB (Civilized Software, Bethesda, MD).
 /*This program fits the McGhee and von Hippel model to data pairs where column 1 is the free ligand concentration in micromolar units and column 2 is the value of theta. K is the association constant in micromolar. ww is used as the symbol for the cooperativity parameter in this routine. $ww > 1$ indicates positive cooperativity and $1 > ww > 0$ indicates negative cooperativity. The program may fail with $ww = 1$ but $ww = 1.01$ is generally fine. n is the number of actin protomers forming a single ligand-binding site. Note that the symbol v is used for theta in this subroutine.*/
 DELETE W

```

/*Make initial guesses for n, K, and ww. It is best to have good data at high ligand
concentrations so that the value of n is well constrained.*/
n = 4; K = 2; ww = 1.01
/*Create a data set of two columns with values of free ligand concentration (L)
in micromolar in column 1 and corresponding values of ligand bound/total actin
(v) in column 2. Name the file with a suffix .txt or .dat. The Read statement calls
the data file and defines it as having 36 rows and 2 columns.*/
DD=READ(dataname.txt,36,2)
CONC=DD COL(1)
/*Place reasonable limits on all values in the constraints statement.*/
CONSTRAINTS Q={K>0,K<100, ww >0, ww<100,n>4,n<8}
/*The McGhee and von Hippel equation is defined below. Each function is associ-
ated with a variable given in parentheses after the function name. The function
v(L) contains the ROOT operator that determines the roots of the equation
E(Z) - Z/L = 0. Z is a dummy variable used for theta in place of the v.*/
FUNCTION F(v) = 1-n*v
FUNCTION G(v) = v*(n+1)
FUNCTION R(v) = SQRT((1-G(v))*(1-G(v))+(4*ww*v*F(v)))
FUNCTION H(v) = ((2*ww-1)*F(v)+v-R(v))/(2*(ww-1)*F(v))
FUNCTION J(v) = (1-G(v)+R(v))/(2.0*F(v))
FUNCTION E(v) = K*F(v)*(H(v)^(n-1))*J(v)*J(v)
FUNCTION v(L) = ROOT(Z,0.001,1/n-0.001,E(Z)-Z/L)
/*The following statements define the range over which the functions are evaluated.
POINTNUM is the number of points you wish to include in the simulated curve.*/
STARTPOINT=0.5*MINV(CONC)
ENDPOINT=1.1*MAXV(CONC)
STEP=ENDPOINT/POINTNUM
/*The fit statement below defines which parameters are to be adjusted. FIT(K,
ww, n) allows all three variables to be adjusted while FIT(K, ww) allows only K
and ww to float.*/
FIT(K, ww, n), v TO D, CONSTRAINTS Q
/*The POINTS operator generates a matrix of points of theta and L using the
parameters obtained from the fit.*/
CV=POINTS(v,STARTPOINT:ENDPOINT:STEP)
/*The following statements produce a graph containing the original data, D, and
the fitted curve, CV.*/
DRAW D pt circle lt none color yellow
DRAW CV color yellow lt (.01,.0075,.013,0,0,.0075,0)
top title "McGhee & vonHippel Simulation" font 7 color yellow
bottom title "Free Ligand in microMolar" font 16
left title "Bound Ligand/Total Protein" font 16
frame color grey
imagebox color yellow
view

```

11. MLAB (Civilized Software, Bethesda, MD) routine for the Hill model.
 /*The model of Hill, Eisenberg, and Greene for the binding of myosin S1 to actin-tropomyosin or actin-tropomyosin-troponin. Concentrations are in units of μM and association constants are in units of μM^{-1} .*/
 DELETE W
 STARTCONC=0.001
 ENDCONC=5
 STEPS=(ENDCONC-STARTCONC)/1000
 /*Enter initial guesses for all parameters.*/
 K1=0.1; K2=2; Y=1.01; LPRIME=1.01
 /*Enter constraints or limits for the parameter values.*/
 CONSTRAINTS Q = {Y>1,Y<1000,LPRIME>1,LPRIME<100,K2>0.1,K1>0}
 /*The binding function is defined below.*/
 FUNCTION A(C) = (1 + K2*C)^7/(LPRIME *((1+K1*C)^7))
 FUNCTION S(C) = SQRT ((1-A(C))^2 + 4*A(C)/Y)
 FUNCTION P2(C) = 2*A(C)/Y /(S(C)* (1-A(C) + S(C)))
 FUNCTION P1(C) = 1-P2(C)
 FUNCTION THETA1(C) = K1*C/(1+K1*C)
 FUNCTION THETA2(C) = K2*C/(1+K2*C)
 FUNCTION THETA(C) = THETA1(C)*P1(C) + THETA2(C)*P2(C)
 /*The following statement reads a set of data with 1000 rows and 2 columns. Column 1 contains free S1 concentrations and column 2 contains corresponding values of theta.*/
 D1 = READ(dataname.txt,1000,2)
 /*The following statement fits the Hill model to the data. Values in parentheses are allowed to float. Fits are insensitive to K1 so the value of K1 is held constant.*/
 FIT(LPRIME,Y,K2), THETA TO D1, CONSTRAINTS Q
 /*The following statement produces a matrix defining the best fit of the model to the data.*/
 cv1 = POINTS(THETA, STARTCONC:ENDCONC:STEPS)
 /*The following statements produces plots of the data and of the fitted line (D1).*/
 DRAW D1, LINETYPE NONE, POINTTYPE CIRCLE color white
 DRAW CV1, color white
 /*The following statements define the plot.*/
 top title "Fixed value of Y" font 7 color yellow
 bottom title "[S1]free" font 16
 left title "[S1]bound/[Actin]total" font 16
 frame color grey
 Frame 0.001 to 0.999, 0.001 to 0.999
 framebox
 imagebox color pink
 view

Acknowledgments

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Analysis of Calcium/Calmodulin Regulation of a Plant Kinesin Using Co-Sedimentation and ATPase Assays

Anireddy S.N. Reddy

Summary

Kinesins, a superfamily of microtubule motor proteins, are implicated in regulating a number of fundamental cellular and developmental processes including intracellular transport of vesicles and organelles, mitotic and meiotic spindle formation and elongation, chromosome segregation, germplasm aggregation, microtubule (MT) organization and dynamics, and intraflagellar transport. Analysis of all the completed genomes of eukaryotes has revealed that *Arabidopsis*, a flowering plant, has more kinesins than any other organism. Although a complete inventory of kinesins in a number of organisms has been reported, the function and regulation of kinesins in general and plant kinesins in particular are poorly understood. In our screen of an expression library with a labeled calmodulin, we isolated a novel plant kinesin (kinesin-like calmodulin-binding protein, KCBP) from plants, which interacts with calmodulin in a calcium-dependent manner. This chapter describes the methods used in elucidating the regulation of this motor protein by calcium/calmodulin.

Key Words: calcium; calmodulin; KCBP, co-sedimentation assay; ATPase assay; kinesin; motor protein; microtubule (MT)

1. Introduction

Calcium, a universal messenger in eukaryotes, regulates a plethora of physiological and developmental processes in plants (1). It has been implicated in mediating the action of many hormonal and environmental signals (2). Many signals that affect plant growth and development have been shown to elevate cytosolic calcium. These changes in cytosolic calcium are transmitted to the metabolic machinery of the cell via calcium sensors. These sensors either directly or indirectly regulate cellular processes. In recent years, a large number (more than 250) of putative calcium sensors have been identified (3). Some of

these are enzymes (e.g., calcium-dependent protein kinases), and the activity of these enzymes is modulated directly by calcium. Other calcium sensors, such as calmodulin, have no enzymatic activity but interact and regulate the activity of a large number of diverse proteins including protein kinases, phosphatases, transcription factors, and cytoskeletal proteins in a calcium-dependent manner. To understand the calcium/calmodulin-regulated process in plants, much focus has recently been aimed at identifying the proteins that interact with activated (calcium-bound) calmodulin. These efforts have resulted in identification of more than 150 calmodulin target proteins (2,4). In our screens of expression libraries with labeled calmodulin, we isolated a novel calmodulin-binding kinesin from *Arabidopsis* and a number of other plants (5,6), raising the possibility that this kinesin is regulated by calcium via calmodulin. The methods described below have allowed us to demonstrate that activated calmodulin inhibits motor interaction with microtubules (7–9), suggesting that the elevated levels of calcium inactivate kinesin-like calmodulin-binding protein (KCBP).

2. Materials

2.1. Constructs of *Arabidopsis* KCBP and Calmodulin

1. Full-length KCBP is 1261 amino acids (aa) long. Two truncated versions of KCBP fused to different tags (7,10) are used in these experiments: (i) 1.5 C in pET-32 (aa 821–1261) and (ii) 1.0 C in pET-28 (aa 860–1210) (see Fig. 1 for the tags on each of these fusions). *Escherichia coli* BL21(DE3) was used to express these proteins.
2. pET-5 constructs of *Arabidopsis* calmodulin2 (CaM2), calmodulin4 (CaM4), and calmodulin6 (CaM6) isoform in *E. coli* BL21(DE3) (11).

2.2. Expression and Purification

2.2.1. CaM Isoforms

1. NZY medium: Dissolve 10 g NZ amine, 5 g NaCl, 2 g $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ in 1 L, adjust pH to 7.0, and sterilize by autoclaving for 20 min on liquid cycle.
2. Ampicillin: 50 mg/mL in water.
3. Isopropyl-1-thio- β -D-galactopyranoside (IPTG): 100 mM in water.
4. Buffer A: 50 mM Tris-HCl, pH 7.5.
5. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 2 mM Na_2 ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 200 $\mu\text{g/mL}$ lysozyme.
6. DNase.
7. Saturated $(\text{NH}_4)_2\text{SO}_4$. Dissolve 69.7 g ammonium sulfate in 10 mL water.
8. DTT: 1 M stock in water.

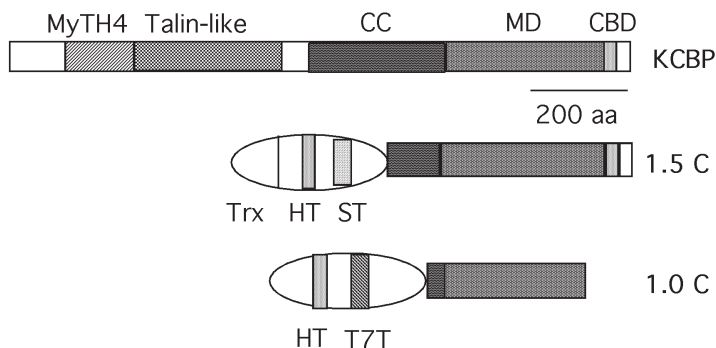


Fig. 1. Schematic representation of fusion proteins of *Arabidopsis* kinesin-like calmodulin-binding protein. KCBP, the full-length *Arabidopsis* kinesin-like calmodulin-binding protein, showing various domains that are identified based on sequence similarity or functional analysis of *Escherichia coli*-expressed protein (5,18); MyTH4, a domain present in the tail region of some members of the myosin superfamily (VIIa, IV, X, and XII); CC, α -helical coiled-coil region; MD, motor domain; CBD, calmodulin-binding domain; 1.5C, the carboxy-terminal fusion protein (amino acids 821–1261) of KCBP containing motor and calmodulin-binding domains and a limited coiled-coil stalk; 1.0C, the carboxy-terminal fusion protein (amino acids 860–1210) containing a short coiled-coil stalk and motor domain but without calmodulin-binding domain; HT, His.tag for affinity purification of fusion protein; T7T, T7.tag; Trx, thioredoxin for increased solubility of fusion protein; ST, S tag.

9. Buffer 1: 50mM Tris-HCl, pH 7.5, 0.1mM CaCl_2 , and 0.5mM DTT.
10. Buffer 2: 50mM Tris-HCl, pH 7.5, 0.1mM CaCl_2 , and 0.5mM DTT, 5mM NaCl.
11. Buffer 3: 50mM Tris-HCl, pH 7.5, 0.1mM ethyleneglycoltetraacetic acid (EGTA), and 0.5mM DTT.

2.2.2. KCBP

1. Luria-Bertani (LB) medium: 1% bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl. Adjust pH to 7.0 with NaOH. Sterilize the medium by autoclaving for 20min on liquid cycle.
2. Ampicillin: 50mg/mL in sterile water.
3. Kanamycin: 50mg/mL in sterile water.
4. Isopropyl-1-thio- β -D-galactopyranoside (IPTG): 100mM in water.
5. Calmodulin-Sepharose (Pharmacia).
6. His-bind matrix (Novagen).
7. Complete protease inhibitor tablets (Boehringer Mannheim).

2.3. Co-Sedimentation of Microtubules with Motors

1. Tubulin (cytoskeleton): 10 mg/mL.
2. 5X microtubule preparation buffer: 400 mM piperazine-*N,N*-bis[2-ethanesulfonic acid] (PIPES), pH 6.8, 5 mM MgCl₂, 5 mM EGTA, 5 mM GTP, 5 μ M taxol, and 50% glycerol.
3. 10X co-sedimentation assay buffer: 200 mM PIPES, pH 6.9, 10 mM MgCl₂, 10 mM DTT, 1.5 M NaCl, 200 μ M taxol, 5 mM AMP-PNP.
4. Calcium chloride.
5. Arabidopsis CaM isoform.
6. Bovine CaM (Calbiochem).
7. Taxol (paclitaxel; Sigma T-7402): 6 mM in dimethylsulfoxide (DMSO).

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 30% acrylamide/bisacrylamide solution; 29.2% acrylamide, 0.8% bisacrylamide. Acrylamide is a neurotoxin. Wear a mask when weighing acrylamide. Wear gloves in handling this solution. This solution can be stored for a month.
2. 10% ammonium persulfate solution: Prepare fresh solution before use or freeze in aliquots at -20°C .
3. 1.5 M Tris-HCl, pH 8.8.
4. 0.5 M Tris-HCl, pH 6.8.
5. 2X sample loading buffer: 0.125 M Tris HCl, pH 6.8, 4% SDS, 10% β -mercapto-ethanol, 20% glycerol, 0.015% bromophenol blue.
6. 5X electrode buffer: 125 mM Tris base, 960 mM glycine, and 0.5% (w/v) SDS. Use mask while weighing SDS. Do not adjust pH. Store at room temperature.
7. Staining solution: 0.25% Coomassie blue R250, 40% methanol, and 7.5% acetic acid.
8. Destaining solution: 30% methanol and 7% acetic acid.

2.5. ATPase Assay

1. ATPase reaction buffer: 15 mM imidazole, pH 7.0, 2 mM MgCl₂, and 1 mM dithiothreitol (DTT).
2. MgATP: 100 mM in water. Make it fresh.
3. 20% (v/v) Triton X-100. Make it fresh.
4. Malachite green reagent (MGR): Dissolve 340 mg malachite green (Sigma) in 75 mL deionized water and dissolve 10.5 g ammonium molybdate in 250 mL 4N HCl. Mix these two solutions and bring the volume to 1 L with water. Keep this solution on ice for at least 1 h to clear the solution. Filter the solution through Whatman paper and store at 4°C . It can be stored up to 2 months.
5. MGR/Triton X-100: Mix 50 mL MGR prepared as above with 250 μ L freshly prepared 20% (v/v) Triton X-100.
6. 34% (v/v) citric acid: Make fresh.

3. Methods

3.1. Expression and Purification of Different CaM Isoforms

1. Inoculate 3 mL NZY medium containing 50 µg/mL ampicillin with a single colony or 5 µL glycerol stock of a CaM isoform construct and grow overnight at 37°C.
2. Add overnight culture to 500 mL fresh NZY medium containing 50 µg/mL ampicillin and incubate with shaking at 37°C until the OD₆₀₀ reaches 0.6.
3. Add IPTG to a final concentration of 1 mM (5 mL from 100 mM stock) and continue incubation for an additional 3 h at 37°C.

Perform all the following procedures at 4°C.

4. Harvest the cells by centrifugation at 5000g for 5 min (see **Note 1**).
5. Wash the cells by suspending the pellet in 10 mL buffer A and collect the cells as in **step 4**.
6. Resuspend the cell pellet in the lysis buffer, incubate for 30 min, and add DNase to 50 U/mL and MgCl₂ to 3 mM to remove DNA. Incubate for an additional 30 min.
7. Centrifuge the extract at 27,000g for 30 min.
8. To the supernatant, add saturated (NH₄)₂SO₄ dropwise to obtain 55% saturation and stir gently while adding the saturated solution (see **Note 2**). Continue stirring for 30 min to precipitate proteins.
9. Collect the precipitate by centrifugation at 27,000g for 30 min, suspend it in 50% (v/v) H₂SO₄ to bring the pH to 4, and stir it for another 30 min (see **Note 3**).
10. Collect the precipitated protein by centrifugation at 27,000g for 30 min.
11. Resuspend the pellet in 2.5 mL buffer A containing 1 mM DTT.
12. Dialyze the protein for 1 h against 1 L deionized water and then for another 1 h against 1 L buffer A containing 100 mM NaCl, 0.5 mM EGTA, and 1 mM DTT.
13. Following dialysis, centrifuge the protein at 27,000g for 15 min.
14. Add calcium chloride to a final concentration of 5 mM, mix, and load it onto a Phenyl-Sepharose CL-4B column (5 mL bed volume) that was equilibrated with buffer 1. Wash the column with the same buffer until A₂₈₀ is less than 0.01.
15. Wash the column with buffer 2 until the A₂₈₀ = 0.
16. Elute the bound protein with buffer 3.
17. Pool the peak fractions and dialyze first against 20 mM NH₄HCO₃ and then against deionized water (see **Note 4**).
18. Check the purity and concentration of protein on an SDS gel (**Fig. 2**).
19. Aliquot and freeze the dialyzed fractions and store at -80°C or -20°C. About 20 mg purified protein is obtained from 1 L culture.

3.2. Expression and Purification of 1.5C and 1.0C Constructs of KCBP

1. Inoculate 3 mL LB medium containing either 50 µg/mL ampicillin (for pET 32 constructs) or 50 µg/mL kanamycin (for pET28 constructs) with a single bacterial

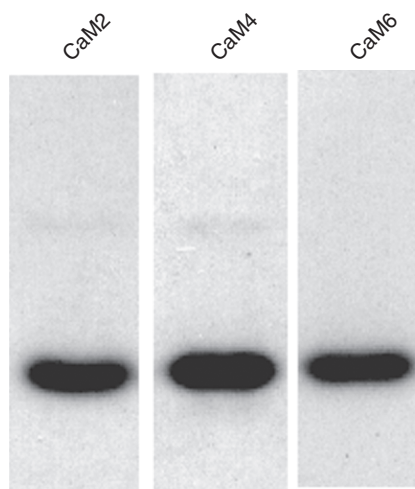


Fig. 2. Stained gel showing the purity of *Arabidopsis* calmodulin (CaM) isoforms. Purified CaM isoforms were analyzed by electrophoresis and stained with Coomassie blue (19).

colony from a freshly streaked plate or 5 μ L glycerol stock and grow the cells overnight at 37°C.

2. Add 2 mL overnight culture to fresh 50 mL LB containing the appropriate antibiotic in a 250-mL Erlenmeyer flask. Incubate the flask at 37°C with shaking (250 rpm) until the A_{600} is about 0.6.
3. Induce the protein by adding IPTG to a final concentration of 0.5 mM (add 0.25 mL IPTG from 100 mM stock) (see Note 5).
4. Grow the culture for another 5 h at 30°C (see Note 6).
5. Harvest the cells by centrifugation at 5000g for 5 min at 4°C (see Note 7).
6. Resuspend the pellet in 5 mL 50 mM Tris-HCl, pH 8.0, and collect the cells by centrifugation as above.
7. Add 5 mL lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM Mg ATP, 0.5 mM β -mercaptoethanol, 0.5 mM DTT, 100 mM lysozyme, and complete protease inhibitor) and place the cells on ice for 30 min (see Notes 8 and 9).
8. Sonicate three to four times, 10 s each, and centrifuge at 12,000g for 30 min at 4°C.
9. Purify the protein containing the calmodulin-binding domain using a calmodulin-Sepharose 4B affinity column and the protein without the calmodulin-binding domain using a His-bind affinity column (see Note 10).

3.2.1. Purification of Calmodulin-Binding Domain (CBD)-Containing KCBP (1.5 C) Using Calmodulin-Sepharose 4B Affinity Column

The binding of calmodulin to its targets occurs mostly through hydrophobic interactions. The hydrophobic regions of calmodulin are exposed in the

presence of calcium. Hence, calmodulin binds to its target proteins with high specificity only in the presence of calcium. Protein extracts containing calmodulin-binding proteins are prepared in a calcium-containing buffer and passed through calmodulin-Sepharose, which allows calmodulin target proteins to bind to the affinity matrix. Following washing, the bound calmodulin-binding protein can be eluted with a buffer containing a calcium-chelating agent such as EGTA. Because of the high specificity and affinity of calmodulin to its target proteins, highly pure protein can be obtained by one-step purification. Perform all operations at 4°C.

1. Prepare a calmodulin-Sepharose 4B column (10 mL bed volume). Equilibrate the column with binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM Mg-ATP, and 1 mM CaCl_2) by passing at least 50 mL binding buffer (*see Note 11*).
2. Load the protein extract from **step 8** above onto the column and allow it to pass through the column.
3. Wash the column thoroughly with the binding buffer until there is no protein in the flow-through ($A_{280} = 0$).
4. Elute the bound protein in elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM Mg-ATP, and 1 mM EGTA) in 2-mL fractions. EGTA strips the calcium from calmodulin and reverses the conformational change, thereby releasing the bound protein.
5. Pool the peak fractions and dialyze against motor buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM Mg-ATP) to remove EGTA.
6. Check the purity and concentration of the protein by running about 30 μL from each fraction on denaturing gels.

3.2.2. Purification of KCBP Without a CBD (1.0C) Using the His-Bind Column

The KCBP (1.0C) that lacked the CBD contains a His-tag at the N-terminus (*see Fig. 1*). The presence of a stretch of histidines permits one-step purification of protein to near homogeneity using an affinity matrix (e.g., His-bind column) that binds the His-tag.

1. Prepare His-bind affinity (10 mL bed volume) column. Equilibrate the column with TN buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 0.5 mM Mg-ATP) containing 5 mM imidazole. Pass at least 50 mL binding buffer (*see Note 9*).
2. Load the protein extract from **step 8** above onto the column and allow it to pass through the column.
3. Wash the column thoroughly with the TN buffer containing 20 mM imidazole until there is no protein in the flow-through.
4. Elute the bound protein in TN buffer containing 100 mM imidazole. Collect 2-mL fractions. A high concentration (100 mM) of imidazole strips the bound protein from the His-bind matrix.

5. Pool the peak fractions and dialyze against motor buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.5mM Mg-ATP) to remove EGTA.
6. Check the purity and concentration of the protein by running about 30μL from each fraction on denaturing gels. Use freshly purified protein for microtubule-binding and ATPase assays (*see* **Note 12**).

3.3. Analysis of KCBP Interaction with Microtubules

3.3.1. Calcium/Calmodulin Regulation of KCBP Interaction with Microtubules

Co-sedimentation of kinesin with microtubules (MTs) in the presence of ATP or nonhydrolyzable ATP analogs has been widely used to study the interaction of motors with MTs (**12,13**). In this simple yet powerful assay, the purified motor protein is mixed with taxol-stabilized MTs, incubated for some time, and centrifuged at high speed to separate MTs (pellet) from unbound kinesin (supernatant). If the motor is bound to MTs, it co-sediments with MTs. The presence of motor in the pellet and supernatant is then analyzed by SDS-PAGE. This method was used to identify MT-binding domains in motors and other MT-interacting proteins as well as to study the regulation of interaction between kinesins and MTs. We have used the co-sedimentation assay to study calcium/calmodulin regulation of KCBP and to identify a second MT-binding domain in the nonmotor region of KCBP (**7–9**).

3.3.2. Preparation of Microtubules

1. To 100μL (10mg/mL) tubulin (*see* **Note 13**), add 10μL 10X MT preparation buffer plus 90μL deionized water and incubate at 35°C for 30 min.
2. Then add 2μL 6mM taxol (*see* **Note 14**), 46μL 50% glycerol, 6μL 10X MT preparation buffer, and 6μL water.
3. Incubate at 35°C for 30 min.
4. Add 5μL 6mM taxol (*see* **Note 14**) and 0.6μL MT preparation buffer.
5. Incubate at 35°C for 5 min.
6. Add another 5μL 6mM taxol (*see* **Note 14**) and 0.6μL MT preparation buffer.
7. Incubate at 35°C for 4h.
8. Sediment microtubules at 10,000g at 35°C for 30 min.
9. Dissolve MT pellet in 200μL 1X co-sedimentation assay buffer to obtain about 5μg/μL concentration (*see* **Note 15**).

3.3.3. Co-Sedimentation Assays

Perform the MT-motor (KCBP) binding assays in a reaction volume of 100μL.

1. Add 10μL 10X pelleting buffer, 2.5μM purified KCBP (*see* **Note 16**), and 10μM taxol-stabilized MTs (co-sedimentation buffer in controls), and bring the final

volume to 100 μ L. Run assays with MT alone and protein alone in parallel as controls. To test the effect of calcium (100 μ M), calmodulin (15 μ M), and calcium/calmodulin on motor binding to MTs, add these test compounds to appropriate assay tubes.

2. Incubate the reaction mixture at 22°C for 20 min.
3. Centrifuge the tubes for 20 min at 100,000g at 35°C.
4. Collect the supernatant (*see Note 17*) and add an equal volume of 2X SDS sample loading buffer. Dissolve the pellet in 1X SDS sample loading buffer.
5. Boil the protein samples for 5 min and analyze them by SDS-PAGE.
6. Visualize the amount of motor in the pellet and supernatant by Coomassie blue staining.

3.4. Denaturing SDS-PAGE

1. Assemble the glass plate sandwiches with two clean glass plates and two 1-mm-thick spacers for each gel and lock them into a casting stand.
2. Prepare 10% separating solution by mixing 15 mL 1.5 M Tris-HCl, pH 8.8, 20.1 mL 30% acrylamide/bisacrylamide solution, 600 μ L 10% SDS, 24.3 mL deionized water, and degas this solution for 10 min. Then add 300 μ L 10-ammonium persulfate and 30 μ L TEMED (tetramethylethylenediamine), and mix the solution by swirling gently (*see Note 18*).
3. With a pipet pour 4.5 mL separating gel solution along the edge of one of the spacers in each minigel, and with a Pasteur pipet gently overlay the separating gel solution with water-saturated isobutyl alcohol; this will leave enough space for the stacking gel. Allow the gel to polymerize for 30–60 min.
4. While the separating gel solution polymerizes, prepare 4% stacking solution by mixing 5 mL 0.5 M Tris-HCl, pH 6.8, 2.6 mL 30% acrylamide/bisacrylamide solution, 200 μ L 10% SDS, 12.2 mL deionized water, and degas this solution for 10 min. Then add 100 μ L 10-ammonium persulfate and 20 μ L TEMED, and mix the solution by swirling gently (*see Note 18*).
5. Pour off the isobutyl alcohol and rinse the top of the separating gel with water to completely remove any residual isobutyl alcohol.
6. Using a Pasteur pipet, pour the stacking solution and insert 1-mm-thick combination into the stacking solution. Allow stacking solution to polymerize for about 30 min.
7. Prepare 1X electrode buffer by diluting 100 mL 5X buffer with 400 mL deionized water.
8. Carefully remove the comb from the stacking gel, rinse the wells with 1X electrode buffer, and fill the wells with the same buffer.
9. Attach the gel sandwich to the upper buffer chamber and place it in the lower buffer chamber with 1X electrode buffer so that the electrode wire is immersed in the buffer. Then fill the upper chamber with 1X electrode buffer.
10. Carefully load the protein samples (prepared as above) into the wells. Load prestained molecular weight markers in one of the lanes (*see Note 19*).

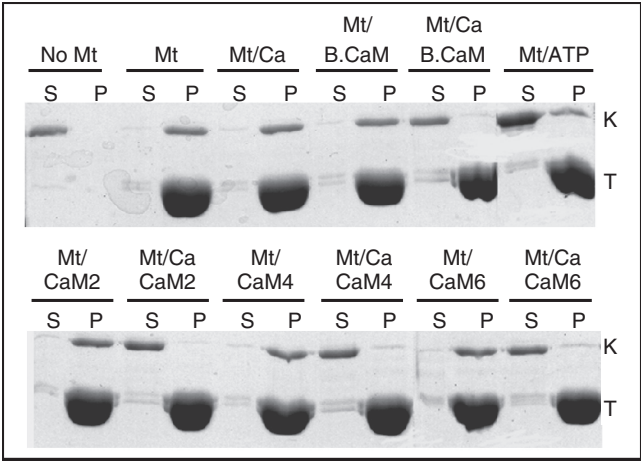


Fig. 3. *Arabidopsis* CaM isoforms regulate the interaction of KCBP containing the CBD with MTs. Purified 1.5C KCBP with the CBD (K) was incubated with MTs (Mt) for 20 min at room temperature (RT) in the presence of Ca^{2+} (Mt/Ca), various CaMs (Mt/B.CaM, Mt/CaM2, Mt/CaM4, or Mt/CaM6) alone or in the presence of both Ca^{2+} and various CaMs (Mt/Ca B.CaM, Mt/Ca CaM2, Mt/Ca CaM4, or Mt/Ca CaM6). Following centrifugation, the supernatant (S) and pellet (P) fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained (stained gel). Except in the Mt/ATP reaction, in which ATP was used, all reactions were performed in the presence of AMP-PNP. No Mt, motor protein without MTs; CaM2, CaM4, and CaM6, three isoforms of *Arabidopsis* CaM; B.CaM, bovine CaM; Mt, microtubules; K, motor; T, tubulin subunits (9).

- 11. Connect the power supply to the electrophoresis apparatus and run at 50 V per gel until the bromophenol blue reaches the bottom of the separating gel.
- 12. Disconnect the power supply, remove the upper buffer chamber with the attached gels, and discard the buffer.
- 13. Remove the gel sandwich from the casting stand and lay it on a paper towel. Carefully pry open the glass plates, and cut a small triangle at one corner of the gel to mark the orientation of the gel.
- 14. To visualize the proteins, immerse the gel in Coomassie blue staining solution for 1 h with gentle shaking, and then briefly rinse the gel with deionized water and transfer it to destaining solution until the gel is completely destained. **Figure 3** shows the binding of 1.5C KCBP to microtubules in the presence of calcium, calmodulin, and calcium/calmodulin.

3.5. Calcium/Calmodulin Regulation of ATPase Activity of KCBP

There are a number of methods (colorimetric and radioactive) to quantify ATPase activity of an enzyme (14,15). Some colorimetric assays are based on

the properties of the complex formed between the inorganic phosphate and molybdate under acid conditions. These assays are useful to study the regulation of MT-dependent and MT-independent ATPase activity of kinesins by other macromolecules that interact with motors. The ATPase assay described here is a simple colorimetric assay based on the change in malachite green with the release of Pi from ATP. The hydrophobic surface of the phosphomolybdate complex binds malachite green dye and shifts the wavelength for the maximum absorbance (16,17). We have used this assay to demonstrate that the MT-dependent ATPase activity of KCBP is inhibited by activated calmodulin.

1. Perform the ATPase assays in a final volume of 50 μ L (*see Note 20*). Perform all assays in triplicate in plastic tubes (*see Note 21*).
2. To each tube, add 5 μ L 10X ATPase reaction buffer, 1.5 μ L 100 mM Mg-ATP (final concentration of ATP is 3 mM), and 2 μ M Taxol-stabilized microtubules and 50–300 nM purified motor protein (KCBP). Bring the volume of all assays to a final volume of 50 μ L. Control assays have no motor protein.
3. To test the effect of EGTA (2 mM), calcium (100 μ M), calmodulin (1 μ M), and calcium/calmodulin on microtubule-dependent and -independent activity of the motor, add these test compounds in the appropriate tubes.
4. Incubate the tubes at 30°C for 20 min.
5. Then add 800 μ L of freshly prepared malachite green reagent/Triton X-100 (*see Note 22*) and 100 μ L 34% citric acid in this order and mix the solutions by vortexing.
6. Incubate the tubes at room temperature for 10 min.
7. Measure the O.D. at 660 (*see Notes 23 and 24*).
8. Subtract the O.D. value of the control (no motor protein) from all assays with motor protein.
9. Calculate the average of triplicates and estimate the released Pi using $1 \text{ OD}_{660} = 9.45 \text{ nmoles Pi}$ and use this value to calculate the specific activity of the motor (i.e., micromoles of Pi released per milligram of motor protein per minute).

4. Notes

1. The cell pellet can be stored at –20°C for several days.
2. Use freshly prepared solution. It is important to gently stir the solution while adding the saturated ammonium sulfate solution to the protein solution; this eliminates spatial nonuniformity in the salt concentration.
3. It takes only a drop or two to lower the pH to 4.
4. Because of the low content of aromatic amino acids, the UV absorbance of CaMs is low. Do not discard the fractions that have low absorbance. Check the concentration of protein by running an aliquot on SDS gels and staining with Coomassie blue. Run a known concentration of bovine serum albumin (BSA) in parallel to estimate the concentration of protein.

5. Induction at room temperature is important to obtain good quantities of motor in the soluble fraction. A high level of protein is induced at 37°C, but most of it goes into the insoluble fraction.
6. Limit the induction time to 3–4 h. Longer induction times result in the loss of protein from the soluble fraction.
7. The cell pellet can be frozen at –80°C for several days.
8. Inclusion of complete protease inhibitor cocktail is necessary to prevent the degradation of motor protein.
9. It is important to include ATP in the buffer to keep the motor protein active.
10. Do not store the protein extract at this stage. Storing of the protein at this step leads to degradation and inactivation of the motor.
11. Inclusion of 150 mM NaCl eliminates nonspecific binding of the proteins to the column.
12. Freezing and thawing of purified motor results in precipitation of protein and loss of MT binding and ATPase activities.
13. Use highly purified tubulin that is devoid of any microtubule-associated proteins (MAPs). The presence of MAPs interferes with the motor activity.
14. Taxol is sparingly soluble in water. Hence, to avoid precipitation mix the solution after adding taxol.
15. Prepare MTs on the day of co-sedimentation or ATPase assays. Polymerized MTs are stable for several hours at 25°C.
16. Before co-sedimentation assays, centrifuge the purified motor protein at 100,000g for 1 h at 4°C to remove any precipitated protein.
17. The MT pellet is very small. Care should be taken to not disturb the pellet while collecting the supernatant. Small (1.5-mL) tubes are better than the larger tubes for these assays.
18. Separating and stacking gel solutions should be prepared fresh. These solutions should be used immediately to prevent polymerization before pouring the gels. For running CaM, prepare 12% running gel.
19. Add 1X sample buffer to any empty wells to prevent distortion of lanes.
20. If needed, the volume can be scaled up to 100 µL.
21. Use disposable plastic reaction tubes as they are devoid of any phosphate residues.
22. Prepare this solution fresh during the incubation time of ATPase assays. Avoid pipeting any precipitated material in the MGR bottle in preparing this reagent.
23. Take the O.D. within 3 min. Microtubules precipitate after 13 min.
24. Use disposable plastic cuvetts for taking the O.D. Reusable glass or quartz cuvetts tend to accumulate color stain on the walls.

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In Vitro and In Vivo Analysis of Microtubule-Destabilizing Kinesins

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Summary

Cellular microtubules are rigid in comparison to other cytoskeletal elements (1,2). To facilitate cytoplasmic remodeling and timely responses to cell signaling events, microtubules depolymerize and repolymerize rapidly at their ends (3). These dynamic properties are critically important for many cellular functions, such as spindle assembly, the capture and segregation of chromosomes during cell division and cell motility. Microtubule dynamics are spatially and temporally controlled in the cell by accessory proteins. Molecular motor proteins of the kinesin superfamily that act to destabilize microtubules play important roles in this regulation (4).

Key Words: Kinesin; microtubule; MCAK; cytoskeleton.

1. Introduction

The kinesin superfamily of proteins has recently been classified into 14 subfamilies based on primary protein structure (5). The proteins that comprise many of these subfamilies utilize the energy from ATP hydrolysis to transport along microtubules. However, at least 2 kinesin subfamilies, the kinesin-13s and kinesin-14s, contain motors with microtubule-destabilizing activity (6–8). Detailed mechanistic studies of the microtubule-destabilizing activity exhibited by these motors have significantly contributed to our understanding of their cellular functions.

In this chapter, we discuss assays for measuring the microtubule-destabilizing activity of kinesins both in a purified system and within the context of mammalian cells. We first describe a turbidity assay that can be used to determine whether a kinesin is a bona fide microtubule destabilizer and to

quantitatively compare the activities of different kinesin preparations. In addition, we report a fluorescence microscopy assay for analyzing the effect of kinesin expression on microtubule stability in cells. We have applied these approaches in combination with site-directed mutagenesis to study the microtubule-destabilizing activity of MCAK (mitotic centromere-associated kinesin), one of the founding members of the kinesin-13 family (9–12). For many of these assays, MCAK protein or constructs provide an admirable positive control for microtubule-destabilizing activity.

2. Materials

2.1. Microtubule Turbidity Assay

1. Purified kinesin: Recombinant kinesin should be expressed in bacteria or insect cells and purified using standard chromatography techniques. Elute the kinesin from the final purification column in motor storage buffer and store frozen in working aliquots.
2. Motor storage buffer: 300 mM KCl, 1 mM MgCl₂, 200 mM imidazole-HCl, pH 7.0, 5 μM ATP, and 20% glycerol in ddH₂O.
3. Tubulin: Purified bovine brain tubulin can be purchased from Cytoskeleton, Inc. (Cat. #T237) as a 10 mg/mL (100 μM) stock solution.
4. GMP-CPP: GMP-CPP can be obtained from Jena Biosciences as a 10 mM stock.
5. BRB80 buffer: 80 mM piperazine-*N,N*-bis[2-ethanesulfonic acid] (PIPES), 1 mM MgCl₂, 1 mM ethyleneglycoltetraacetic acid (EGTA) (pH 6.8 with potassium hydroxide). Sterile-filter and store at 4°C. BRB80 can also be made and stored as a 5X stock.
6. Poly-L-lysine-coated glass coverslips: Coat glass coverslips in bulk by soaking in a 0.1% solution of poly-L-lysine (Sigma) for 15 min. Remove coverslips and allow them to dry individually on a clean Kimwipe or paper towel. Store in a small covered dish or beaker at room temperature until use.
7. Antibody dilution buffer (Abdil): 1% bovine serum albumin (BSA) (IgG-free, protease-free), 0.1% Triton X-100, and 0.02% sodium azide in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl).
8. Nitrocellulose membranes: Use 25-mm-diameter nitrocellulose membranes with 0.45-μm pores.
9. A screen or frit support that allows suction to be applied to filters while collecting flow-through for radioactive waste disposal is needed.
10. Mg-ATP: A stock solution of 100 mM ATP and 100 mM MgCl₂ in water should be sterile-filtered and stored at –20°C in working aliquots.
11. α-³²P-ATP: α-³²P-ATP stock should be purchased from a reputable source as a 10 mCi/mL stock.
12. Turbidity buffer: BRB80 buffer supplemented with 250 μM Mg-ATP, 75 mM KCl, 1 mM dithiothreitol (DTT), and 200 μg/mL BSA.

2.2. *In Vivo* Microtubule Depolymerization Assay

1. CHO cell culture: CHO (Chinese hamster ovary) cells are cultured using standard sterile tissue culture techniques in minimal essential medium- α (MEM- α) medium supplemented with 10% fetal bovine serum (FBS) and grown in a 37°C, 5% CO₂ incubator.
2. Kinesin expression plasmid: Choose a well-characterized vector that expresses the fluorescent protein of choice (*see* **Note 1**).
3. Transfection reagent: Any commercially available transfection reagent or procedure suitable for transfecting plasmid DNA into mammalian cells is usable.
4. Glass coverslip preparation: Wash 12-mm-diameter glass coverslips in 1 M HCl at room temperature for 2 h, stirring occasionally. Rinse five times with ddH₂O. Wash five times with 95% ethanol. Store in 70% ethanol. Flame coverslips in laminar flow hood before use.
5. Phosphate-buffered saline (PBS): Prepare a 1X stock solution of 0.2 M monobasic sodium phosphate, 0.2 M dibasic sodium phosphate, and 150 mM NaCl in ddH₂O. PBS can be prepared as a 10X stock and stored at room temperature after autoclaving.
6. Mounting media: Use an antifade cell mounting media containing a DNA stain such as 4',6-diamidino-2-phenylindole (DAPI).
7. Image analysis software: An image analysis software package that allows quantification of fluorescence intensity in user-defined regions of an image is needed. We routinely use ImageJ, which is available for free download at <http://rsb.info.nih.gov/ij>. The specific commands given for quantifying fluorescence in **Subsection 3.2.3** are for the Macintosh version of ImageJ 1.33.

3. Methods

3.1. *In Vitro* Microtubule Turbidity Assay

Assembled microtubules cause detectable light scattering at 350 nm. This characteristic turbidity can be utilized to assay kinesin-mediated microtubule disassembly (**12**). The turbidity assay is best performed using GMP-CPP-stabilized microtubules. Longer taxol-stabilized microtubules tend to be bundled by added protein, and bundling of microtubules will artificially contribute to light scattering. We describe here how to prepare GMP-CPP microtubules and carry out the turbidity assay. To facilitate accurate comparisons between different preparations or variants of depolymerizing kinesins using the turbidity assay, we also describe a method used to determine the concentration of active motors competent to release ADP and bind ATP.

3.1.1. Preparation of GMP-CPP Microtubules

Tubulin has a higher affinity for guanosine triphosphate (GTP) than it does for GMP-CPP (**13**). Thus, to grow microtubules with an uninterrupted GMP-CPP lattice, a method is required for removing the GTP that is commonly

present in solution with purchased tubulin. To accomplish this, we recommend cycling the tubulin once in GMP-CPP, as noted in **steps 1–4**.

1. Prepare a 200- μ L reaction on ice containing 20 μ M tubulin and 1 mM GMP-CPP diluted in BRB80. Incubate on ice for 10 min. Polymerize reaction at 37°C for 30 min.
2. Sediment the reaction at 150,000 *g* in a small-volume ultracentrifuge at room temperature for 10 min.
3. Resuspend the pellet in BRB80 and disassemble microtubules on ice for 20 min. Sediment at 13,000 *g* for 2 min at 2°–4°C to remove insoluble protein.
4. Remove the supernatant and dilute with BRB80 to achieve a final concentration of 20 μ M tubulin. Add GMP-CPP to 1 mM final concentration and incubate on ice for 10 min.
5. Polymerize the reaction at 37°C for 30 min. Sediment the microtubules at 150,000 *g* for 10 min at 25°C.
6. Resuspend the resulting pellet to a tubulin concentration of 89 μ M. The original tubulin concentration before assembly can be determined by measuring the absorbance (*A*) at 280 nm (*see Note 2*).
7. Aliquots of these cycled GMP-CPP assembled microtubules can be frozen in liquid nitrogen and stored at –80°C. A fresh aliquot must be thawed for each experiment, because assays for depolymerase activity are sensitive to the concentration of microtubule ends and aliquots of assembled GMP-CPP microtubules will undergo end-to-end annealing over time. Thaw one aliquot and calibrate it for microtubule length and end concentration (as described in **Subsection 3.1.2**).

3.1.2. Determination of Microtubule-End Concentration

1. Spin down 100 μ L GMP-CPP microtubule solution in a small-volume ultracentrifuge at 150,000 *g* onto poly-L-lysine-coated glass coverslips (*see Note 3*).
2. Fix coverslips in methanol, rehydrate by rinsing in PBS, and block with 20% donkey serum.
3. Label with antitubulin primary antibody and appropriate fluorescent secondary antibody.
4. Mount coverslips microtubule side down in a drop (1–2 μ L) of antifade mounting medium.
5. Record images of fluorescently labeled microtubules from multiple locations on the coverslip using a wide-field fluorescent microscope equipped with a charge-coupled device (CCD) camera.
6. Measure the average microtubule lengths using an image analysis program. Scale by the magnification factor of the microscope and lens used to convert the microtubule lengths to micrometers. We find that GMP-CPP microtubules prepared as described in **Subsection 3.1.1** are approximately 2.5 μ m in length.
7. Calculate the microtubule-end concentration in the GMP-CPP stock solution (*see Note 4*).

3.1.3. Determination of Kinesin ATP-Binding Site Concentration

1. Wash nitrocellulose filters in 0.4N KOH for 10 min at room temperature; rinse five times with ddH₂O and two times with BRB80 (*see Note 5*). Let stand at room temperature for at least 60 min.
2. Supplement motor storage buffer with 33 μ M cold ATP and 110 nM α -³²P-ATP, correcting for ³²P decay (*see Note 6*).
3. Thaw protein to be assayed on ice. Mix well 2 μ L protein with 18 μ L solution from **step 2**. This solution is the assay mixture. Also prepare a buffer blank with 3 μ L motor storage buffer and 27 μ L solution from **step 2**. Incubate at room temperature for 10–20 min and then place on ice (*see Note 7*).
4. Apply suction to a washed nitrocellulose filter. Spot 5 μ L assay mixture from **step 3** on the filter and immediately wash with 0.5 mL ice-cold BRB80. Remove filter with forceps and place in a scintillation vial (*see Note 8*).
5. Apply 1 μ L buffer blank solution from **step 3** to each of three dry filters and, without filtering or washing, place these in scintillation vials. These filters will provide the total signal for 1 μ L 100 nM α -³²P-ATP.
6. Add scintillation cocktail to all filters and count each for 1 min. Calculate mean values for each reaction.
7. Calculate ATP-binding activity as follows: Subtract the counts per minute (cpm) value for the washed buffer blank from the protein assay value to get blank-subtracted cpm. Multiply this value by the dilution factor used when setting up the binding reaction (20 μ L/2 μ L = 10X) and any intermediate dilution made of the protein stock. Next, divide by the number of microliters spotted on each filter (5 μ L) to give the calculated signal per microliter of protein stock solution. Then multiply by the ratio of cold:hot ATP in **step 4** (50X), divide by the mean cpm from the unwashed filters prepared in **step 5**, and multiply by the concentration of hot ATP in **step 5** (100 nM). For an example calculation, *see Note 9*.

3.1.4. Microtubule Turbidity Assay

1. Thaw an aliquot of GMP-CPP tubulin and dilute it to a concentration of 300 nM in turbidity buffer. The ionic strength of this solution is optimal for microtubule depolymerization assays using MCAK. Equilibrate the solution to 23°C in a quartz cuvette before the addition of motor.
2. Add purified motor that has been calibrated for ATP binding (*see Subsection 3.1.3.*) and mix rapidly using a P1000 pipettor (*see Note 10*).
3. Monitor turbidity in real time by measuring the absorbance at 350 nm at 5-s intervals using a spectrophotometer.
4. Measure the final extent of depolymerization by the addition of 5 mM CaCl₂ to the reaction (*see Note 11*).
5. Normalize turbidity traces from absorbance units to tubulin polymer concentration using a standard curve (*see Note 12* and example in **Fig. 1**).

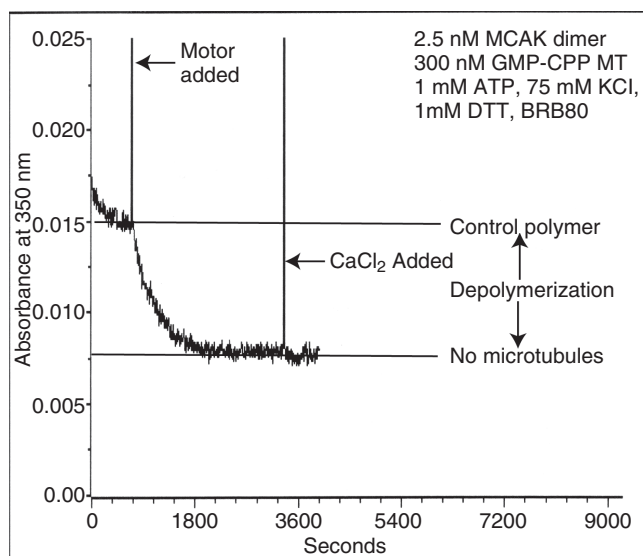


Fig. 1. Example of a typical raw data trace of MCAK-dependent microtubule depolymerization as assayed by turbidity (or absorbance at 350 nm).

3.2. *In Vivo* Assay for Microtubule Depolymerization

In addition to measuring microtubule depolymerization *in vitro*, it is important to determine the ability of a kinesin to depolymerize microtubules *in vivo*. This *in vivo* depolymerization assay depends in large part on the tubulin autoregulatory system. When microtubules are depolymerized by nocodazole or transiently transfected MCAK, the decrease of microtubule polymer and increase of tubulin dimer causes the degradation of β -tubulin mRNA (10,14,15). Over a period of 24 h, this causes an overall reduction of tubulin in the cell, which can be detected by a decrease in tubulin immunofluorescence. To measure microtubule depolymerization of a known or suspected depolymerizing kinesin *in vivo*, tubulin immunofluorescence intensity can be measured after kinesin expression and compared to tubulin immunofluorescence in control cells (10). We utilize Chinese hamster ovarian (CHO) cells for this assay because they consistently express transfected DNA quickly (i.e., within 12–24 h).

3.2.1. Preparation of Cultured Cells

1. CHO cells should be grown using standard sterile cell culture techniques to 70%–90% confluency (see Note 13) and then plated onto glass coverslips (see Note 14).

2. Transfect cells with a recombinant expression plasmid that will result in a high level of GFP-kinesin fusion protein expression (*see Note 1*). As a negative control, an empty EGFP (enhanced green fluorescent protein) vector should be transfected into cells on another coverslip in parallel. Transfection can be carried out with any procedure suited for transfection of mammalian cells.
3. Incubate cells in a 37°C, 5% CO₂ incubator for 24 h before fixation.

3.2.2. Fixing and Staining Cells for Tubulin

1. Fix cells with 1% paraformaldehyde in -20°C methanol for 10 min (*see Note 15*).
2. Wash cells twice with PBS for 5 min each at room temperature.
3. Block for 30 min in Abdil supplemented with 20% donkey serum (*see Note 16*).
4. Briefly rinse coverslips in PBS.
5. Stain cells with primary antibodies against alpha-tubulin (mouse anti-DM1 α ; Sigma) diluted 1:100 in Abdil. Incubate with rotation for 1 h.
6. Wash coverslips for 5 min in PBS.
7. Incubate cells with Texas Red conjugated donkey antimouse secondary antibody (Jackson Immunoresearch Laboratories) diluted 1:75 in Abdil. Incubate with rotation for 1 h.
8. Wash coverslips for 5 min in PBS.
9. On a slide, lay coverslips cell-side-down in 4 μ L antifade mounting media containing a DNA stain such as DAPI.
10. Seal coverslip edges with nail polish.
11. When the nail polish is dry (10–15 min), slides can be viewed immediately or stored at -20°C.

3.2.3. Quantification of Fluorescence

1. When the coverslips are fixed, stained, and mounted, the cells are ready to be assayed using a wide-field fluorescent microscope equipped with a CCD camera. For this analysis, choose only interphase cells that are well spread on the coverslip and which have similar levels of GFP expression (*see examples in Fig. 2*).
2. Starting with untransfected cells, determine an exposure length for tubulin in a normal cell (*see Note 17*). Use this exposure length for all tubulin images analyzed. (With our antibodies and fixation/staining procedure, typical exposure times are between 200 and 400 ms.)
3. Determine the expression level of kinesin that gives complete microtubule depolymerization without protein aggregation (clumps of GFP). Use this exposure length for all GFP images.
4. Acquire images of at least 50 transfected cells per experiment for each construct being tested. Make sure that for each transfected cell imaged there is an untransfected cell in the same frame that has similar size and shape. Depending on transfection efficiency and cell density, one to three coverslips per condition may be needed to find enough usable cells. Organize image files in a way that is optimized for analysis later (*see Note 18*).

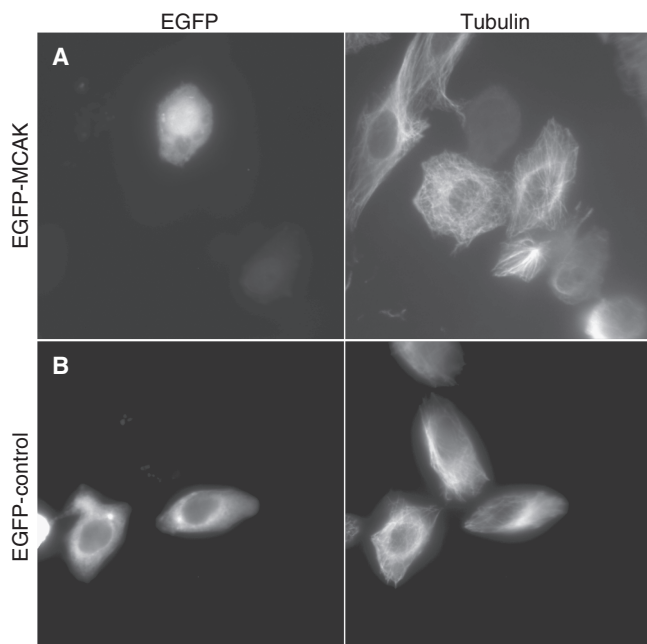


Fig. 2. Example of in vivo microtubule depolymerization by MCAK. CHO cells were fixed and stained for tubulin 24h after transfection with either EGFP-MCAK (**A**) or EGFP-control (**B**) plasmids. (**A**) The average pixel intensity for the tubulin immunofluorescence of the MCAK-transfected cell (TF) is 24.0 arbitrary units. The average pixel intensity for the tubulin immunofluorescence of a neighboring untransfected cell (UTF) is 48.9. The tubulin intensity ratio (TF/UTF) is 0.49. The average green fluorescent protein (GFP) intensity is 137.3. (**B**) The average pixel intensity for the tubulin immunofluorescence of the control transfected cell is 102.5. The average pixel intensity of a neighboring untransfected cell is 114.4. The tubulin intensity ratio is 0.90. The average GFP intensity is 100.8.

5. Once all the images are acquired, open them in ImageJ or similar image analysis software. (File-Import-Image Sequence-Image Folder, then select the first image in the folder and click Open.) Select Reduce to 8-bit and open all files in the folder (see **Note 19**). Set up measurement parameters to collect the following data: area of the cell, average fluorescence intensity, and maximum fluorescence intensity (see **Note 20**).
6. For the first cell, select the area of the cell (use the freehand tool in ImageJ) while viewing either the GFP image or the tubulin image, whichever gives a better outline of the cell. Go to the GFP image and measure the area of the cell and GFP fluorescence intensity, then measure the same parameters in the tubulin image (see **Note 21**).

7. Repeat **step 7** for an untransfected cell in the same frame.
8. Copy the mean GFP and tubulin pixel intensity for both the transfected and untransfected cell and paste these values into a spreadsheet.
9. Divide the tubulin fluorescence of the transfected cell by the tubulin fluorescence of the untransfected cell to obtain a tubulin fluorescence ratio.
10. Repeat **steps 8–11** for the rest of the photographs for a given construct, and repeat the entire procedure for each expression construct being analyzed.
11. Calculate the average and standard deviation of the GFP intensities. Determine the average and standard deviation of the tubulin fluorescent ratios calculated in **step 9**. Comparison of the data obtained from GFP-expressing and GFP-kinesin-expressing cells indicates the effectiveness of microtubule depolymerization (*see Note 22*).

4. Notes

1. We routinely use pEGFP-C1 (Clontech). This vector drives strong, quick expression of EGFP fusion proteins under the control of a CMV promoter. The EGFP coded in the vector folds quickly and bleaches slowly, which allows for quick expression and stable photographing, respectively. Additionally, this vector has an f1 single-strand DNA origin for replication that is useful for fast production of multiple mutant transgenes. N-terminal versus C-terminal placement of the transgene relative to the fluorescent protein should be considered and tested to determine whether placement has an effect on expression and function.
2. The original tubulin concentration before assembly can be measured by measuring the absorbance (A) at 280 nm, assuming an extinction coefficient for tubulin of $115,000 \text{ mol}^{-1} \text{ cm}^{-1}$. Using this value, a 1 mM solution of tubulin would possess an $A_{280} = 1.15$. This extinction coefficient is noted in Hyman et al. (1991) (16).
3. Coverslips are mounted inside the centrifuge tube. Depending on the type of centrifuge tubes used, it may be necessary to machine a small fitting for mounting the coverslips securely at the bottom of the tube. It is important that the coverslip fits securely into the tube to ensure all microtubules are pelleted onto the glass. We routinely spin microtubules onto 5-mm-diameter coverslips in 8-mm (outer diameter) centrifuge tubes using a Beckman airfuge. As an alternative to a machined fitting for the centrifuge tubes, a small amount of epoxy can be placed in the bottom of the tube and then spun while the epoxy is curing; this will generate a flat-angled surface upon which to lay the coverslip.
4. Measure the average number of microtubules per frame, multiply this number by the total coverslip surface area, and divide by the surface area of one frame; this gives the total number of microtubules on the coverslip. Next, divide by the initial starting volume to determine the initial microtubule concentration. Multiply this number by two to determine the microtubule-end concentration.

5. For a typical ATP-binding assay, prepare three nitrocellulose filters per protein preparation being assayed and three for the buffer blank control. Additionally, we routinely wash a few spare membranes in case some break during the procedure.
6. α - ^{32}P -ATP, 800 Ci/mmol, 10 mCi/mL, or 12.5 μM α - ^{32}P -ATP on reference date. Concentration on date of assay is $A(t) = A_0 * e^{-lt}$ where A_0 = concentration on reference date, t = number of days after reference date, and $l = \ln 2$ (1/half-life) or 0.0485 day $^{-1}$ for ^{32}P .
7. Note that the hot:cold ATP ratio in this solution is assumed to be 1:300. If the stock concentration of active protein much exceeds 5 μM , the cold ATP carried by the protein will be significant, and a dilution of protein to 1–2 μM in motor storage buffer +5 μM ATP should be made before the assay.
8. Each step in this process should be done as quickly and consistently as possible. Process three filters for each assay mixture or buffer blank.
9. Example of ATP-binding activity calculation:
 2 μL MCAK, average of $3 \times 5 \mu\text{L}$ samples: 20,667 cpm
 Blank reaction, average of $3 \times 5 \mu\text{L}$ samples: 1,587 cpm
 100 nM hot ATP, average of $3 \times 1 \mu\text{L}$ samples: 198,690 cpm
 $20,667 \text{ cpm} - 1,587 \text{ cpm} = 19,080 \text{ cpm} \times (20 \mu\text{L}/2 \mu\text{L}) \times (1/5 \mu\text{L}) \times 300 \text{ cold:hot ATP} = 1.15 \times 10^7 \text{ cpm}/\mu\text{L}$ corrected for sample volumes and dilution
 $1.15 \times 10^7 \text{ cpm}/\mu\text{L} \times (1 \times 10^{-7} \text{ M} \times 1 \mu\text{L}/198,690 \text{ cpm}) = 5.8 \mu\text{M}$ active ATP-binding sites.
10. A typical test reaction would employ 300 nM GMP-CPP microtubules and a 3 nM full-length MCAK dimer, but optimal motor concentration of other kinesins should be determined empirically.
11. Addition of CaCl_2 to a final concentration of 5 mM will disassemble all GMP-CPP microtubule polymers (17); this is a useful control to determine the A_{350} of fully disassembled microtubule polymer. When modest concentrations of active motor are added to microtubules, destabilizers such as MCAK will disassemble the microtubules to a new steady-state polymer concentration. It is unwise to assume that the lowest A_{350} reading represents fully disassembled microtubules.
12. Turbidity traces can be converted to tubulin polymer concentration using a standard curve in which the zero time point corresponds to the concentration of tubulin in the assembled microtubules (i.e., 300 nM) and the turbidity after complete disassembly in CaCl_2 is equal to 0 nM assembled tubulin.
13. Confluency refers to the density of the cells adhered to the plate. For example, 100% confluency means cells that have adhered to and spread out on the plate are dense enough that they are touching each other and taking up 100% of the plate surface.
14. One day before transfection, we plate CHO cells in a 24-well plate containing one acid-washed 12-mm coverslip in each well. Under these conditions, seed cells at a density of 3.5×10^4 cells per well in 500 μL culturing media. If cells are

seeded too densely, analysis of tubulin fluorescence is difficult because cells do not spread properly (**Section 3.2.3**).

15. We fix and wash our cells in 250-mL plastic beakers containing 100 mL fix solution or PBS. The coverslips are held vertically in a coverglass staining rack, and the cell-containing side of each coverslip is carefully tracked through the procedure.
16. We block and stain cells by placing coverslips cell-side-up on a piece of parafilm laid flat inside a 25-mm Petri dish. Damp paper towels are rolled and placed around the inside of the dish to provide humidity and prevent drying during incubation periods.
17. Choose cells that do not touch any other cells for this analysis because fluorescence from contacting cells can complicate the quantification procedure. Also, choose cells that provide a good dynamic range with respect to fluorescence intensity and avoid cells that contain areas of fluorescence saturation (at or above the maximal intensity value).
18. We routinely take separate images for microtubules and GFP, and number the files sequentially (e.g., Kif2C_01_GFP; Kif2C__01_MT, where “Kif2C” is the name of the kinesin being analyzed). We keep all the files for each construct in a single folder. This organization is optimal for importing the files as a sequence into ImageJ for analysis.
19. All the photos in the folder will open as a stack, with the GFP channel first and the microtubule channel second. To go forward within the stack, use the period key (.), and to go backward use the comma key (,).
20. In ImageJ, set parameters by opening Analyze-Set measurements. Click the boxes for “Area,” “Mean Gray Value,” “Min and Max Gray Value,” and “Display Label.”
21. In ImageJ, take measurements with the Analyze-Measure command. Toggle between the GFP and tubulin images to take measurements using the same cell outline.
22. The average GFP intensities should be statistically the same for all constructs in the assay by a Student’s *t* test analysis. Cells transfected with GFP should give a microtubule fluorescence ratio of approximately 1, whereas cells expressing a microtubule-depolymerizing kinesin should give a tubulin fluorescence ratio that is less than 1 (for example, see [Fig. 2](#)).

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Approaches to Kinesin-1 Phosphorylation

Gerardo Morfini, Gustavo Pigino, and Scott T. Brady

Summary

Most mammalian proteins undergo reversible protein modification after or during synthesis. These modifications are associated, for the most part, with changes in protein functionality. Protein phosphorylation is the most common posttranslational modification in mammalian cells, regulating critical cellular processes that include cell division, differentiation, growth, and cell–cell signaling as well as fast axonal transport (FAT). Evidence has accumulated that kinesin-1 phosphorylation plays a key regulatory role in kinesin-based FAT. Multiple kinase and phosphatase activities with the ability to regulate kinesin-1 function and FAT have been identified. Moreover, additional pathways are likely to exist for regulating FAT through reversible phosphorylation/dephosphorylation of specific motor protein subunits. The present chapter describes specific biochemical assays to determine, or to perturb experimentally, the phosphorylation status of kinesin-1. These protocols provide assays for characterization of novel effectors (i.e., trophic factors, neurotransmitters, pharmacological inhibitors, pathogenic protein expression, etc.) that affect the phosphorylation status of kinesin-1. Finally, in vitro phosphorylation assays suitable for analyzing the direct effects of specific kinases on kinesin-1 are provided.

Key Words: Kinesin-1; fast axonal transport; phosphorylation; immunoprecipitation; kinase; phosphatase.

1. Introduction

The asymmetrical distribution of membrane proteins in neuronal cells ultimately underlies their ability to receive, process, and transmit information. In mature neurons, membrane proteins needed for proper axonal function need to be transported from their place of synthesis in the neuronal cell body to their final sites of utilization by fast axonal transport (FAT) mechanisms ([1](#)). An additional degree of complexity is added to this remarkable task, because different membrane proteins need to be delivered to specialized subdomains within

axons. Presynaptic terminals and nodes of Ranvier are primary examples of spatially discrete axonal specializations, with little or no overlap in protein composition. The machinery for release and recycling of synaptic vesicles must be assembled at presynaptic terminals, whereas voltage-dependent sodium channels are present at high levels specifically at nodes of Ranvier. The accurate delivery of membrane-bounded organelles (MBOs) to discrete axonal subdomains implies the existence of specific targeting mechanisms (2,3).

Kinesin-1 (also known as conventional kinesin, KIF5) is the most abundant microtubule (MT)-based anterograde motor in the mammalian nervous system (4). A large body of experimental evidence indicates kinesin-1 is responsible for the anterograde trafficking of a large variety of MBOs (4,5), including synaptic vesicles, axolemmal precursors, and mitochondria. Kinesin-1 is a heterotetrameric protein, comprising two heavy chains of 115–130 kDa and two light chains of 62–70 kDa (reviewed in refs. 4,5). Kinesin-1 heavy chains (KHCs) have a conserved globular head domain responsible for both ATP hydrolysis and MT-binding activities, whereas kinesin light chains (KLCs) are involved in kinesin-1 binding to MBOs (6–8). Kinesin-1 primary functions are to interact with the appropriate MBOs and to convert the chemical energy stored in the form of ATP into mechanical forces that allow MBO translocation along MTs.

Despite the recognized importance of FAT for neuronal function, few studies have focused on the issue of how kinesin-1 is subject to regulation *in vivo* (9). However, some regulatory mechanisms have recently been identified that might help to regulate the delivery of correct cargoes by kinesin-1 (10–12). Interestingly, it was found that phosphorylation represents a major mechanism for the regulation of kinesin-1-based motility (9,13,14). Several kinase and phosphatase activities have been identified that regulate FAT through phosphorylation/dephosphorylation of specific motor protein subunits (9). For example, glycogen synthase kinase 3 (GSK3) phosphorylates KLCs and inhibits kinesin-1-based motility by promoting the release of kinesin-1 from MBOs (12). Other kinases, such as JNK kinase, inhibit kinesin-1-based motility through a different mechanism, phosphorylating KHCs, and inhibiting kinesin-1 binding to MTs (10). In addition, some protein kinases have been identified that affect FAT indirectly. For example, inhibition of the neuronal-specific protein kinase cyclin-dependent kinase 5 (CDK5) results in inhibition of kinesin-1-based motility (11,15). However, CDK5 does not directly phosphorylate kinesin-1. This finding ultimately resulted in the identification of a novel, neuronal-specific signaling pathway for the regulation of kinesin-1-based motility. In this pathway, GSK3, rather than CDK5, was found responsible for the direct phosphorylation of kinesin-1 KLCs, and *in vitro* phosphorylation experiments provided further support to this idea (11). All these phosphorylation-

dependent regulatory mechanisms for kinesin-1 are likely to be essential for the proper generation and maintenance of polarized protein distribution in nerve cells. However, the large heterogeneity of MBOs in neuronal cells, and the cytotypic differences in FAT requirements for different neuronal types, suggest that the mechanisms described above only represent a fraction of a large repertoire, with many more mechanisms awaiting identification (9).

Identification of regulatory mechanisms for kinesin-1-driven motility could have implications beyond its normal role in neuronal function. Indeed, recent genetic evidence has linked alterations in kinesin-1-based motility to selective degeneration of neuronal cells (3). Moreover, changes in regulatory pathways for FAT have also been recently implicated in neurodegeneration (2,3). For example, neurons with familial Alzheimer's disease mutations in presenilin-1 (PS1) show increased activation of GSK3 and a concomitant reduction in kinesin-1-based motility (16). Similarly, expansion of a polyglutamine tract in the androgen receptor and huntingtin proteins leads to an inhibition of kinesin-1-based motility (17). Remarkably, these effects were caused by JNK-mediated phosphorylation of kinesin-1 (10). Thus, elucidation of novel regulatory pathways for kinesin-1-based motility could have important implications for the understanding of pathogenic mechanisms in human neurodegenerative diseases.

The present chapter describes specific biochemical assays to determine, or to perturb experimentally, the phosphorylation status of kinesin-1. **Subsection 3.2.1.** describes an assay that can facilitate the identification of novel effectors (i.e., trophic factors, neurotransmitters, pharmacological inhibitors, pathogenic protein expression, etc.) that affect the phosphorylation status of kinesin-1. Finally, **Subsection 3.2.2.** describes in vitro phosphorylation assays that can be used to confirm whether a specific kinase can phosphorylate kinesin-1 *directly*.

2. Materials

2.1. Equipment and Facilities

1. An ultracentrifuge with rotors suitable for submilliliter volumes (i.e., TLA100.3).
2. A tabletop microcentrifuge.
3. A rotary shaker suitable for microcentrifuge tubes.
4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis equipment and a protein transfer apparatus are needed.
5. A standard tissue culture facility.
6. For metabolic labeling studies, ^{32}P incorporation is quantified using both a Phosphorimager system (Molecular Dynamics Typhoon or equivalent) and a scintillation counter. Institutional radiation safety procedures and guidelines should be followed at all times.

2.2. General Reagents

1. Water is ultrapure reagent grade (Millipore MilliQ filtered or equivalent; dH₂O).
2. All electrophoresis reagents are analytical grade.
3. Protein concentration is determined using BCA Protein Assay Reagent (Pierce, Rockford, IL), a detergent-compatible reagent.
4. **Microcentrifuge tubes:** We use low protein-binding microcentrifuge tubes, such as CLP Cat. #3445 (*see Note 1*).
5. **Kinase and phosphatase inhibitors:** Kinase inhibitors are obtained in solid form from Calbiochem. Stock solutions are prepared as 1000X concentrated, following supplier directions. Kinase and phosphatase stocks are stored in small aliquots at -80°C until use.
6. **Protease inhibitor cocktail:** We use mammalian protease inhibitor cocktail from Sigma (Cat. #P8340).
7. **Phosphatase inhibitor (PIC) cocktail:** Obtained from Calbiochem (Cat. #524625). It consists of 200 mM imidazole, 100 mM sodium fluoride, 115 mM sodium molybdate, 100 mM sodium orthovanadate, and 400 mM sodium tartrate dihydrate.
8. **Poly-L-lysine:** A solution of 1 mg/mL is prepared in 0.1 M borate buffer (1.24 g boric acid, 1.90 g sodium tetraborate in 400 mL H₂O), pH 8.5, sterilized by filtration. Individual aliquots are stored at -20°C up to 3 months. Before experiments, dishes are covered with 3–4 mL freshly prepared poly-L-lysine solution and let stand for 12–24 h. Remove poly-L-lysine solution and rinse dishes with sterile water (two washes, 2 h each). After last wash, add 3–4 mL appropriate culture media, supplemented with 5%–10% serum, and place them in an incubator (they can be stored for several days before use, typically 1–5).
9. **5X gel loading buffer:** 0.35 M Tris-HCl, pH 6.8, 10% w/v SDS (Pierce; Sequanal grade), 36% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue.
10. **2X gel loading buffer:** 0.14 M Tris-HCl, pH 6.8, 4% SDS, 14.4% glycerol, 0.01% bromophenol blue.
11. **Antibodies:** Several mouse monoclonal (mAbs), as well as rabbit polyclonal antibodies specific for kinesin-1, have been generated in our laboratory. These antibodies have been rigorously characterized for their specificity using biochemical and molecular biological methods. For metabolic labeling experiments (**Subsection 3.2.1.**), antibodies of choice should have immunoprecipitation activity. A number of antibodies against kinesin-1 are now commercially available that can selectively immunoprecipitate endogenous kinesin-1 ([8,12,18,19](#)). Antibodies against kinesin-1 KHC subunits include mAbs H2, H1 (both available through Chemicon), and SUK4 (available from BabCo; Cat. #MMS-188P). Antibodies against kinesin-1 KLC subunits include mAbs L1, L2 (both available through Chemicon), 63–90, and KLC-All (these latter two are available from our laboratory upon request).

12. **Detergent stocks:** Triton X-100 stocks are prepared as 10% w/v. Long-term storage of detergent solutions is not recommended as this can lead to formation of peroxide radicals.

2.2.1. Metabolic Labeling Experiments

1. **Lysis buffer (LB):** 10mM HEPES, pH 7.4, 0.5% Triton X-100, 80mM β -glycerophosphate, 50mM NaF, and 100mM potassium phosphate.
2. **Complete lysis buffer (CLB):** Prepare immediately before experiments by adjusting the volume of 1X LB buffer needed to 2mM sodium orthovanadate, 100nM staurosporine, 100nM K252a, 50nM okadaic acid, 50nM microcystin, 1/100 dilution of PIC, and 1/100 dilution of mammalian protease inhibitor cocktail (*see Note 2*).
3. **Radiolabeled ^{32}P inorganic phosphate:** Obtained from ICN Radiochemicals (Cat. #6401410).
4. **P81 phosphocellulose paper circles:** Obtained from Whatman (Cat. #3698-321).
5. **100mM phosphoric acid:** Prepared from an 8.7M stock. Store at room temperature.
6. **Agarose conjugates:** Protein G agarose beads are obtained from Pierce. When using rabbit polyclonal Abs, protein A agarose beads (BioRad) can be used instead. Both mouse and rabbit IgG-conjugated sepharose beads can be obtained from Jackson ImmunoResearch. All volumes for agarose and sepharose beads are referred to as 50% resin slurries. Store at 4°C in HEPES 10mM, pH 7.4, 1% (w/v) IgG-free bovine serum albumin (BSA; Jackson ImmunoResearch), and 0.01% (w/v) sodium azide.
7. **Wash buffer I:** HEPES 10mM, 150mM NaCl, Triton X-100 0.5%, sodium azide 0.02%. Adjust pH to 7.4.
8. **Wash buffer II:** HEPES 10mM, NaCl 500mM, Triton X-100 (0.5%), ethylenediaminetetraacetic acid (EDTA) 1mM, sodium azide (0.02%). Adjust pH to 7.4.

2.2.2. In Vitro Phosphorylation of Kinesin-1

1. **Homogenization buffer (HB):** 10mM HEPES, pH 7.4, 150mM NaCl, 1% Triton X-100.
2. **Complete homogenization buffer (CHB):** Prepare immediately before experiments, by adjusting the volume of HB buffer needed to 1/100 dilution of mammalian protease inhibitor cocktail.
3. **Wash buffer II:** Described in **Subsection 2.2.1**.
4. **Kinase buffer (KB):** 10mM HEPES, pH 7.4, 1mM dithiothreitol (DTT), 0.1mM ethyleneglycoltetraacetic acid (EGTA), 10mM MgCl_2 .
5. **Kinase buffer complete (KBC):** Prepare immediately before experiments by adjusting the volume of KB buffer needed to 1 μM protein kinase A (PKA)

inhibitor peptide (Upstate; Cat. #12-151), 50 nM okadaic acid, and 50 nM microcystin (see **Note 16**).

6. **5X ATP stock:** 500 μ M K-ATP. Prepare and store in small aliquots at -20°C up to 6 months. Add radiolabeled γ - ^{32}P -ATP shortly before use.

3. Methods

3.1. Cell Culture and Preparation

A variety of cell lines and primary cultures can be used, including neuronal and nonneuronal cell types. Methods described here have been successfully applied to various cell lines, including SH-SY5Y cells (20), NSC34 immortalized motoneurons (21), immortalized striatal cells (22), N27 dopaminergic cells (23), and both cortical and hippocampal primary cultured neurons (24). The choice of cell type depends on a number of practical and theoretical considerations. For example, the species specificity of available kinesin-1 antibodies can be a strong determinant of cell type choice. In general, primary cultures of hippocampal and cortical neurons are the prime model system for the study of neuronal-specific pathways (11,16,24). These cells however, can prove difficult to transfect with recombinant DNA constructs, and they are generally more sensible and fragile when challenged to experimental manipulations. Cell lines, on the other hand, can generally be transfected more effectively. Moreover, stably transfected subclones expressing various recombinant proteins can be generated (25). Alternatively, cell lines may be selected because they exhibit well-characterized behavioral and biochemical responses to a given factor. For example, PC12 and neuroblastoma N2a cells extend neurite-like processes in response to nerve growth factor (NGF) or FGF-2 through activation of well-studied pathways (26). Similarly, sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells (17,20).

Before an experiment, cell lines are maintained on their preferred substrate. We typically grow cell lines in their appropriate culture media, supplemented with 5%–10% serum, until cells reach 70%–80% confluency. Some cell lines are often changed to low-serum or serum-free medium to limit both cell division and the serum-dependent activation of signaling pathways (i.e., PI3K pathway). To improve cell attachment of some cell types, we coat tissue culture dishes with poly-L-lysine. We usually prepare dishes on the day of an experiment, but dry coated dishes can be stored at 4°C for at least 1 week. Primary cultures of cortical and hippocampal neurons are prepared as described in Banker et al. (24). In some cases, neuronal cultures can also be prepared from wild-type and mutant mouse embryos at day 16 of gestational age (11,16).

3.2. Biochemical Approaches to Study the Regulation of Kinesin-1 Phosphorylation and Kinesin-1 Functional Activities

Methods presented in this subsection can be used to test the effects of candidate regulators on the phosphorylation status of kinesin-1 (**Subsection 3.2.1.**).

3.2.1. Metabolic Labeling Experiments

Kinesin-1 exists as a phosphoprotein *in vivo* ([12,14](#)), and phosphorylation represents a major mechanism for the regulation of kinesin-1-based motility ([2,9](#)). The metabolic labeling protocol described below can help in determining the impact of specific effectors or cellular manipulations on the phosphorylation status of kinesin-1 (*see* **Note 3**). These approaches have proven useful for the identification of novel regulatory pathways for kinesin-1-based motility ([12](#)) and novel pathogenic mechanisms for human neurodegenerative diseases ([16](#)). The combination of defined cell cultures with ^{32}P -labeled inorganic phosphate and specific immunoprecipitation of motor proteins is an effective approach to study the phosphorylation of kinesin *in situ*. As always, institutional radiation safety procedures and guidelines should be followed at all times.

1. Cells are grown in 100-mm-diameter tissue culture dishes (*see* **Note 4**). It is recommended to grow an additional tissue culture dish (“background control”), which will serve as a negative control for radiolabeled phosphoproteins that may bind nonspecifically to either protein G beads or immunoglobulins.
2. Before experiments, 50% of cell culture media (typically, 3 mL) is replaced with phosphate-free Dulbecco’s minimal essential medium (DMEM; Gibco Cat. #11971-025) (*see* **Note 5**).
3. Add 1 mCi radiolabeled inorganic phosphate (^{32}P ; MP Biomedicals; Cat. #64014) stock to each tissue culture dish. Typically, 1 mL media is removed from the plate and combined in a tube with an aliquot of the ^{32}P -phosphate stock from ICN corresponding to 1 mCi. The radioactive media may then be returned to the dish. Carefully swirl media to equilibrate, taking care not to spill the radioactive media.
4. Cells are incubated for 4 h at 37°C in an atmosphere containing 5% CO_2 (*see* **Note 6**).
5. After appropriate experimental treatments, media is discarded by aspiration, and cells are washed once with 1.5 mL prewarmed serum-free DMEM. This step helps to minimize free unincorporated ^{32}P and reduce background.
6. Cells are scraped in ice-cold CLB, with the help of a rubber cell scraper. The volume of buffer to use is typically 1 mL per 100-mm-diameter dish (or 78 cm² tissue culture surface).
7. Cell lysates are collected in a microcentrifuge tube (*see* **Note 1**) and ultracentrifuged at 130,000 g_{max} for 5 min at 4°C. We use a TLA 100.3 Beckman rotor with microcentrifuge tube adapters. Detergent-insoluble pellets are discarded.

8. Supernatants from **step 7** are transferred to new microcentrifuge tubes. Typically, we transfer 950 μ L to avoid aspirating insoluble material.
9. Aliquots of clarified lysates are spotted (in triplicates) onto P81 cellulose phosphate paper circles to allow for measurement of radioactivity incorporated into proteins (*see Note 7*).
10. Samples are normalized for equal counts with CLB to 1 mL final volume.
11. Samples from **step 10** are transferred to 1.5-mL microcentrifuge tubes, each containing a mixture of 20 μ L protein G agarose beads and 30 μ L mouse IgG Sepharose-conjugated mouse. When using polyclonal antikinesin-1 Abs, use 20 μ L protein A agarose beads and 30 μ L rabbit IgG Sepharose-conjugated beads.
12. Incubate at 4°C with rotation for 30 min using a tube mixer. This step results in the removal of most phosphoproteins, which can bind nonspecifically to either agarose-based resins or immunoglobulins.
13. Centrifuge samples at 5,000rpm in a tabletop microfuge for 1 min at room temperature.
14. Carefully transfer the supernatant from **step 13** (typically 900 μ L) to a new 1.5-mL microcentrifuge tube containing 30 μ L protein G agarose beads, plus the appropriate kinesin-1 antibody (*see Note 8*). Discard bead pellets from **step 13**.
15. Incubate at 4°C with rotation for 3 h to overnight using a tube mixer (*see Note 9*).
16. Centrifuge samples at 5,000rpm in a tabletop microcentrifuge for 1 min at room temperature. If needed, save supernatants to immunoprecipitate proteins other than kinesin-1. Otherwise, discard supernatants by aspiration using a fine-pointed tip attached to a vacuum trap.
17. Wash beads by adding 1 mL wash buffer I. Mix by gently inverting the tube five times (*see Note 9*).
18. Centrifuge samples at 5,000rpm in a tabletop microcentrifuge for 1 min at room temperature.
19. Discard supernatants by aspiration using a fine-pointed pipet tip attached to a vacuum trap.
20. Repeat **steps 17–19** for a total of two washes.
21. Wash beads by adding 1 mL wash buffer II. Mix by gently inverting the tube five times.
22. Proceed with washes as in **steps 17–19**, but using buffer wash II instead, for a total of two washes.
23. Wash beads by adding 1 mL HEPES 10 mM, pH 7.4, and mix by inverting the tube five times. This step helps eliminate NaCl present in wash buffer II, which might interfere later with SDS-PAGE electrophoresis.
24. Centrifuge samples at 5,000rpm in a tabletop microcentrifuge for 1 min at room temperature.
25. Discard supernatants by aspiration using a fine-pointed tip attached to a vacuum trap. Dry agarose beads (*see Note 10*).
26. Add 30 μ L 2X gel loading buffer per tube.
27. Before loading gels, boil samples for 2 min.
28. Immunoprecipitates are separated by SDS-PAGE electrophoresis (*see Note 11*).

29. Gels are dried and exposed in a Phosphorimager cassette, then scanned and quantified on a Typhoon (Amersham/Molecular Dynamics) or exposed to X-ray film for autoradiography.

Using this protocol, we determined that stably transfected SH-SY5Y cells expressing a polyglutamine-expanded mutant form of the androgen receptor (AR) display a 50% increase in KHC phosphorylation levels when compared to wild-type AR-expressing forms ([10](#)) ([Fig. 1](#)).

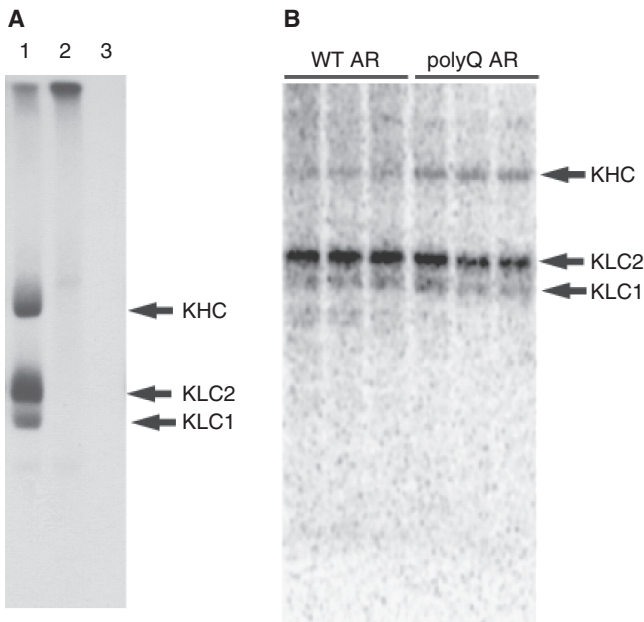


Fig. 1. Kinesin-1 phosphorylation in normal and pathological conditions. **(A)** Typical pattern of kinesin-1 phosphorylation in primary rat cortical cells metabolically labeled with ³²P. Kinesin-1 was immunoprecipitated (IP) with H2, a kinesin-1-specific antibody raised against kinesin-1 heavy chains (KHCs). Note that KHC and both kinesin light chain (KLCs) subunits incorporated ³²P (lane 1). Normal mouse IgG (lane 2) and beads alone (lane 3) are the usual IP controls. Note the presence of a high molecular weight band incorporating ³²P in both lanes 1 and 2 but not lane 3; this indicates the presence of one or more nonspecific phosphoproteins that can bind to beads in the presence of immunoglobulins. **(B)** SH-SY5Y cells expressing WT or polyQ-AR were incubated with ³²P. Representative autoradiogram (³²P) shows immunoprecipitated, radiolabeled kinesin-1 heavy (KHC) and light chain (KLCs) subunits. Note the increase in kinesin-1 heavy chain phosphorylation in polyQ-AR expressing SH-SY5Y cell lines, whereas KLCs are comparable in both cell lines. Total kinesin-1 levels are comparable in the two cell lines ([10](#)).

3.2.2. In Vitro Phosphorylation of Kinesin-1

Assays described in **Subsection 3.2.1.** provide a basis for the identification of signaling pathways that might induce changes in kinesin-1 phosphorylation. Once these changes are confirmed, pharmacological inhibitors can be used to identify specific kinases and phosphatases involved. These approaches, however, do not ensure that a given phosphotransferase is *directly* responsible for the changes observed in kinesin-1 phosphorylation. For example, pharmacological experiments in isolated squid axoplasm first indicated kinesin-1-based motility was dependent upon sustained cyclin-dependent kinase 5 (CDK5) activity ([15](#)), but CDK5 does not directly phosphorylate kinesin-1 ([11](#)).

3.2.2.1. IN VITRO PHOSPHORYLATION SUBSTRATE CHOICE

Endogenous kinesin-1, as well as recombinant KHCs and KLCs subunits, have all been successfully used as a source of material for in vitro phosphorylation experiments ([10,12,13](#)) ([Fig. 2](#)). Recombinant kinesin-1 constructs can also

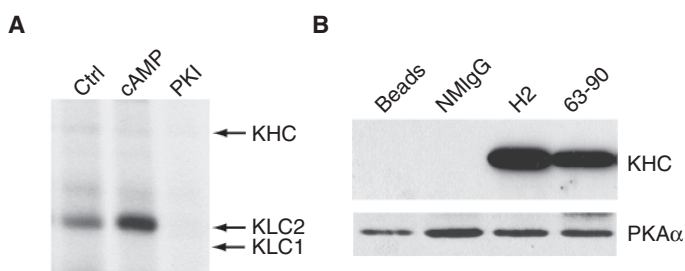


Fig. 2. In vitro phosphorylation assays using immunoprecipitated kinesin-1. **(A)** In the absence of any exogenously added protein kinase, incubation of immunoprecipitated kinesin-1 with radiolabeled γ - ^{32}P -ATP (Ctrl lane) results in strong phosphorylation of KLC2 and, to a minor extent, KHC (better seen in longer exposures; not shown here). These data suggest that one or more kinases can be co-immunoprecipitated with kinesin-1. Pharmacological experiments indicate PKA kinase is responsible for kinesin-1 phosphorylation under these conditions as addition of cAMP strongly increases KLC2 phosphorylation. Moreover, addition of the PKA inhibitor peptide PKI dramatically reduced KLC2 phosphorylation. **(B)** Immunoblot analysis indicates that results in **(A)** correspond to an artifact caused by nonspecific binding of PKA catalytic subunits to agarose beads. PKA catalytic subunit is found in association with either normal mouse IgG (NMIG) or kinesin-1 (H2 and 63–90) immunoprecipitates. Moreover, the PKA catalytic subunit can be found in association with agarose beads (Beads) without addition of immunoglobulins. Kinesin-1 (KHC), on the other hand, is only immunoprecipitated with kinesin-1 antibodies specific for KHC (H2) or KLC (63–90). Under nondenaturing conditions, antibodies to either KHC or KLC will precipitate *both* KLC and KHC.

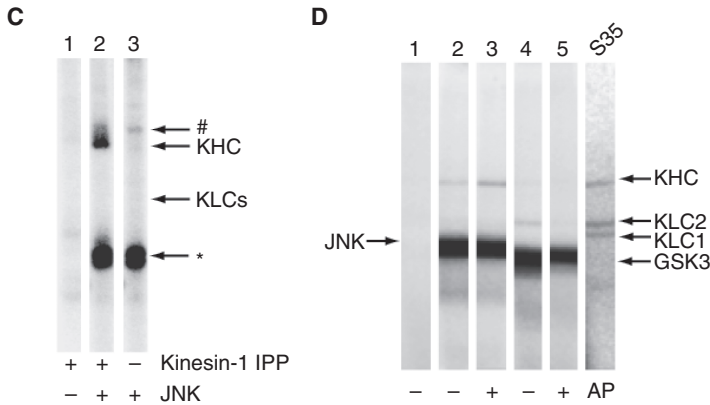


Fig. 2. (Continued) **(C)** Example of a typical *in vitro* phosphorylation experiment, including appropriate controls. Lanes 1 and 2 contain H2-immunoprecipitated kinesin-1; lane 3 corresponds to material associated with agarose beads alone (no antikinesin-1 antibodies added, “background control”). Exogenous JNK kinase has been added (+) in lanes 2 and 3 but omitted in lane 1 (–). Little or no phosphorylation of protein is observed when kinesin-1 immunoprecipitates are incubated in the absence of JNK kinase (lane 1). Addition of JNK kinase results in significant phosphorylation of KHCs but not KLCs (lane 2). Strong autophosphorylation of JNK kinase is observed (*) in lanes 2 and 3, as well as phosphorylation of a protein nonspecifically associated to agarose beads (#). Note that this band is also present in lane 2 and might be confused with a KHC isoform without this background control. **(D)** The phosphorylated status of immunoprecipitated kinesin-1 can affect the outcome of *in vitro* phosphorylation experiments. Lane 1 corresponds to kinesin-1 immunoprecipitate incubated with γ -³²P-ATP in the absence of exogenous kinase. Aliquots of kinesin-1 immunoprecipitate were incubated with (+) or without (–) alkaline phosphatase (AP) before phosphorylation assays *in vitro*. The effects of adding JNK are shown in lanes 2 and 3. Quantitative analysis (not shown) indicates that JNK kinase-mediated phosphorylation of KHC increases twofold when immunoprecipitated kinesin-1 has been dephosphorylated with AP before addition of JNK (compare lanes 2 and 3). This result suggests that some KHC sites that can be phosphorylated by JNK kinase activity *in vitro* were already phosphorylated endogenously in the immunoprecipitated kinesin-1 material. No KLC phosphorylation is observed with JNK in either sample. In contrast to JNK, GSK3 phosphorylates KLC2 but not KHC when nondephosphorylated immunoprecipitated kinesin-1 is used as a substrate (lane 4). Dephosphorylation of kinesin-1 dramatically reduces KLC2 phosphorylation by GSK3 (lane 5). This result is consistent with the requirement of GSK3 kinase for a “priming” phosphorylation at an adjacent site on most GSK3 substrates (12). ³⁵S-metabolically labeled kinesin-1 immunoprecipitated from primary cultured neurons was used as a reference to indicate the position of kinesin-1 subunits (lane 6). Note that both JNK (lanes 2 and 3) and GSK3 (lanes 4 and 5) are autophosphorylated in these assays; this verifies the activity of the added kinase. For kinases that are not autophosphorylated, a specific substrate can be added as a positive control (12).

prove a very useful tool for the exact mapping of specific phosphorylation sites. In addition, mutant protein constructs can be easily generated, to confirm a potential phosphorylation site. However, when recombinant kinesin-1 constructs are the substrate choice, a number of considerations need to be taken. Three genes exist for KHC (kinesin-1a, b, c) (27). At least three genes have also been reported for KLC (KLC1, KLC2, and KLC3) (6,28,29) in mammals, although KLC3 expression appears largely restricted to testes (29). In addition, *in vivo* metabolic labeling experiments indicate that not all KHCs and KLCs are phosphorylated equally (Morfini, Elluru, and Brady, unpublished data). These observations highlight the difficulties associated with the choice of an appropriate recombinant construct. For example, GSK3 was shown to phosphorylate KLCs when endogenous kinesin-1 purified from mouse brain was used as a substrate (12). Subsequently, KLC2 was found to be the preferred KLC phosphorylated by GSK3 (see Fig. 2D), consistent with multiple GSK3 consensus sites found at the carboxy terminus of KLC2. However, recombinant KLC2 was phosphorylated by GSK3 only if KLC2 had been previously phosphorylated by a “priming” kinase (i.e., casein kinase 2) (see Ref. 12). These observations were confirmed using immunoprecipitated kinesin-1 as a substrate (see Fig. 2D). Therefore, negative results from *in vitro* phosphorylation using recombinant kinesin-1 constructs should be interpreted carefully, because the chosen construct might not be the exact final target of a given kinase activity.

3.2.2.2. ENDOGENOUS BRAIN KINESIN-1

Endogenous brain kinesin-1 can be purified to near homogeneity by taking advantage of its unique biochemical properties (30). These methods, however, can be time consuming (up to 3 days), and the yields are modest when using rodent brain as original material (12). Immunoprecipitation-based purification methods, on the other hand, offer a much easier and less costly alternative for studies of kinesin phosphorylation if suitable antibodies are available (see “Antibodies” in Subsection 2.2.). An advantage of using H2-immunoprecipitated kinesin-1 is that all known KHC and all KLC gene products for kinesin-1 are represented in these samples (our unpublished data). In sum, as an initial step, we recommend the use of endogenous mouse brain kinesin-1 as a substrate for *in vitro* phosphorylation assays. Results from these experiments can help in the selection of recombinant constructs to be phosphorylated later. The protocol given below is designed for the use of immunoprecipitated mouse brain kinesin-1 as a substrate for *in vitro* phosphorylation studies.

1. Quickly dissect, or thaw, one mouse or one rat brain (*see Note 12*).
2. Homogenize one mouse brain in 3 mL ice-cold CHB, with 15–20 strokes of a glass homogenizer (*see Note 13*). When using rat brains, use 10 mL ice-cold CHB per brain.
3. Spin brain homogenates at $100,000g_{\max}$ for 5 min at 4°C. We use 3-mL tubes in a TLA 100.3 rotor.
4. Transfer supernatant from **step 3** (SN1) to a new tube.
5. Repeat **step 3**, to obtain supernatant SN2.
6. Transfer SN2 to a 5-mL conical tube containing 100 μ L protein G agarose beads and 200 μ L normal mouse IgG Sepharose beads. When using polyclonal anti-kinesin-1 Abs, use 100 μ L protein A agarose beads and 200 μ L rabbit IgG Sepharose-conjugated beads.
7. Incubate at 4°C with rotation for 30 min using a tube mixer. This step results in the removal of proteins that nonspecifically bind to either agarose-based resins or immunoglobulins.
8. Centrifuge samples at 5,000rpm for 1 min at room temperature.
9. Transfer precleared SN2 to a new tube (*see Note 14*).
10. 300- μ L aliquots are transferred to microcentrifuge tubes and brought to 1 mL with CHB buffer.
11. Add 20 μ L protein G beads and 5 μ g antikinesin-1 antibody (i.e., H2 mAb or other antibody of interest) per tube (*see Note 15*). Incubate. It is recommended to include an additional tube as “background control” (*see Note 18*).
12. Incubate at 4°C with rotation for 3 h to overnight, using a rotary shaker/tube mixer (*see Note 9*).
13. Centrifuge samples at 5,000rpm in a tabletop microcentrifuge for 1 min at room temperature. If needed, save supernatants to immunoprecipitate proteins other than kinesin-1. Otherwise, discard supernatants by aspiration using a fine-pointed tip attached to a vacuum trap.
14. Wash beads by adding 1 mL wash buffer II. Mix by gently inverting the tube five times.
15. Centrifuge samples at 5,000rpm in a tabletop microcentrifuge for 1 min at room temperature.
16. Discard supernatants by aspiration using a fine-pointed tip attached to a vacuum trap.
17. Repeat **steps 13–15** for a total of two washes.
18. Wash beads by adding 1 mL KB. Mix by gently inverting the tube five times.
19. Proceed with washes as in **steps 13–15**, but using KB, for a total of two washes.
20. (Optional.) If needed, immunoprecipitated kinesin-1 can be dephosphorylated at this point (*see Note 19*). Simply add 300 U calf intestine alkaline phosphatase (Calbiochem) and incubate for 30 min at room temperature. Wash kinesin-1 immunocomplexes twice (as in **steps 13–15**) with KB and proceed to **step 20**.
21. Centrifuge samples at 5,000rpm in a tabletop microcentrifuge for 1 min at room temperature.

22. Discard supernatants by aspiration using a fine-pointed tip attached to a vacuum trap. Dry agarose beads (*see Note 10*).
23. Resuspend beads in 45 μ L KBC (*see Note 16*), plus appropriate protein kinase [i.e., 0.5 μ g recombinant JNK3/SAPK1b (Upstate) or other kinase of interest] (*see Note 17*).
24. Start kinase reactions by adding 5 μ L 5X ATP stock.
25. Incubate reactions at the appropriate temperature (typically 30°–35°C) for 30 min.
26. Stop kinase reactions by adding 15 μ L 5X gel loading buffer.
27. Equal volumes of each fraction are separated by SDS-PAGE (*see Note 11*). We typically run 25 μ L of the final reaction.
28. Gels are dried, exposed in a phosphorimager cassette, then scanned and quantified on a Typhoon (Amersham/Molecular Dynamics) or exposed X-ray film for autoradiography.

We recommend routinely running basic controls to determine the specificity of phosphorylation (*see Note 18* and **Fig. 2A,B**). Using the foregoing protocol, we demonstrated that KHCs can be directly phosphorylated by recombinant JNK3 kinase (**10**) and that KLC2 can be directly phosphorylated by recombinant GSK3 kinase (**12**).

4. Notes

1. These microcentrifuge tubes can withstand the recommended g force throughout these protocols. Low protein-binding tubes are required to avoid high background levels in metabolic labeling experiments. Any microcentrifuge tube that can meet these two criteria can be used.
2. Kinesin-1 is subject to modification by multiple enzymatic activities, some of which can be activated during homogenization (**31**), leaving kinesin-1 subject to postlysis phosphorylation (**12,14,31**). To help prevent potential phosphorylation artifacts, we include a mixture of kinase and phosphatase inhibitors. These inhibitors have been chosen for their wide range of inhibition toward most kinases (**32,33**) and phosphatases.
3. Preferentially, effectors with known mechanisms of action should be chosen. For example, some neurotrophic factors and neurotransmitters trigger well-characterized signal transduction responses. Pilot studies should be performed in which a wide range of effectors concentrations are tested. In addition, effects of wild-type and pathogenic protein expression on kinesin-1 phosphorylation can be determined (**10,16**).
4. As described in **Subsection 3.2.**, cell lines are typically grown until they reach 80% confluency. In most cases, cell lines are switched to serum-free culture media 1–2 h before treatment. For primary cultured neurons, we plate 6×10^6 cells per 100-mm-diameter dish and treat the cells after 5–6 days in culture.
5. This step allows for increased incorporation of radiolabeled ^{32}P into phosphoproteins. In our experience, cells do not appear to be affected by reductions of three-

fourths of the phosphate content in the media of choice. We have also grown primary cultures of neurons under these conditions without any obvious effects on cell viability.

6. This time of incubation has been determined empirically to allow for steady-state levels of kinesin-1 phosphorylation. However, incorporation of radiolabeled ^{32}P into kinesin-1 has been detected as early as 15 min of incubation using primary cultures of cortical neurons. Incorporation of radiolabeled ^{32}P into cellular phosphoproteins reaches steady-state levels within 3–4 h in these cells (Morfini and Brady, unpublished observations).
7. ^{32}P radioactivity incorporation into phosphoproteins is determined by scintillation counting as follows: 10- μL aliquots of clarified lysates (from **step 8**) are spotted onto P81 phosphocellulose paper circle lysates. Papers are air-dried for 5 min at room temperature. Once dried, P81 papers are washed with 500 mL 100 mM phosphoric acid for 10 min. This wash step is repeated four times. Finally, papers are washed once with acetone for 1 min and allowed to dry at room temperature. P81 papers are finally placed into individual scintillation vials, and 1 mL scintillation cocktail is added. Relative ^{32}P incorporation is measured by scintillation counting.
8. Kinesin-1 antibody should be excluded from the “background control” tube described in **step 1**. The exact amount of antibody to use for immunoprecipitation depends upon multiple variables (i.e., relative kinesin-1 abundance, antibody affinity, etc.) and should be empirically determined. We typically use 5 μg H2 antibody per tube, but lesser amounts of antibody can also be used successfully.
9. We have observed substantial nonspecific binding of proteins to the walls of microcentrifuge tubes upon long incubation (i.e., more than 10 h); this can result in higher background levels, which might obscure signals from immunoprecipitated kinesin-1. When immunoprecipitations are performed overnight, we typically transfer agarose beads–antibody complexes to a new tube (1.5-mL microcentrifuge tube) after the first wash with wash buffer I (**step 17**).
10. Dry agarose beads by aspirating the remaining buffer with a 30-gauge syringe needle attached to a vacuum trap. The small diameter of this syringe helps to avoid loss of agarose beads during aspiration.
11. SDS-PAGE conditions might need to be optimized, particularly when separation of KLC1 and KLC2 is desired (**11**). Good separation of KLCs is obtained by electrophoresing samples on 7.5%–16% Tris-glycine (BioRad) acrylamide gels. Alternatively, 4%–12% (Bis-Tris) NuPage acrylamide gels (Invitrogen) can be used using MOPS-based running buffer.
12. Liquid nitrogen-frozen rodent brains can be also be used; these can be obtained from commercial sources, such as Pel-Freez Biologicals (<http://www.pelfreez-bio.com>). When using frozen tissue, care must be taken to avoid long postmortem periods before freezing during which kinases, phosphatases, and proteases may all be active.
13. We recommend rinsing brains with ice-cold 10 mM HEPES, pH 7.4, before homogenization. This simple step eliminates a significant amount of rodent

- blood, which contains IgGs that can reduce kinesin-1 immunoprecipitation efficiency.
14. At this point, the concentration of brain lysates is approximately 4 mg/mL. Aliquots of precleared brain lysates can be stored at -80°C and used for future experiments.
 15. The exact amount of antibody to use for immunoprecipitation depends upon multiple variables (i.e., relative kinesin-1 abundance, antibody affinity, etc.) and should be empirically determined. We typically use 5–10 μg H2 antibody per tube.
 16. The abundant protein kinase PKA has been shown to co-precipitate with a variety of agarose-based resins (12) (and see Fig. 2A,B), and some have reported phosphatase activities in association to kinesin-1 immunoprecipitates (34). To avoid nonspecific phosphorylation/dephosphorylation of kinesin-1, PKA inhibitor peptide, okadaic acid, and microcystin are included in KB assay buffer (see Fig. 2B). In immunoprecipitation experiments, PKA mostly phosphorylates KLC2, because this KLC has multiple consensus sites for PKA in its carboxyl terminus (see Fig. 2A). Although PKA can directly phosphorylate kinesin-1 (35), this phosphorylation event does not affect kinesin-1-based motility (12).
 17. A variety of active protein kinases may be routinely obtained in recombinant form from multiple sources. We routinely use kinases prepared by Upstate Biotechnologies. It is recommended to include a tube containing an appropriate kinase substrate for every tested kinase (11). This positive control for protein kinase activity is particularly important when immunoprecipitated endogenous kinesin-1 fails to be phosphorylated.
 18. Proteins that nonspecifically bind to agarose beads can also act as substrates for protein kinases. In addition, some kinases display high levels of autophosphorylation (10–12). Thus, a “background” control tube should be included from which antikinesin-1 antibodies have been omitted (see Fig. 2C,D). Finally, some protein kinases can be nonspecifically co-immunoprecipitated with kinesin-1 (12) (see Note 16). Thus, even when using PKA inhibitors in the phosphorylation reaction, a tube should be included that contains immunoprecipitated kinesin-1 alone but not protein kinase (Fig. 2C).
 19. By dephosphorylating kinesin-1 before assay, a higher increase of phosphorylation can be achieved when using some, but not all, kinases (see Fig. 2D). Presumably, this is because a portion of immunoprecipitated kinesin-1 has been already phosphorylated at a given specific site before assay. However, kinases such as GSK3 require a “priming” phosphorylation event, which is required to phosphorylate some of its substrates. For such kinases, dephosphorylation of kinesin-1 can result in failure to phosphorylate kinesin-1.

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Protein Modification to Probe Intradynein Interactions and In Vivo Redox State

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Summary

Dyneins are highly complex molecular motors containing multiple components that contribute motor, regulatory and cargo-binding activities. Within cilia/flagella, these enzymes comprise the inner and outer arms associated with the doublet microtubules. In this chapter, we describe how to purify the outer dynein arm from flagella of the unicellular green alga *Chlamydomonas*, which is one of the best characterized members of this motor class. We also detail the methods that we use to identify interactions involving dynein components by chemical cross-linking and a recently developed technique to assess the in vivo redox state of thioredoxin-like proteins that are associated with axonemal dyneins from a wide range of organisms. Finally, we describe how to purify highly specific antibodies from serum by blot purification using recombinant proteins. Although designed for analysis of *Chlamydomonas* flagellar dyneins, these approaches should be readily adaptable to the study of other systems.

Key Words: *Chlamydomonas*; cilia; cross-linking; dynein; flagella; redox poise; thioredoxin.

1. Introduction

Dyneins are microtubule-based motors that function in the cytoplasm and cilium/flagellum. They are required for a wide range of essential cellular processes such as mitosis, vesicular transport, ciliary assembly, and motility. These massive enzymes are highly complex, consisting of one or more heavy chain units that exhibit ATPase and motor activity as well as intermediate, light-intermediate, and light chains that are involved in motor assembly, cargo recognition, and motor regulation (*see* **ref. 1** for review). For example, the outer dynein arm from the *Chlamydomonas* flagellum contains three different motor units as well as 2 intermediate chains and 11 light chains (**2–4**). Furthermore,

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at least two additional substructures including a trimeric docking complex (5) and the Oda5p/adenylate kinase complex (6) are necessary for assembly of this motor within the axonemal superstructure. One key challenge in analysis of dyneins is to define the overall architecture of these 1- to 2-MDa complexes and understand how the multiple components are arranged and function. Furthermore, motor activity is subject to multiple levels of regulation, which in cilia/flagella appears to involve phosphorylation, Ca^{2+} , and redox-based signaling pathways.

In this chapter, we describe how to purify axonemes and intact outer-arm dynein with the associated docking complex from *Chlamydomonas* flagella and the covalent modification methods we use to probe protein–protein interactions within the flagellar dynein particle; we also detail sulfhydryl derivatization methods, originally designed for study of the bacterial DsbA–DsbB system (7), that enable the *in vivo* redox state of dynein-associated thioredoxins (8,9) to be defined. Finally we describe the method, based on the original description by Olmsted (10), that we routinely employ for the blot purification of highly specific polyclonal antibodies from serum.

Although the methods described here are aimed at the analysis of flagellar axonemes and dyneins from *Chlamydomonas*, they should, with minimal alteration (such as changing salt concentrations in buffers), be readily adaptable to dyneins from other organisms and indeed to other macromolecular complexes in general. For example, proteomic studies indicate that the flagellum is built from nearly 500 different proteins (11); cross-linking holds particular promise for defining how these various components interact with each other.

2. Materials

2.1. Preparation of Flagellar Axonemes and Dynein

1. 10 mM HEPES, pH 7.5, 1 mM dithiothreitol (DTT).
2. HMS: 30 mM HEPES, pH 7.5, 5 mM MgSO_4 , 1 mM DTT, 4% (w/v) sucrose.
3. HM25%S: 30 mM HEPES, pH 7.5, 5 mM MgSO_4 , 1 mM DTT, 25% (w/v) sucrose.
4. 5.3% (w/v) CaCl_2 .
5. 25 mM dibucaine · HCl in water.
6. HMEK buffer: 30 mM HEPES, pH 7.5, 5 mM MgSO_4 , 1 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM DTT, and 25 mM KCl (*see Note 1*).
7. 10% (v/v) Igepal CA-630 (replaces Nonidet P-40, which is no longer commercially available) in HMEK.
8. HMEA: 30 mM HEPES, pH 7.5, 5 mM MgSO_4 , 0.5 mM EGTA, 25 mM KAc, 1 mM DTT, 1 mM phenylmethyl sulfonyl fluoride (PMSF) (*see Note 2*).

9. HME0.6K: 30 mM HEPES, pH 7.5, 5 mM MgSO₄, 0.5 mM EGTA, 0.6 M KAc, 1 mM DTT, 1 mM PMSF.
10. HME0.6Na: 30 mM HEPES, pH 7.5, 5 mM MgSO₄, 0.5 mM EGTA, 0.6 M NaCl, 1 mM DTT, 1 mM PMSF.
11. HMEA buffer containing 5% and 20% (w/v) sucrose.

2.2. Derivatization with AMS

1. 100% (w/v) trichloroacetic acid (TCA): This reagent is obtained as a hygroscopic solid. To prepare 100% (w/v) TCA solution, add 227 mL water to a 500-g unopened bottle of TCA. Caution: this reagent is highly corrosive.
2. 20 mM 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid (AMS) (Cat. #A485; Molecular Probes, Eugene, OR) in 0.1 M Tris-HCl, pH 7.4, 1% sodium dodecyl sulfate (SDS).
3. HMEKAc buffer: 30 mM HEPES, pH 7.4, 5 mM MgSO₄, 1 mM EGTA, and 50 mM potassium acetate.
4. Nonreducing 2X SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer: 0.1 M Tris-HCl, pH 6.8., 4% SDS, 20% glycerol, 0.01% bromophenol blue.

2.3. Cross-Linking Between Primary Amines

1. 0.5 M 1,5-difluoro-2,4-dinitrobenzene (DFDNB) in methanol plus appropriate dilution series (*see Note 3*).
2. 0.5 M dimethyl pimelimidate (DMP) in methanol plus appropriate dilution series.
3. 0.5 M disuccinimidyl suberate (DSS) in dimethylformamide plus appropriate dilution series.
4. 100 mM triethanolamine, pH 8.2.
5. 50 mM ethanolamine.
6. 0.25 M Tris-HCl, pH 6.8.
7. 5X SDS-PAGE sample buffer (prepared from 3 mL 20% SDS, 3 mL glycerol, 0.45 g Tris, pH to 6.8).

2.4. Zero-Length Cross-Linking with EDC

1. 0.5 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in HMEK and appropriate dilutions made in the same buffer. This reagent is highly water soluble and should be prepared fresh for each experiment.
2. 0.1 M 2-mercaptoethanol in HMEK buffer (*see Note 4*).

2.5. Blot Affinity Purification of Polyclonal Antibodies

1. 0.2% Ponceau S in 1% acetic acid.
2. Tris-buffered saline: (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% Tween 20.
3. 0.2 M glycine, pH 2.1.
4. 1.5 M Tris-HCl, pH 8.8.

3. Methods

3.1. Preparation of Flagellar Axonemes and Dynein

1. The procedure detailed here for purification of intact *Chlamydomonas* outer-arm dynein is modified from that described by Nakamura et al. (12) and King et al. (13).
2. Grow 4×9 L carboys of *Chlamydomonas* strain *ida1* (see Note 5) and concentrate to ~1200 mL using a Pellicon tangential flow filtration system or low-speed centrifugation. For methods to grow and harvest large quantities of *Chlamydomonas* and media requirements, etc., the reader is referred to ref. 14.
3. Aliquot ~200 mL cells into each of six 250-mL bottles.
4. Centrifuge at 2,600 *g* for 4 min at 20°C using a GSA rotor in a Sorvall centrifuge.
5. Drain supernatant and resuspend each cell pellet in 200 mL 10 mM HEPES, pH 7.5.
6. Repeat steps 4 and 5 twice.
7. Drain supernatant and resuspend each cell pellet in 20 mL cold HMS. All subsequent steps are to be carried out on ice.
8. Add 0.5 mL 5.3% CaCl_2 and 10 mL 25 mM dibucaine-HCl to each bottle, and mix with gentle swirling.
9. Using a 10-mL plastic pipette with the tip broken off, triturate 10 times and check that the cells are deflagellated by phase-contrast microscopy.
10. Add 60 mL cold HMS plus 0.3 mL 100 mM EGTA to each bottle and mix by swirling.
11. Centrifuge at 2,600 *g* for 5 min at 4°C using a GSA rotor in a Sorvall centrifuge.
12. Transfer the supernatant containing flagella to 50-mL round-bottomed tubes.
13. Centrifuge at 12,000 *g* for 10 min at 4°C using a SS34 rotor in a Sorvall centrifuge.
14. Resuspend the flagella pellets with one 20-mL aliquot HMS.
15. Split the 20-mL sample into two and overlay each 10-mL aliquot on top of 10 mL HM25%S in Beckman ultraclear centrifuge tubes.
16. Spin using an SW28 rotor (Beckman) in the ultracentrifuge at 400 *g* (1,500 rpm) for 15 min at 4°C. Remaining cell bodies will pellet while flagella remain in the upper layer and at the interface between the two sucrose solutions.
17. Remove the upper layer, which contains flagella, to two 50-mL round-bottomed tubes. Add 1/10 volume of 10% Igpal CA630 detergent (Sigma) in HMEK to a final detergent concentration of 1%.
18. Centrifuge at 12,000 *g* for 10 min at 4°C.
19. Remove supernatant and resuspend each pellet in 10 mL HMEK plus 1% Igpal CA630.
20. Centrifuge at 12,000 *g* for 10 min at 4°C.
21. Remove supernatant and resuspend each pellet in 10 mL HMEK.
22. Centrifuge at 12,000 $\times g$ for 10 min at 4°C.
23. Repeat steps 21 and 22, twice.

24. Remove supernatant, resuspend each axoneme pellet in 1 mL HMEK containing 1 mM PMSF, and keep at 4°C.
25. Divide the axoneme sample equally among two 2-mL round-bottomed tubes and centrifuge at 12,000 *g* for 10 min at 4°C.
26. Resuspend axonemes in 2 × 1 mL HMEA and centrifuge at 12,000 *g* for 10 min at 4°C.
27. Discard supernatant and resuspend axonemes in 2 × 1 mL HME0.6K (*see Note 6*). Incubate for 15 min on ice. Centrifuge at 12,000 *g* for 10 min at 4°C.
28. Repeat **step 27**.
29. Discard supernatant and resuspend axonemes in 2 × 1 mL HME0.6Na (*see Note 7*). Incubate for 15 min on ice and centrifuge at 29,000 *g* for 10 min at 4°C.
30. Retain the supernatant as crude dynein and repeat **step 29**.
31. Concentrate the 4 mL crude dynein extract to less than 0.6 mL using a Centriplus 30 ultrafiltration unit.
32. Overlay each ~200 μ L extract onto a 6.5 mL 5%–20% linear sucrose gradient made in HMEA (*see Note 8*).
33. Centrifuge at 110,000 *g* (30,000 rpm) for 10 h at 4°C in a SW55 rotor (Beckman).
34. Collect 300- μ L fractions. Outer-arm dynein sediments at ~23 S toward the bottom of the gradient (**Fig. 1**).

3.2. Derivatization with AMS

1. To 150 mL of *Chlamydomonas* culture ($\sim 3 \times 10^6$ cells/mL), add 4 mL 1 M acetic acid (final, ~25 mM) for deflagellation. Mix rapidly.
2. Immediately add 7.5 mL 100% TCA (final, ~5% v/v) with rapid mixing to fix the redox state.
3. Place 50 mL in a clean plastic tube, cap, and centrifuge at 2,200 *g* for 3 min at 20°C to pellet the cell bodies but not the flagella.
4. Decant the supernatant into a fresh tube and centrifuge again under the same conditions.
5. Decant the supernatant from the second spin and centrifuge at 27,000 *g* 12 min at 20°C in a SS34 fixed-angle rotor (Sorvall).
6. Resuspend the pellet in 1 mL acetone and transfer to 1.5-mL microfuge tube.
7. Centrifuge at 17,500 *g* 10 min at room temperature. Wash pellet twice with acetone.
8. Air-dry the pellet for ~30 min.
9. Add 100 μ L 20 mM AMS in 0.1 M Tris-HCl, pH 7.4, 1% SDS, and resuspend the pellet (*see Note 9*).
10. Once the pellet has been thoroughly resuspended, incubate for 30 min at room temperature.
11. Add 100 μ L 2X SDS sample buffer without any reducing reagents and incubate at 37°C for 30 min.
12. Separate the proteins by SDS-PAGE (10 μ L per lane on a minigel should provide a good signal for immunoblotting).

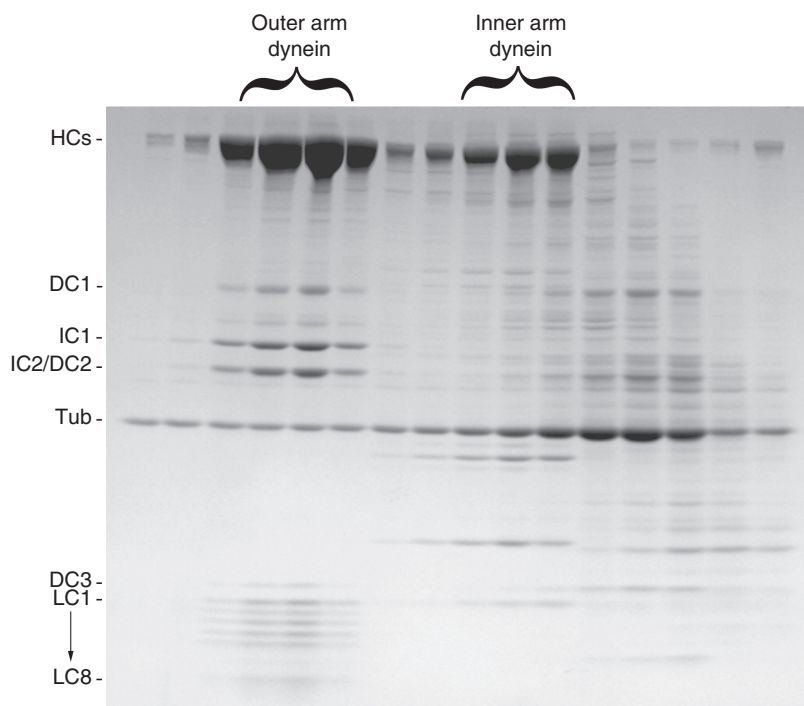


Fig. 1. Sucrose gradient purification of intact outer-arm dynein. The high salt extract from *ida1* axonemes, which lack inner arm II, was sedimented in a 5%–20% sucrose density gradient at low hydrostatic pressure in the presence of Mg^{2+} . Equal volumes of each fraction were electrophoresed in a 5%–15% acrylamide gradient gel and stained with Coomassie blue (bottom of the gradient is at the left). The location at which the inner and outer arms migrate is indicated at top; the individual components of the outer arm are marked on the left.

13. It is necessary to electrophorese fully oxidized and reduced control samples at the same time. These are obtained by treating isolated axonemes ($\sim 6 \mu\text{g}/\mu\text{L}$) in 250 μL HMEK buffer with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB; final concentration, 10 mM) and DTT (final concentration, 10 mM) in HMEKAc, respectively, at room temperature for 30 min. For the oxidized control, it is necessary to omit DTT from the buffers.
14. Add 13 μL 100% TCA acid (final, $\sim 5\%$ v/v), mix well, and centrifuge at 17,500 g for 10 min.
15. Wash three times with 500 μL acetone and dry for 30 min.
16. Add 100 μL AMS solution and incubate at room temperature for a further 30 min.
17. Add 100 μL 2X nonreducing SDS sample buffer, incubate at 37°C for 30 min, and then subject to SDS-PAGE (a 5- μL sample should give a sufficient signal).

3.3. Cross-Linking Between Primary Amines

1. Resuspend demembranated flagellar axonemes in HMEK buffer or 100 mM triethanolamine, pH 8.2 (*see Note 10*) at a concentration of ~3 mg/mL. Use 45 μ L per sample, which allows for ~150 μ g protein to be loaded into a single lane of a polyacrylamide gel in a final volume of 100 μ L (*see Note 11*). For purified dyneins, the sample should be at 0.25–0.5 mg/mL, although lower concentrations can be used if necessary. Nucleotides and various analogs may be included (*see Note 12*).
2. Prepare a series of microfuge tubes containing the appropriate volume of axonemes (*see Note 13*).
3. Prepare a cross-linker dilution series containing the reagent at 10-fold the final desired concentration (the structures of the various cross-linkers routinely used in our laboratory are shown in **Fig. 2**). Methanol is the standard solvent used

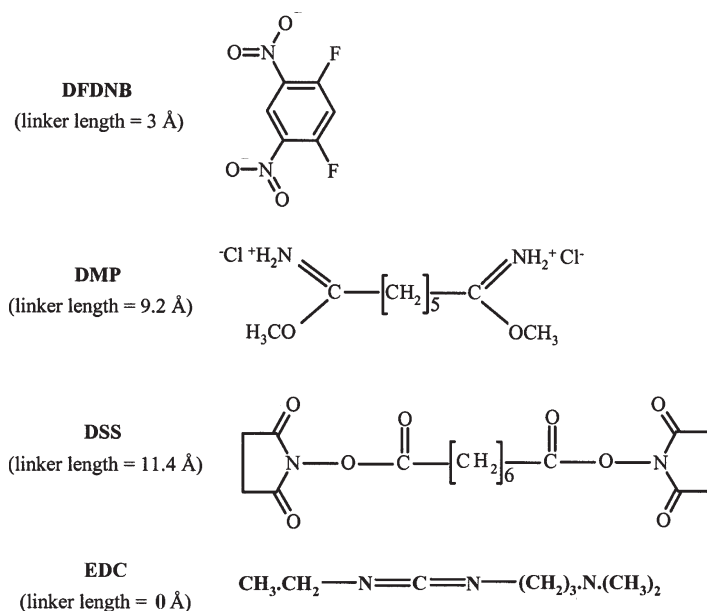


Fig. 2. Cross-linking reagents. Chemical structures of three reagents used for generating covalent linkages between primary amines and of the water-soluble carbodiimide that links carboxyl and amino groups. These reagents employ different chemistries and have different linker lengths; the length of the final linkage (in Å) is indicated. When first investigating a novel system, it is useful to try several reagents as often products may be obtained with one reagent but not another. DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DMP, dimethylpimelimidate; DSS, disuccinimidylsuberate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

for DFDNB and DMP, whereas DSS can be solubilized in dimethylformamide. We normally use final linker concentrations in the range 0.1–20mM (*see Note 14*).

4. Add 1/10 volume of each of the cross-linker dilution series to an axoneme sample. Mix by gentle vortexing and incubate for 30–60 min at room temperature.
5. Quench the reaction by addition of 5X gel sample buffer and heat at 90°–95°C for 5 min. Alternatively, if protein denaturation is not required (for example, if one intends to examine the enzymatic consequences of modification), the reaction may be quenched with 50mM ethanolamine or 0.25M Tris-HCl, pH 6.8.

3.4. Zero-Length Cross-Linking with EDC

1. The methods that we use for EDC cross-linking of primary amines to carboxyl groups are essentially identical to those detailed above for cross-linking primary amines. The use of a carbodiimide generates a zero-length attachment where the cross-linker forms a urea derivative and provides no atoms to the final cross-linked product. This implies that the groups covalently attached must have been in direct contact within the macromolecular complex under study.
2. The EDC reaction may be terminated by addition of 0.1M 2-mercaptoethanol before preparation of the sample for electrophoresis. This step is especially helpful if the effect of cross-linking on enzyme activity is to be assayed. The effects of EDC treatment on *Chlamydomonas* axonemes and the quenching activity of 2-mercaptoethanol are shown in [Fig. 3](#). The use of immunoblotting to identify specific protein products in axonemes and purified dynein samples is illustrated in [Fig. 4](#).

3.5. Blot Affinity Purification of Polyclonal Antibodies

1. Using standard SDS-PAGE methods, electrophorese a large amount (~1 mg) of the recombinant protein of interest. We usually do not include a comb when casting the stacking gel. Rather, we leave a gap between the top of the stacking gel and the edge of the glass plate and simply load the entire sample so that it runs as a single broad band.
2. Blot the gel to a nitrocellulose membrane.
3. Allow the blot to air-dry for a few minutes.
4. Stain with Ponceau S solution and destain with water to visualize all the proteins on the blot. Excise the band of interest as a single thin strip.
5. Place the strip in a 15-mL disposable plastic tube and wash with ~12 mL Tris-buffered saline (TBS) containing 1% Tween 20.
6. Add ~200 μ L serum. Cap the tube well and incubate overnight at 4°C with constant gentle agitation.

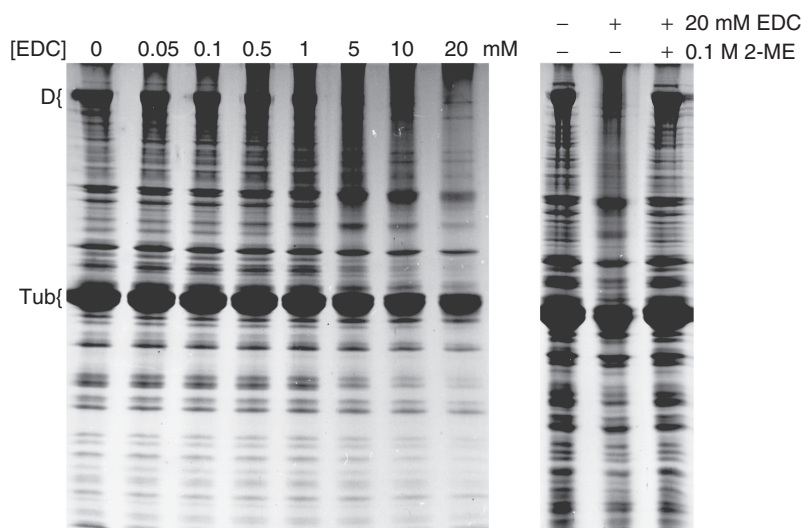


Fig. 3. Zero-length cross-linking of *Chlamydomonas* flagellar axonemes. Left: *Chlamydomonas* axonemes ($\sim 200\mu\text{g}$) in HMEK buffer containing 1 mM ATP and $50\mu\text{M}$ vanadate were treated with the indicated concentrations of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for 60 min. Samples were electrophoresed in a 5%–15% acrylamide gradient gel. The location of dynein heavy chains (D) and tubulin (Tub) are indicated at far left. Right: Axonemes were incubated in the presence or absence of 20 mM EDC with or without addition of 0.1 M 2-mercaptoethanol. Cross-linking is clearly evident following EDC treatment but is quenched by the addition of 2-mercaptoethanol. (Reprinted and modified from [ref. 17](#). Copyright ©1991 American Society for Biochemistry and Molecular Biology.)

7. Remove antibody solution and wash the strip three times (for 20 min each) with TBS containing 1% Tween 20.
8. Remove the wash buffer and place the tube upside down for ~ 5 min to allow excess liquid to drain out.
9. Add 1 mL 0.2 M glycine, pH 2.1, and agitate the tube by hand for 50 s–1 min to elute antibody bound to the immobilized protein.
10. Rapidly remove the strip to a fresh 15-mL tube containing wash buffer.
11. Immediately add 1 mL 1.5 M Tris-HCl, pH 8.8, to the antibody-containing solution to neutralize the acidic conditions (*see Note 15*).
12. Blot-purified antibodies may be used immediately or stored at -20°C . For immunoblotting, we have found that a dilution of 1/50–1/100 provides a robust signal (*see Note 16*).

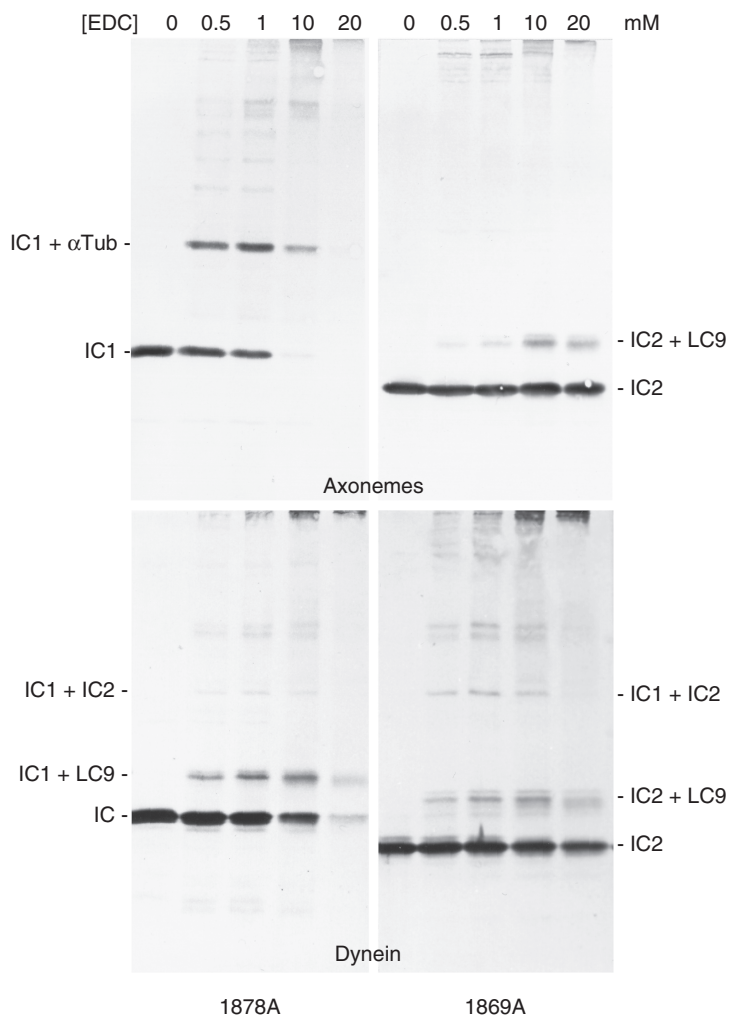


Fig. 4. Identification of a dynein-microtubule interaction. Intact axonemes (upper panels) or purified dynein (lower panels) were treated with the indicated concentrations of EDC, electrophoresed, and blotted to nitrocellulose membrane. The blots were probed with monoclonal antibodies 1878A and 1869A that specifically react with the IC1 and IC2 intermediate chains, respectively. IC2 is cross-linked to one light chain (now known to be the Tctex1 protein LC9; DiBella et al. 2005, **ref. 4**) in both axonemes and the purified enzyme. In contrast, IC1 is cross-linked directly to α -tubulin in axonemes but to LC9 in dynein. Furthermore, a small amount of IC1-IC2 product is evident in the dynein samples. (Reprinted and modified from **ref. 17**. Copyright ©1991 American Society for Biochemistry and Molecular Biology.)

3. Notes

1. *Chlamydomonas* dyneins are normally purified under relatively low salt conditions (i.e., 25 mM KCl). If adapting this procedure for dyneins from other species, it is important to test whether this salt concentration is sufficient. For example, dyneins from sea urchin sperm flagella require a salt concentration of ~200 mM for solubility (15).
2. PMSF is prepared as a 100 mM stock in isopropanol. Keep cold and dark. When first added to aqueous media, precipitation will occur. However, the precipitate will slowly dissolve.
3. Although DFDNB is readily soluble in methanol, it is highly insoluble in aqueous solution. With a final organic solvent concentration of 10% (v/v), significant precipitation occurs at and above ~10 mM DFDNB.
4. This reducing reagent is a mercaptan with a very strong smell that most people find offensive. Use in a fume hood whenever possible.
5. This strain is defective for inner dynein arm II (subspecies f) that migrates very close to the outer dynein arm in sucrose density gradients. Use of this strain allows for a more highly purified preparation of outer-arm dynein.
6. 0.6 M NaCl or KCl is necessary to extract outer-arm dynein from the axoneme. However, initial treatment with these salts also solubilizes many other axonemal components that can contaminate the dynein preparation and make purification more difficult. The preextraction step using 0.6 M potassium acetate extracts many of these components but not dyneins (12).
7. We routinely use 0.6 M NaCl to extract outer dynein arms, although 0.6 M KCl may also be used if desired. In this case, however, it is necessary to remove the KCl by dialysis before loading the extract onto the sucrose density gradient as the high KCl content buffer will not float on top of 5% sucrose.
8. The outer-arm dynein particle with associated docking complex remains intact only if the sucrose gradient step is performed at low hydrostatic pressure (using a SW55 rotor) in the presence of Mg^{2+} (16). When subject to ultracentrifugation in the absence of Mg^{2+} using a SW41 rotor, the complex dissociates to yield an 18 S $\alpha\beta$ subparticle (containing the α - and β - heavy chains, both intermediate chains and multiple light chains), a 12 S γ subparticle (containing the γ heavy chain and two light chains), and a 7 S trimeric docking complex (2,5,13).
9. TCA-fixed pellets are hard to resuspend, and it is helpful to use a pipette tip of which the end has been cut off.
10. Although many buffers other than those employed here are likely to be acceptable, it is essential to ensure that primary amines (e.g., from buffers containing Tris) are completely excluded as they will immediately quench the reaction.
11. Although for high-yield products this amount is large, the use of such quantities does allow for the detection of more minor products that are often of considerable interest.
12. It may be of interest to add ATP, vanadate, and/or nonhydrolysable nucleotide analogs. For example, we previously observed that treatment with EDC in the presence/absence

- of ATP and vanadate had significant effects on ATPase activity (17). Note that all samples should be treated with 1 mM artemisinol before assay to reduce the vanadate.
13. The volume of axonemes needed per cross-linker concentration depends on the number of samples required. Usually, we prepare enough sample to run one gel for Coomassie blue staining and three or more for immunoblot analysis.
 14. It is important to recognize that different cross-linking reagents generate different product yields. Yields can vary based on a wide variety of factors including: chemistry of the active groups, linker length (short lengths may have serious steric impediments), and buffer composition and pH. For example, DFDNB has a short linker length (3 Å) and in our hands (18) generates much lower product yields than do imido ester or *N*-hydroxysuccinimide ester derivatives such as DMP and DSS, which have linker lengths of 9.2 and 11.4 Å, respectively. It is important to use a wide concentration range, at least initially, because certain products are observed only at high concentrations whereas others form very readily (see Fig. 3) and can become incorporated into higher-order cross-linked species that are hard or impossible to analyze. It is also essential to include a no-cross-linker control sample.
 15. It is essential that the antibodies do not remain in the glycine elution buffer at pH 2.1 for more than 1–2 min as the highly acidic conditions rapidly denature the protein. Thus, it is critical to add 1.5 M Tris buffer at pH 8.8 immediately to return the pH to near neutrality. Occasionally this purification procedure fails to produce functional antibody. In nearly all cases, failure may be traced to overly extended periods in acidic conditions.
 16. Although this method usually results in greatly increased antibody specificity when compared to the original serum, it is important to realize that the total protein concentration is low. Therefore, relatively small dilution factors are employed. This parameter may have to be optimized for particular antibody preparations.

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Methods to Study the Interactions of the Dynein Light Chains and Intermediate Chains

Kevin W.-H. Lo and K. Kevin Pfister

Summary

The cargo-binding domain of the cytoplasmic dynein complex consists of an intermediate chain, a light-intermediate chain, and three families of light chains. These five subunits form the base of the dynein complex. Variations in the composition and interactions of these subunits play an important role for selecting a particular cargo and regulating dynein function. By using several complementary binding methods, we have investigated the protein–protein interaction in the cargo-binding domain of the cytoplasmic dynein.

Key Words: Cytoplasmic dynein; motor protein; microtubule; cytoskeleton; intermediate chain; light chain; protein–protein interaction.

1. Introduction

Cytoplasmic dynein 1 is a motor complex responsible for the transport of membranous vesicles and different cargo proteins toward the minus ends of microtubules (1,2). This dynein complex is involved in multiple cellular processes including mitosis, nuclear migration, Golgi and centrosome localization, organelle and viral transport, and axonal transport (3–6). Cytoplasmic dynein 1 is a large multisubunit assembly (~1.5MDa) that is composed of two functional domains. The motor domain comprises two copies of the heavy chain, DYNC1H1, which contain the microtubule-binding sites and the ATPase activity essential for force production (7). The cargo-binding domain of the dynein complex contains two intermediate chains, DYNC1I, two light intermediate chains, DYNC1LI, and two copies of each of three light chain families: DYNLL (LC8), DYNLT (Tctex1), and DYNLRB (Roadblock) (1,3,8). The intermediate chain serves as a scaffolding protein. It binds to the dynein heavy chain and to

all the cytoplasmic dynein light chain subunits, and to the p150 subunit of the cargo adaptor protein dynactin (9). Thus, the intermediate chain serves as a link between the dynein motor domain and its cargo. Tctex1, Roadblock, and LC8 light chains were shown to bind directly to the intermediate chain (10–12) by using various in vitro binding study methods. By using pair-wise yeast two-hybrid analysis and blot overlay assay, we found that the Roadblock binding domain on the intermediate chain is significant downstream of the LC8 and Tctex1 binding sites (12).

In this chapter, we discuss two other methods for studying the subunit interactions in the dynein complex, the glutathione-S-transferase (GST) pull-down assay and co-immunoprecipitation of overexpressed proteins (13). An analysis of the interaction of a light chain, Roadblock 1 (Robl-1), and an intermediate chain, 2C (IC-2C), is used as the example of the GST pull-down method. The interaction of two intermediate chains is shown as an example of the co-immunoprecipitation method. The methods described in this chapter should be adaptable for studying dynein–cargo interaction.

2. Materials

2.1. Plasmids, Expression, Purification, and Transfection Reagents

1. Bacterial expression plasmid pGEX4T-1 (Amersham Biosciences) containing mouse Roadblock isoform 1 gene (pGEX4T-1-Robl-1) (available from the author).
2. Bacterial strain: *Escherichia coli* BL21 (DE3) (Stratagene).
3. L-broth (Luria-Bertani, LB) bacterial growth medium: 10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl. Autoclave at 121°C for 30 min. Store at room temperature.
4. Isopropyl- β -D-thiogalacopyranoside (IPTG): 1 M in water, store at –20°C.
5. Ampicillin: 100 mg/mL in water. Store at –20°C.
6. Glutathione Sepharose beads (Amersham Biosciences).
7. 293T human cell line (American Type Culture Collection). (The 293T cell line is a potential biohazard, and use of this cell line falls within Biosafety level 2 criteria. You should consult the health and safety guidelines at your institution regarding use and handling of the 293T cell line.)
8. Dulbecco's modified essential medium (Invitrogen) supplemented with 10% (v/v) calf serum (HyClone) + 1% sodium pyruvate (Invitrogen) + 0.1% gentamicin.
9. Trypsin/EDTA: Dilute the 10X trypsin/ethylenediaminetetraacetic acid (EDTA) (Invitrogen) to 1X with Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (Invitrogen).
10. Mammalian expression vectors pCMV-Myc (Clontech) and pCMV-HA (Clontech) containing wild-type full-length IC-2C (pCMV-Myc-FL-2C) or full-length IC2C with deletion of the Roadblock binding site (pCMV-Myc-FL-2C Δ Robl) (available from the author).

11. Fugene 6 transfection reagent (Roche Molecular Biochemicals) (*see Note 1*).
12. Lysis buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 µg/mL leupeptin, 1 µg/mL pepstatin A, 1 µg/mL aprotinin, and 1 mM phenyl methyl sulfonyl fluoride (PMSF). (PMSF is extremely destructive to the mucous membranes of the respiratory tract, eyes, and skin. It should be handled with great care.) (*See Note 2*.)
13. 1X phosphate-buffered saline (PBS): 0.137 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, adjust pH to 7.4.

2.2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Hoefer SE250 minigel system.
2. Resolving gel buffer: 1.5 M Tris-HCl, pH 8.8. Store at room temperature.
3. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8. Store at room temperature.
4. SDS: prepare 10% solution in water and store it at room temperature.
5. Ammonium persulfate: prepare 10% solution in water and store at 4°C.
6. 30% acrylamide/bis solution, 29:1 (acrylamide is neurotoxic and should be handled with care), and *N,N,N,N'*-tetramethyl-ethylenediamine (TEMED; BioRad).
7. Running buffer: 0.025 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% SDS.
8. Prestained molecular markers, broad range (Bio-Rad). Store at -20°C.
9. 2X SDS-PAGE sample buffer: 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.28 mM β-mercaptoethanol, and 20 µg/mL bromophenol blue. Store at -20°C.
10. Coomassie blue protein stain: 0.1% (w/v) Coomassie brilliant blue R250, 50% (v/v) methanol, 10% (v/v) acetic acid. Store at room temperature.
11. Destain buffer: 10% (v/v) acetic acid.

2.3. Western Blot Analysis

1. Hoefer Transphor Electrophoresis Unit.
2. Transfer buffer: 2.21 g/L N-cyclohexyl-3-aminopropane-sulfonic acid (CAPS), pH 11, 10% (v/v) methanol. Store at 4°C.
3. Polyvinylidene difluoride (PVDF) membrane (Bio-Rad).
4. Tris-buffered saline with Tween (TBS-T): 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20.
5. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
6. Bovine serum albumin (BSA) blocking buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% BSA (Sigma), 0.05% Tween-20. It can be stored up to 1 month at 4°C.
7. Monoclonal anti-myc antibody (9E10) (Lymphocyte Culture Center, University of Virginia): 1:1,000 dilution in BSA blocking buffer.
8. Secondary antibody: horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Amersham Biosciences).
9. Enhanced chemiluminescent (ECL) reagents (Pierce) and X-ray film (Midwest Scientific).

2.4. *In Vitro* GST Pull-Down Assay

1. Binding buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA. Store at 4°C.
2. Tris-buffered saline (TBS): 50 mM Tris-HCl, pH 7.4, 150 mM NaCl.
3. Glutathione Sepharose beads.
4. Monoclonal anti-GST antibody (Zymed): 1:1000 dilution in BSA blocking buffer. The antibody can be reused and stored up to 1 month at 4°C with 0.02% sodium azide. (Sodium azide is poisonous. It should be handled with great care.)
5. BSA protein standards (2 mg/mL) (Pierce).

2.5. Co-Immunoprecipitation

1. Monoclonal anti-hemagglutinin (HA) antibody (10 µg) (Lymphocyte Culture Center, University of Virginia).
2. Protein A Sepharose 4B (Zymed).
3. Washing buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA.

3. Methods

3.1. Expressing the 293T Cells

3.1.1. Culture Conditions

293T cells grow very rapidly. Split the cells every 3–4 days by 1:40 dilution and culture under 5% CO₂ (see **Note 3**).

3.1.2. Preparation of DNA for Transfection

DNA used for transfection can be prepared by a Qiagen miniprep kit. DNA concentration should be accurately determined by measuring its optical density at 260 nm. An O.D. reading of 1 at 260 nm corresponds to 50 µg/mL.

3.1.3. Transfection of 293T Cells

The following protocol is for cells grown in a 6-cm dish with 5 mL medium.

1. Plate cells the previous day to give ~70% confluence at the day of transfection (see **Note 4**).
2. Dilute 6 µL Fugene 6 reagent to 200 µL serum-free medium without supplements (see **Note 5**).
3. Incubate for 5 min at room temperature.
4. Add 2 µg plasmid DNA to diluted Fugene 6 reagent from **step 2**.
5. Incubate for 20 min at room temperature.
6. Add the transfection reagent:DNA complex to the cells in a drop-wise manner.
7. Return the cells to 37°C incubator supplemented with 5% CO₂. Incubate the cells for 24–36 h.

3.1.4. Cell Lysis

1. Remove the growth medium from the cells to be assayed.
2. Rinse the cells with an excess of phosphate-buffered saline (PBS).
3. Collect the cells into a 1.5-mL microcentrifuge tube and centrifuge for 1 min at 5,000g.
4. Decant the supernatant and discard.
5. Resuspend the cell pellets in 100 μ L ice-cold lysis buffer.
6. Incubate the cells for 10 min on ice.
7. Centrifuge the lysed cells for 20 min at 15,800g to pellet the cellular debris.
8. Remove the protein-containing supernatant to a chilled microcentrifuge tube. For immediate use, keep on ice. Otherwise, store the protein at -80°C .

3.2. In Vitro GST Pull-Down Assay

3.2.1. Expression and Purification of GST-Roadblock-1

3.2.1.1. GST-ROADBLOCK-1

1. Use a single colony of the host BL21 (DE3) strain transformed with pGEX4T-1-Robl-1 grown on a fresh agar plate to inoculate a culture tube containing 10 mL LB medium and 100 μ L/mL ampicillin. Incubate the tube overnight at 37°C in an orbital shaker (250 rpm).
2. Dilute the culture from **step 1** into 500 mL LB medium containing 100 μ g/mL ampicillin and incubate with orbital shaking (250 rpm) at 37°C until OD_{600} reaches approx. 0.8 (mid-log phase) (*see Note 6*). At this point, induce GST-Roadblock-1 expression by adding IPTG to a final concentration of 0.5 mM.
3. Incubate the culture at 37°C for 3 h with shaking (*see Note 7*).
4. Harvest the cells by centrifuging the culture at 5000g at 4°C , and store the cell pellets at -80°C for future use.

3.2.1.2. PURIFICATION OF GST-ROADBLOCK-1

All the purification procedures are carried out in a cold room.

1. Thaw the frozen cell pellets from 0.5 L culture in 10 mL lysis buffer (PBS buffer, pH 7.4, containing 1 mM PMSF, 1 mM EDTA, 1 μ g/mL leupeptin, 1 μ g/mL aprotinin). Make sure the cells are completely resuspended.
2. Lyse cells by sonication (3 min at full power at 50% duty cycle) (Fisher Scientific 60 Sonic Dismembrator). Keep the cell suspensions on ice during the sonication to avoid heating of the mixtures (*see Note 8*).
3. Centrifuge the suspensions at 30,000g for 30 min and keep the supernatants.
4. Load the GST-Roadblock-1 crude mixture to a 1-mL GSH-Sepharose column preequilibrated with PBS buffer containing 1 mM EDTA.
5. Wash the column extensively with the same buffer.
6. Remove the GST-Roadblock-1-containing beads from the column and transfer to a prechilled microcentrifuge tube. Add equal volume of buffer (PBS containing

1 mM EDTA, 1 mM PMSF, 1 μ g/mL leupeptin, and 1 μ g/mL aprotinin) to the beads to generate 50% slurry. The GST-Roadblock-1-containing glutathione agarose bead slurry can be stored up to 2 weeks at 4°C.

7. To assay the amount of GST-Roadblock-1, take 10 μ L of the GST-Roadblock-1-containing glutathione agarose bead slurry and resuspend in 10 μ L 2X SDS-PAGE sample buffer. Also prepare BSA protein standards (1 μ g, 2 μ g, 5 μ g, 10 μ g, and 20 μ g) in 2X SDS-PAGE sample buffer. Boil the samples for 5 min.
8. Electrophoresis the boiled samples (see **Subsection 3.2.1.**). Estimate the amount of GST-Roadblock-1 by comparing the intensity of the GST-Roadblock-1 band (~39 kDa) to the BSA band (~66 kDa) in a Coomassie blue-stained SDS-PAGE gel.

3.2.2. Combining GST-Roadblock-1 with the Myc-Tagged IC2C-Containing Lysates

1. Prepare GST-Roadblock-1 bound on beads (~10 μ g for each reaction) in a microcentrifuge tube (see **Note 9**).
2. Add 10 μ L of the transfected 293T cell lysates containing overexpressed myc-tagged IC2C (from **step 7** under **Subsection 3.1.4.**).
3. Add the binding buffer to bring the volume to 0.5 mL.
4. Mix the contents of the tube and incubate the mixture on a rocking plate for 1 h at 4°C (see **Note 10**).
5. Centrifuge the mixture for 1 min at 15,800g at 4°C to pellet the glutathione agarose beads with the bound GST-Roadblock-1 and the myc-tagged IC2C. (Lower centrifugation speeds can easily be used as the agarose beads are large and will settle to the bottom of the tube within 5–10 min on the bench top.)
6. Wash the beads with 0.5 mL ice-cold binding buffer and centrifuge for 1 min at 15,800g at 4°C. Repeat this step twice.
7. Elute the GST fusion protein complex from the beads using 2X SDS-PAGE sample buffer.
8. Boil the sample for 5 min.
9. Load the entire sample onto a SDS-PAGE gel (see **Subsection 3.4.1.**).
10. Western blot analysis (see **Subsection 3.4.2.**) of the myc-tagged IC2C that was pulled down by the GST-Roadblock-1 (**Fig. 1**).

3.3. In Vivo Co-Immunoprecipitation of Epitope-Tagged IC2C

3.3.1. Preparation of Antibody-Linked Beads

1. To 1 mL TBS, add 50 μ L 50 % slurry of protein A Sepharose 4B beads.
2. Add 10 μ L anti-HA antibody (10 μ g).
3. Rock at 4°C for 1 h.
4. Pellet the beads in a microcentrifuge and wash three times with 1 mL TBS.

3.3.2. Co-Immunoprecipitation

1. Cotransfection of 293T cells with both HA-tagged and myc-tagged vectors containing IC-2C genes (see **Subsection 3.1.3.**).

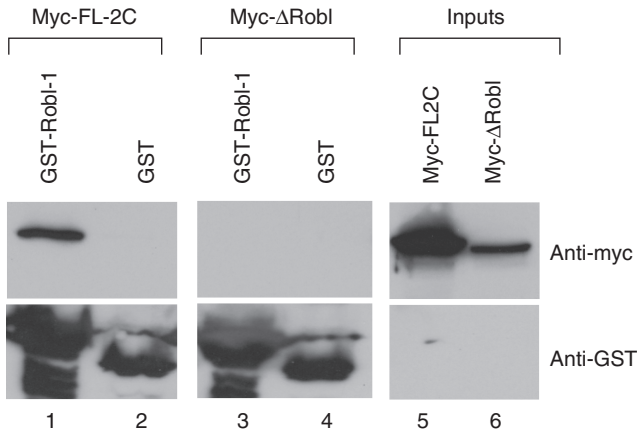


Fig. 1. Roadblock (Robl)-1 binds to full-length intermediate chain 2C, but not to an intermediate chain 2C deletion mutant, in an *in vitro* glutathione-*S*-transferase (GST) pull-down assay. To identify the intermediate chain domain necessary for binding to the Roadblock light chain, we deleted a 39-residue fragment of IC-2C and assayed the binding activity to the Roadblock-1 in a GST pull-down assay. Lane 1 shows that myc-tagged full-length intermediate chain 2C (Myc-FL-2C) expressed in 293T cells binds to and co-pellets with GST-Roadblock-1. Lane 3 shows that deletion of the 39-residue fragment from the full-length IC-2C (Myc-ΔRobl) completely abolished its binding to the GST-Roadblock-1. Purified GST was used as the negative control (lanes 2 and 4) (*see Note 11*). Lanes 5 and 6 show the inputs used in the pull-down assays. The anti-GST blot shows the presence of the GST and GST-fusion proteins in the binding assay (lower row).

2. Carry out the cell lysis in the same manner as described in **Subsection 3.1.4**.
3. Add the cell lysates to the antibody-linked beads (*see Subsection 3.3.1.*).
4. Rock at 4°C for 3 h (also *see Note 10*).
5. Pellet the beads by centrifugation (15,800g for 1 min) and wash three times with the washing buffer. Resuspend the beads with 2X SDS-PAGE sample buffer. Boil the samples for 5 min.
6. Electrophorese the boiled sample (*see Subsection 3.4.1.*) and analyze by Western blotting (*see Subsection 3.4.2.*). *See Fig. 2* for example.

3.4. SDS-PAGE and Western Blotting Analyses of the GST Pull-Down and Co-Immunoprecipitation Assays

3.4.1. SDS-PAGE

1. Assemble the glass plates according to the manufacturer's instructions (Hoefer).
2. Prepare 12.5% acrylamide solution for the separating gel as follows: 4.2mL acrylamide, 2.5mL resolving gel buffer, 100μL 10% SDS, 3.13mL deionized

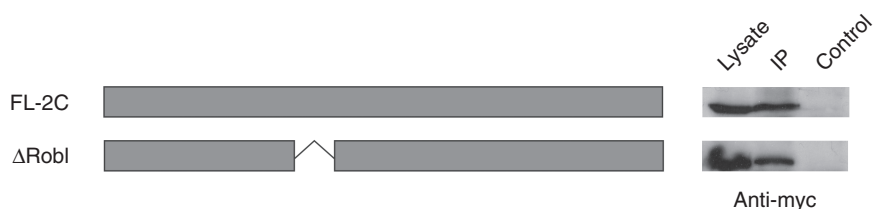


Fig. 2. Co-immunoprecipitation of intermediate chain 2C. When co-expressed in 293T cells, both HA- and myc-tagged full-length intermediate chain 2C (FL-2C) could be co-immunoprecipitated with anti-HA antibody (IP lane, upper row). This result demonstrates that full length intermediate chain 2C self-associates to form oligomers. Myc- and HA-tagged Δ Robl intermediate chain constructs were co-transfected into cultured cells. The myc-tagged Δ Robl intermediate chain was co-immunoprecipitated with the anti-HA antibody (IP lane, lower row). The result indicates that the Roadblock light chain binding domain is not required for the oligomerization. In single expression controls that were transfected with myc-tagged intermediate chain alone, no myc-tagged intermediate chains were immunoprecipitated with the antibody to HA (Control lane). The lysate lane shows the expression of the myc-tagged intermediate chains in the assay.

water, 100 μ L ammonium persulfate, 10 μ L TEMED. This recipe will make three mini-gels.

3. Pour the acrylamide solution into the gap between the glass plates. Leave sufficient space for the stacking gel.
4. Overlay the acrylamide solution with 100% ethanol and place the gel in a vertical position at room temperature. About 15 min is required for complete polymerization.
5. Pour off the overlay after the polymerization is complete. Wash the top of the gel several times with deionized water.
6. Prepare the stacking gel as follows: 0.67 mL acrylamide, 1.25 mL stacking buffer, 50 μ L 10% SDS, 3 mL deionized water, 50 μ L ammonium persulfate, 5 μ L TEMED.
7. Pour the stacking gel solution immediately on the top of the polymerized separating gel. Insert a comb into the stacking gel solution.
8. Once the stacking gel has set, carefully remove the comb and wash the polymerized wells with deionized water to remove the excess acrylamide solution.
9. Mount the gel in the electrophoresis apparatus according to the manufacturer's instructions. Add the running buffer to the top and bottom of the reservoirs.
10. Load protein samples into the bottom of the wells. Attach the electrophoresis apparatus to an electric power supply. Apply 200 V for 1 h.

3.4.2. Immunochemical Analysis

3.4.2.1. WESTERN BLOTTING

1. After electrophoresis, soak the gel in transfer buffer for 5 min.
2. Immerse the PVDF membrane in 100% methanol for a few seconds.
3. Proteins are transferred electrophoretically to the PVDF membrane in transfer buffer at 100 V for 1.5 h.
4. Keep the PVDF membrane wet in transfer buffer for immediate use. Otherwise, dry the membrane at room temperature for future use.

3.4.2.2. PROBING OF WESTERN BLOT

1. Incubate the membrane in blocking buffer for 1 h at room temperature. (If the membrane is dry, immerse the membrane in 100% methanol for a few seconds before this step.)
2. Subsequently, incubate with primary antibody (anti-myc or anti-GST) for 1 h at room temperature.
3. Wash the membrane three times with TBS-T.
4. Incubate the membrane with HRP-conjugated goat antimouse IgG (1:2500 dilution in BSA blocking buffer) for 30 min at room temperature.
5. Wash the membrane three times with TBS-T.
6. Develop the membrane with ECL reagent and expose to X-ray film.

4. Notes

1. We find that the Fugene 6 reagent is easy to use. It also gives high transfection efficiency compared to other transfection reagents tested.
2. PMSF is labile in aqueous solution. It should be added from a stock solution just before the lysis buffer is used.
3. Because 293T cells attach loosely, cells can be detached with brief trypsinization or trituration. Do not overtreat with trypsin.
4. The efficiency will decrease if the cells reach 90% confluence.
5. To avoid adversely affecting transfection efficiency, do not allow the Fugene 6 reagent to come into contact with the plastic surfaces other than the pipette tips.
6. To obtain high-level expression of GST-Roadblock-1, it is necessary to induce protein expression at the mid-log phase. We have found that some GST-Roadblock-1 was expressed as inclusion bodies when we induce the protein expression after the mid-log phase.
7. In general, we have found that the best results for the proteins described here are obtained when the GST fusion protein induction occurs at 37°C. In some cases, GST fusion protein expression occurs best at room or even lower temperature such as 16°C. Therefore, initial studies that examine time and temperature of induction are essential.
8. In principle, one can use lysozyme or French press method to replace sonication.

9. We have found that treatment of the beads with BSA blocking buffer for 1 h at 4°C at this step can minimize the background caused by nonspecific interaction between the binding sites of the bead and the proteins in the cell lysates.
10. Incubation time can be varied, i.e., 1 h, 2 h, 3 h, or even overnight. In most of our cases, we obtained positive pull-down or co-immunoprecipitate reactions in less than 3 h incubation at 4°C.
11. In all pull-down assays, carefully designed control experiments are absolutely necessary for generating biologically significant results. In this experiment, a negative control experiment consisted of purified GST protein (affinity support of the GST-Roadblock-1) and the lysates containing myc-tagged IC2C. Purified GST protein can be obtained by using the protocol described in **Subsection 3.2.1.**

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Identification of Motor Protein Cargo by Yeast 2-Hybrid and Affinity Approaches

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Summary

Identification of the molecular composition of the cargo transported by individual kinesin motors is critical to an understanding of both motor function and regulation of the proper intracellular placement of numerous cellular components including proteins, RNA, and organelles. In this chapter, we describe methods to identify the motor tail sequences responsible for cargo binding by expression of green fluorescent protein (GFP)-motor tail fusion proteins in mammalian cells. In addition, we detail two complementary approaches to identify specific proteins associated with these targeting sequences: a yeast 2-hybrid screen and affinity chromatography.

Key Words: GFP; yeast 2-hybrid; affinity chromatography; kinesin; motor protein; KIFC1; protein–protein interaction.

1. Introduction

Kinesin molecular motors transport a wide variety of cargos along cytoplasmic microtubules in eukaryotic cells. Kinesins contain a highly conserved motor domain that is responsible for ATP hydrolysis and binding to microtubules, enabling these proteins to walk along polymer tracks dragging their attached cargo to its proper intracellular destination (reviewed in **refs. 1–4**). Although the motor domain of kinesins is well conserved, the tail domains are quite divergent and contain binding sites for specific cargo molecules including tubulin, nuclear transport receptors, transcription factors, membrane receptors, scaffolding proteins, and lipid rafts (**5–13**). In many of these cases, however, the exact sequences involved in the motor–cargo binding and how these interactions may be regulated are poorly understood.

KIFC1 and related motor KIFC5A are members of the kinesin-14 sub-family of C-terminal kinesins along with the spindle motors *Drosophila ncd* and *Xenopus XCTK2* (14–16). KIFC1 and KIFC5A are 87% identical along their entire length; however, they display quite different subcellular localizations. KIFC1 was initially identified in brain (17) and is widely expressed in other tissues, including the testis, where it is associated with the elongating spermatid nucleus (18,19). KIFC5A, on the other hand, is associated with the mitotic spindle and has been implicated in centrosome separation (6,20). The primary difference between these two motors is the sequences in their tail domains (6). The KIFC1 tail is almost identical to that of KIFC5A except for the absence of two domains: an N-terminal extension and an insert in the stalk domain. In addition, KIFC1 contains a unique 19-amino-acid (aa) sequence not found in KIFC5A (6). We have used these two motors as a model system to investigate the cargo-binding specificities of different tail domains. In this chapter, we describe the identification of targeting sequences by localization of green fluorescent protein (GFP) fusions and detection of binding proteins by two complementary approaches: interaction in a yeast 2-hybrid assay and affinity chromatography.

2. Materials

2.1. GFP-Motor Fusion Targeting

2.1.1. DNA Manipulations and Bacterial Growth

1. Plasmids: pEGFP-N2 is used for construction of GFP fusion proteins for expression in mammalian cells (Clontech, Mountain View, CA) and T-Vector (Promega, Madison, WI) is used for ligation of polymerase chain reaction (PCR) fragments before transfer to expression vectors. The motor tail of interest is obtained by reverse transcriptase (RT)-PCR from the appropriate tissue cDNA.
2. Oligonucleotides: 100 μ M in 10 mM Tris-HCl, pH 8.0, 0.1 mM ethylenediamine-tetraacetic acid (EDTA). The oligonucleotides should flank the region of interest and be designed to contain restriction enzyme sites at their 5'- and 3'-ends such that the annealed oligos form cohesive ends.
3. 10X TE buffer: 100 mM Tris-HCl, pH 7.5, 10 mM EDTA. Autoclave.
4. 10X annealing buffer: 100 mM Tris-HCl, pH 7.5–8.0, 10 mM EDTA, 500 mM NaCl.
5. 5X ligation buffer: 250 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000 (Cat. #46300-018; Invitrogen, Carlsbad, CA).
6. T4 DNA ligase (Cat. #15224-017; Invitrogen).
7. 10X first-strand RT buffer: 200 mM Tris-HCl, pH 8.4, 500 mM KCl.
8. RNase Block ribonuclease inhibitor (Stratagene, La Jolla, CA).

9. 100 mM deoxyribonucleotide triphosphate (dNTP) solution for reverse transcription.
10. 10X PCR buffer: 10X Ex Taq buffer with Mg^{+2} (Takara, Tokyo, Japan).
11. Taq polymerase: Ex Taq (Takara).
12. 2.5 mM dNTP solution for PCR.
13. Reverse transcriptase: Superscript II (Invitrogen).
14. Competent bacterial cells for transformation: Max Efficiency DH5 α F'IQ (Cat. #18288-019; Invitrogen).
15. Ampicillin: 100 mg/mL in water. Filter sterilize. Store as aliquots at -20°C .
16. Kanamycin stock: 100 mg/mL in purified water. Filter sterilize. Store as aliquots at -20°C .
17. 0.1 M isopropyl-beta-D-thiogalactopyranoside (IPTG): 1.2 g IPTG in 50 mL final volume of water. Filter sterilize and store as aliquots at -20°C .
18. X-Gal stock: Dissolve 100 mg in 2 mL DMF (*N,N*'-dimethyl formamide). Store at -20°C in the dark. Use caution handling DMF as it is harmful by inhalation, ingestion, or skin contact.
19. L-broth (Luria-Betrani, LB) bacterial growth media, agar plates: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl in 800 mL water. Adjust to pH 7.0 with NaOH; bring to 1 L final volume, and autoclave. Add ampicillin to 100 μg /mL for selective media (LB-amp). For agar plates, add 15 g Bacto-agar per liter before autoclaving. Pour 30–35 mL per 85-mm Petri dish, let stand at room temperature to allow agar to harden, invert, and let plates dry overnight at room temperature. To prepare selective plates for ampicillin resistant plasmids, add 1 mL 100 mg/mL ampicillin stock to 1 L autoclaved media after cooling the agar to less than 50°C . For kanamycin selective plates (LB-kan), add 1 mL 100 mg/mL kanamycin stock per liter cooled agar. To use blue/white color selection for recombinant T-vector, add IPTG (final concentration, 0.5 M) and X-Gal (final concentration, 80 μg /mL) to ampicillin-supplemented agar media before pouring onto plates. Alternatively, spread 100 μL 100 mM IPTG and 20 μL 50 mg/mL X-Gal on ampicillin plates and allow liquid to penetrate media for 1–2 h at room temperature before use. Store plates for 1 month at 4°C .
20. Gel Extraction Kit: Qiagen.
21. Plasmid DNA preparation: Wizard Plus SV Miniprep and Wizard Plus SV Maxi-prep kits (Promega, Madison, WI).
22. PCR machine: We use a PTC-200 DNA Engine (MJ Research, BioRad, Waltham, MA).

2.1.2. Cell Culture, Transfection, and Microscopy

1. NIH 3T3 cells: American Type Tissue Collection (ATTC, Manassas, VA).
2. Cell culture media: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotics (penicillin-streptomycin; Invitrogen).

3. 10X phosphate-buffered saline (PBS): 1.37 M NaCl, 26.8 mM KCl, 81 mM Na₂HPO₄, 14.7 mM KH₂PO₄. Dissolve 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄ anhydrous, 2 g KH₂PO₄ anhydrous, in 800 mL water. Bring volume to 1 L.
4. Trypsin stock solution: 2.5% in 1X PBS (Invitrogen).
5. Trypsin working solution: 0.05% is diluted with 1X PBS.
6. Culture flasks and plates (Fisher Scientific, Fair Lawn, NJ).
7. CO₂ cell culture incubator (Forma Scientific, Marietta, OH).
8. Transfection reagent: Lipofectamine (Invitrogen). Store at 4°C.
9. Six-well culture plate and coverslips (Fisher).
10. Tris-buffered saline with Triton (TBSTx): 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100. Mix 10 mL 1 M Tris-HCl, pH 7.5, 4.39 g NaCl, in 300 mL purified water. Add 0.5 mL Triton X-100 and mix well. Bring volume to 500 mL with purified water and store at 4°C.
11. Fixation solution: 50:50 mixture of acetone/methanol at room temperature.
12. Vectashield mounting media containing 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Vector, Burlingame, CA).
13. Microscope: Fluorescence microscope equipped with 100X objective and appropriate filters (Nikon E600).

2.2. Yeast 2-Hybrid

2.2.1. DNA Manipulations and Yeast Growth Media

1. Plasmid vectors: pGBKT7 (Clontech) is used for construction of fusion proteins for expression in yeast. pGBKT7 contains the GAL4 DNA BD (binding domain) upstream of a multiple cloning site such that the protein of interest is expressed as a fusion to the GAL4 DNA BD (the bait). pGBKT7 contains the kanamycin resistance gene for selection in bacteria and the TRP1 gene for yeast selection. The cDNA library is cloned into pACT2 such that the proteins are expressed as fusions to the GAL4 AD (activation domain). pACT2 is ampicillin resistant and contains the yeast LEU2 gene.
2. YPD yeast growth media, agar plates: 20 g Difco peptone, 10 g yeast extract in 1 L water. Autoclave. Add 20 g agar per liter for plates, autoclave, and pour 30–35 mL per 85-mm Petri dish. YPDA is YPD with adenine hemisulfate added to a final concentration of 0.003% (see **Note 1**).
3. Adenine hemisulfate stock: 0.2% adenine hemisulfate (Cat. #A-9126; Sigma-Aldrich, St. Louis, MO). Filter sterilize.
4. Yeast strains: AH109, Y187, Y187 pretransformed with desired cDNA library (Clontech).

2.2.2. Yeast Transformation

1. 50% polyethylene glycol (PEG) stock solution: Dissolve PEG (average molecular weight, 3350) (Cat. #P-3640; Sigma-Aldrich) in sterile purified water and warm to 50°C if necessary to dissolve.

2. 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich).
3. 1 M LiAc: Dissolve 10.2 g in 100 mL water, adjust to pH 7.5 with dilute acetic acid and autoclave.
4. PEG/LiAc solution: 8 mL 50% PEG 3350 (polyethylene glycol), 1 mL 10X TE, 1 mL 1 M LiAc.
5. Herring testis carrier DNA: Purchased from Clontech (Cat. #K1606-A). Just before use, denature DNA by placing in boiling water and immediately cooling on ice.

2.2.3. Yeast Mating, Selection, and Characterization of Interacting Clones

1. SD yeast minimal growth media, agar plates: Dissolve 6.7 g yeast nitrogen base without amino acids, 100 mL 10X sterile appropriate dropout solution for nutrient selection in 1 L water. Add 20 g agar, mix, and autoclave. The yeast 2-hybrid screen requires 50 150-mm plates whereas maintenance, transformations, and mating efficiency tests require 85-mm plates (*see Note 2*). To prepare indicator plates to detect expression from the α -galactosidase reporter gene, add 2 mL 20 mg/mL X- α -Gal to 1 L cooled agar medium before pouring onto plates. Alternatively, dilute X- α -Gal stock to 4 mg/mL in DMF and spread 200 μ L onto a 150-mm plate using glass beads. Allow plates to dry at least 15 min at room temperature before use.
2. YPDA/Kan: YPDA with 50 μ g/mL kanamycin. Kanamycin is used to reduce bacterial contamination during overnight growth of bait containing yeast cells.
3. 2X YPDA: 40 g Difco peptone, 20 g yeast extract, 15 mL 0.2% adenine hemisulfate in 1 L water. Add 20 g per liter for plates, autoclave, and pour 30–35 mL per 85-mm Petri dish.
4. 0.5X YPDA: 10 g Difco peptone, 5 g yeast extract, 15 mL 0.2% adenine hemisulfate in 1 L water. Add 20 g agar per liter for plates, autoclave, and pour 30–35 mL per 85-mm Petri dish.
5. Kanamycin stock: 50 mg/mL in purified water; filter sterilize and store in aliquots at -20°C .
6. X- α -Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Cat. #MESP-1995, Growcells.com, Irvine, CA): 20 mg/mL in DMF (*N,N*-dimethylformamide). Store at -20°C in the dark. Use caution handling DMF as it is harmful by inhalation, ingestion, or skin contact.
7. Pretransformed control yeast strains: YH109 containing pGBKT7-53 (YH109[pGBKT7-53]), AH109 containing pGBKT7-Lam (AH109[pGBKT7-Lam]), and Y187 containing pTD1 (Y187[pTD1]) (Clontech).
8. Yeastmaker yeast plasmid isolation kit (Cat. #630441, Clontech).
9. Plating beads (Cat. #5000-550; Qbiogene, Carlsbad, CA).
10. Replica plating apparatus and velvet pads (Cat. #5000-004, 5000-009; Qbiogene).

2.3. Affinity Purification of Motor Complexes

2.3.1. Tissue Lysate

1. Extraction buffer: 100mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.75, 1 mM ethyleneglycoltetraacetic acid (EGTA), pH 7.0, 1 M MgCl_2 , 4 M glycerol (store at 4°C).
2. Protease inhibitor cocktail for mammalian cell and tissue (Sigma): store in 200- to 300- μL aliquots at -20°C.
3. Nonidet (NP-40; now called IGEPAL CA-630; Cat. #I-8896, Sigma).
4. Potter-Elvehjem tissue grinder (Fisher).
5. Pro 200 Homogenizer (PRO Scientific, Oxford, CT).

2.3.2. Affinity Column

1. One empty Poly-prep column: holds 2 mL resin and 10 mL total volume (BioRad).
2. Resin with prebound peptide. The peptide is synthesized attached to a methacrylate resin (Protein Sequence and Peptide Synthesis Facility, UNC-Chapel Hill). Alternatively, the peptide is linked to an agarose gel via a sulfhydryl group on the peptide using the Sulfolink kit from Pierce.
3. Adams Nutator mixer (BD Diagnostic Systems, Bedford, MA).
4. 0.1 M potassium phosphate buffer: 16 mL 0.2 M KH_2PO_4 , 84 mL 0.2 M K_2HPO_4 in 200 mL H_2O .
5. Bead binding buffer: 50 mM potassium phosphate buffer, pH 7.5, 150 mM KCl, 1 mM MgCl_2 , 1% Triton X-100, and 10% glycerol. Store at 4°C.
6. PBS with 0.02% sodium azide (degassed).
7. 100 mM glycine, pH 2.5.
8. 1 M Tris-HCl, pH 9.5.
9. 5 M guanidine-HCl.
10. 10% acetic acid.
11. 50% (w/v) trichloroacetic acid (TCA).
12. 2% deoxycholic acid.
13. Acetone.

2.3.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. SDS-PAGE electrophoresis equipment (Hoefer model SE400).
2. Separating gel buffer: 1.5 M Tris-Cl, pH 8.8. Store at room temperature.
3. Stacking gel buffer: 0.5 M Tris-Cl. Store at room temperature.
4. SDS stock solution: 10% w/v in water. Store at room temperature.
5. 30% acrylamide/0.8% bisacrylamide. Acrylamide is a neurotoxin and should be handled wearing gloves and mask.
6. Ammonium persulfate: 10% (w/v) in water. This solution is best if made fresh but can be stored at 4°C for several days.
7. TEMED (*N,N,N',N'*-tetramethylethylenediamine; Invitrogen).
8. 6X SDS sample buffer: 100 μL 10% SDS, 7 mL stacking buffer, 3 mL 100% glycerol, 0.6 M DTT (dithiothreitol), and 0.012% bromphenol blue in a total of 10 mL. Store in aliquots at -20°C.

9. 1X SDS electrophoresis buffer: 0.25M Tris-HCl, pH 8.3, 0.12M glycine, 0.1% SDS. Store at room temperature.
10. Coomassie blue protein stain: 0.1% (w/v) Coomassie brilliant blue R250, 50% (v/v) methanol, 10% (v/v) acetic acid. Store at room temperature.
11. Destain buffer: 10% (v/v) acetic acid.
12. BenchMark prestained protein ladder (Invitrogen; Cat. #10748-010).

2.3.4. Western Blotting

1. Blotting apparatus (BioRad transblot or similar).
2. Transfer buffer: 25mM Tris-base, 192M glycine, 15% methanol. Combine 30.3 g Tris base, 144 g glycine, in 5 L water. Add 1500 mL methanol and bring final volume to 10L. Chill.
3. Immuno-Blot PVDF (BioRad).
4. 3MM chromatography paper.
5. Tris-buffered saline with Tween (TBST): 100mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1% Tween-20. Mix 50mL 1M Tris-HCl, pH 7.5, and 4.35 g NaCl in 300mL purified water. Add 500μL Tween-20. Mix well and bring to 500mL with purified water. Store at 4°C.
6. Blocking buffer: 5% nonfat dry milk in TBST (*see Note 3*). Add 0.02% sodium azide to prevent bacterial growth. Sodium azide is a poison and should be handled with caution.
7. Primary antibody: motor or cargo specific antibody, diluted in blocking buffer as appropriate.
8. Secondary antibody: Horseradish peroxidase (HRP)-conjugated donkey antirabbit or antimouse (Cat. #711-035-152 or 715-035-150; Jackson ImmunoResearch, West Grove, PA).
9. Chemiluminescence detection reagent: SuperSignal West Pico Chemiluminescence substrate (Pierce, Rockford, IL).

3. Methods

3.1. GFP-Motor Fusion Targeting

3.1.1. Construction of GFP-Motor Tail Fusion

We describe a method here for isolating the desired motor tail sequence from source tissue or cultured cell mRNA by RT-PCR followed by insertion into the GFP vector.

1. Mix 10μg total RNA [or 0.1–1 μg poly(A) RNA] with 3μL downstream gene-specific primer and adjust the final volume of the reaction to 41μL with RNase-free water. Mix gently.
2. Incubate the reaction at 65°C for 5 min to denature primers and RNA. Cool each reaction slowly at room temperature for 10 min to allow the primers to anneal to the RNA.

3. Add the following components to the reaction in order: 5 μ L 10X first-strand buffer, 1 μ L RNase Block Ribonuclease Inhibitor (40 U/ μ L), 2 μ L 100 mM dNTPs, 1 μ L StrataScript reverse transcriptase (50 U/ μ L).
4. Mix the reaction gently and incubate each reaction at 42°C for 1 h followed by incubation at 90°C for 5 min to stop the reaction.
5. Place the completed first-strand cDNA synthesis reaction on ice for PCR amplification or store at 4°C.
6. Mix 1–3 μ L of the first-strand cDNA mixture (or 0.5–1 μ g of a plasmid containing the motor cDNA), 2.5 μ L 10X buffer, 2 μ L 2.5 mM dNTP, 1 μ M each primer, and 0.5 μ L Taq polymerase in a volume of 25 μ L.
7. Preheat the reactions in the PCR block for 5 min at 95°C.
8. Set up three-step cycling conditions according to the annealing parameters of the primers (see **Note 4**).
9. Separate the PCR amplification products by electrophoresis through an agarose gel. View briefly under long-wave UV and remove desired DNA band using a scalpel. Cut slice into small pieces and transfer gel pieces to microcentrifuge tube. Use appropriate UV protection for eyes and skin.
10. Purify the PCR product by extraction from the gel using the Qiaex kit from Qiagen.
11. Resuspend the PCR fragment in a small volume of water. Confirm presence of purified fragment by analysis of a small aliquot by gel electrophoresis. We typically clone PCR fragments into pGEM-T vector first, and then transfer the insert into pEGFP-N2 because of the high ligation efficiency of PCR fragments into T-vector.
12. Mix 50–100 ng vector, 150–300 ng DNA fragment, 5 μ L 2X rapid ligation buffer (for T-vector) or 2 μ L 5X ligation buffer (for pEGFP), and 3 units of T4 DNA ligase in 10 μ L volume.
13. Incubate ligation reaction at 4°C overnight (see **Note 5**).
14. Remove competent cells from –70°C freezer; thaw on ice.
15. Gently mix the cells, then aliquot 50- μ L competent cells per ligation reaction into chilled 17 $\times$ 100 mm polypropylene tube (Falcon 2059).
16. Add 3 μ L ligation reaction to competent cells in polypropylene tube; gently mix and incubate on ice for 30 min.
17. Heat-shock cells 45 s in a 42°C water bath, then place on ice for 2 min.
18. Add 0.9 mL room temperature Difco NZYM Broth medium and incubate the cells for 1 h at 37°C with shaking (250 rpm).
19. Spread 100 μ L culture evenly onto plate. Use LB/ampicillin/IPTG/X-Gal plates for cells transformed with pGEM-T and kanamycin plates for pEGFP-N2.
20. Invert and incubate plates at 37°C overnight.
21. Choose single colonies from transformation plate and inoculate individual 2-mL cultures. Grow overnight at 37°C with shaking.
22. Prepare plasmid DNA using either the Wizard Plus SV Miniprep or Maxiprep kits (Promega).
23. Identify successful recombinants containing motor tail sequence by digestion with appropriate restriction enzymes followed by agarose gel electrophoresis.

3.1.2. Cell Culture Transfection and Microscopy

1. Two days before transfection, seed NIH 3T3 cells onto coverslips in 6-well plates in complete growth medium (DMEM, 10% FBS).
2. Incubate at 37°C in a CO₂ incubator until the cells are 50%–80% confluent. Just before transfection, remove media and rinse cells three times with PBS.
3. For each well in a transfection, dilute 2–20 µL lipofectamine and 0.8–1.6 µg DNA into 100 µL medium without serum in individual microcentrifuge tubes. Mix gently.
4. Combine diluted DNA and diluted lipofectamine, mix gently, and incubate at room temperature for 40 min to allow DNA–liposome complexes to form. The solution may appear cloudy, but this will not impede the transfection.
5. For each transfection, add 0.8 mL DMEM without serum to the tube containing the complexes. Mix gently and overlay the rinsed cells with the diluted complex solution followed by incubation for 3–6 h at 37°C.
6. Following incubation, add 1 mL growth medium containing twice the normal concentration of serum.
7. Incubate the cells at 37°C and observe with a fluorescence microscope at various time points. The time at which GFP fluorescence becomes visible is protein specific but it usually appears between 4 and 48 h.
8. Remove culture media at end of incubation period and rinse three times with PBS.
9. Incubate cells with 2 mL 4% paraformaldehyde in PBS for 30 min at room temperature (*see Note 6*).
10. Rinse three times with PBS. Carefully remove coverslips from plate and note which side of coverslip contains cells. Drain excess PBS from the coverslips by touching the edge of the coverslip to a Kimwipe.
11. Place drop of Vectashield mounting media containing DAPI on glass microscope slide. Lay coverslip carefully onto mounting media with cells facing down. Try to avoid bubbles as coverslip is slowly lowered onto slide. Seal the coverslips with clear nail polish and observe with a fluorescence microscope fitted with a fluorescein isothiocyanate (FITC)-sensitive filter.

3.2. Yeast 2-Hybrid

3.2.1. Construction of Bait Vector

Determine the sequence or protein domain of interest by physiological methods (such as targeting of GFP fusions as described in **Subsection 3.1**). We were able to narrow this region in the KIFC1 tail domain to a 19-aa sequence and chose to use complementary oligonucleotides encoding the targeting sequence to generate the specific bait sequence.

1. Mix 1 µL sense oligonucleotide, 1 µL antisense oligonucleotide, 1 µL 10X annealing buffer, and 7 µL sterile water.
2. Incubate mixture at 95°C in heating block for 10 min.

3. Remove block from heating element and allow to cool slowly to room temperature to allow oligonucleotides to anneal.
4. Mix 6 μ L annealed oligonucleotide mixture, 100 ng pGBKT7 vector digested with the appropriate enzymes, 2 μ L 5X ligation buffer, and 1 μ L T4 DNA ligase in a total volume of 10 μ L with sterile water.
5. Incubate overnight at 16°C.
6. Transform 3–5 μ L ligation mixture into competent cells according to manufacturer's instruction. Plate cells on LB agar plates containing 100 μ g/mL kanamycin to select for transformants.
7. Choose single colonies from transformation plate and inoculate separate 2 mL LB-amp cultures containing 100 μ g/mL kanamycin. Grow cultures overnight at 37°C in a rotary shaker (225–250 rpm).
8. Isolate plasmid DNA from each culture using a commercially available miniprep kit (WizardPlus SV purification system; Promega) or according to established protocols (21).
9. Confirm presence of insert by digestion of miniprep DNA with appropriate restriction enzymes followed by agarose electrophoresis.
10. Prepare DNA representing the correct motor tail fusion from recombinant using commercial maxiprep kit (Promega).

3.2.2. Yeast Transformation

The motor tail fusion plasmid can be introduced into yeast using either electroporation or the standard lithium acetate procedure according to standard protocols (21) or commercially available kits (Yeastmaker yeast transformation system 2, Clontech, or similar).

1. Streak fresh YPDA plate with yeast host strain YH109 to obtain single colonies. Incubate at 30°C until colonies are 2–3 mm in diameter (about 3 days).
2. Inoculate a 2-mL culture of YPD with a single colony from the fresh plate and grow overnight at 30°C.
3. Inoculate a 50-mL YPD with 2 mL overnight yeast culture and grow 16–18 h with shaking at 250 rpm. OD₆₀₀ of the culture should be greater than 1.5 (see **Note 7**).
4. Inoculate 300 mL YPD in 1-L flask with 50 mL yeast culture. Incubate with shaking for 2–3 h until the OD₆₀₀ is between 0.4 and 0.6; this is enough cells for 10–15 transformations.
5. Collect cells by centrifugation at 1,000g for 5 min at room temperature.
6. Remove supernatant and resuspend the pellet in sterile TE or water to a volume of 25–30 mL.
7. Collect cells a second time at 1,000g for 5 min at room temperature. Remove supernatant.
8. Resuspend the cell pellet in 1.5 mL fresh 1X TE/1X LiAc.
9. For each transformation, mix 0.1 μ g plasmid DNA, 0.1 mg herring testis carrier DNA, with 0.1 mL competent yeast cells in sterile 1.5-mL tube. Mix well by vortexing.

10. Add 0.6 mL sterile PEG/LiAc solution and vortex vigorously for 10 s.
11. Incubate at 30°C for 30 min with shaking at 200 rpm.
12. Add 70 μ L DMSO and mix well by gently inverting tubes, but do not vortex.
13. Heat shock tubes for 15 min in a 42°C water bath. Chill on ice for 1–2 min.
14. Centrifuge cells for 5 s at 16,000g at room temperature. Remove the supernatant.
15. Resuspend cells in 0.5 mL sterile TE buffer.
16. Plate 100 μ L undiluted cells and cells diluted 1:10, 1:100, and 1:1000 on selective media. The selective media will depend on the auxotrophic marker contained in the yeast expression vector (*see Note 2*).
17. Yeast cells containing the bait fusion vector in pGBKT7 (YH109[bait]) should grow in the absence of tryptophan (SD/Trp drop-out media).
18. Streak out positive transformants on selective media. Before proceeding with the screen for interacting clones, it is very important to determine that the motor-tail fusion does not activate transcription from the reporter gene, that its expression is not toxic to yeast, and that it does not significantly reduce mating efficiency (*see Note 8*).

3.2.3. Yeast Mating, Selection, and Characterization of Interacting Clones

Interaction between the motor-tail domain and proteins encoded by a cDNA library are detected by mating between yeast containing the bait plasmid and pretransformed libraries (purchased from Clontech or prepared by the investigator) followed by selection for expression of auxotrophic markers. Detailed protocols for manipulation of yeast (protocol PT3024-1), construction of pretransformed libraries (protocol PT3529-1), and yeast 2-hybrid screening (protocol PT3247-1) are available from the Clontech Website. Include control mating reactions for yeast mating efficiency and protein-protein interaction (YH109[pGBKT7-53] $\times$ Y187[pTD1]).

1. Streak out (YH109[bait]) obtained in **Subsection 3.2.2.** on selective media (SD/Trp) at 30°C until colonies are 2–3 mm.
2. Inoculate 2 mL selective media (SD/Trp) with a single colony, vortex, and grow with shaking (250 rpm) overnight at 30°C.
3. Inoculate 50 mL SD/Trp in a 250-mL flask with the overnight yeast culture. Continue growth overnight at 30°C with shaking.
4. Check that the OD₆₀₀ of culture is greater than 0.8. Transfer cells to centrifuge tubes and spin down cells at 1000g for 5 min at room temperature. Decant supernatant and resuspend cell pellet in approximately 4 mL residual media.
5. Just before setting up the yeast mating, thaw one aliquot of the pretransformed library (~1 mL) in a room temperature water bath. Gently vortex and reserve 10 μ L on ice in a microcentrifuge tube for later titering (*see Note 9*).

6. Combine the yeast cells containing the bait plasmid from **step 4** and the pretransformed library culture in a 2-L sterile flask and add 45 mL 2X YPDA/Kan. Rinse the library tube two times with 1 mL 2X YPDA/Kan and add to 2-L flask. Swirl gently and incubate 20–24 h at 30°C with gentle shaking (30–50 rpm) to allow the yeast to mate.
7. The next morning, check a drop of the mating culture by phase-contrast microscopy. If zygotes are still present (three-lobed structures), continue incubation for 4 h.
8. Transfer the mating culture to sterile 50-mL centrifuge tube. Spin down cells at 1000g for 10 min at room temperature.
9. While the cells are spinning down, rinse the 2-L flask that contained the mating culture two times with 50 mL 2X YPDA/Kan. Combine the rinses and use them to resuspend the pellet.
10. Spin down the cells again at 1000g for 10 min at room temperature. Resuspend the cell pellet in 0.5X YPDA/Kan and measure the total volume of cells and media.
11. Spread 100 μ L of 1:10,000, 1:1,000, 1:100, and 1:10 dilutions of both experimental and control mating mixture on SD/–Leu, SD/–Trp, and SD/–Leu/–Trp to measure mating efficiency (*see Note 10*).
12. Spread the remaining experimental mating mixture on about 50 150-mm plates (200 μ L per plate; *see Note 11*). For stringent selection of protein–protein interaction, plate cells on SD/–Ade/–His/–Leu/–Trp (quadruple drop-out, QDO). For less stringent selection, plate mating culture on triple drop-out media (TDO) SD/–His/–Leu/–Trp. Both types of plates should contain X- α -Gal to test for expression of the MEL1 reporter gene.
13. Invert plates and incubate at 30°C until colonies appear: approximately 3–8 days for TDO media and 8–21 days with QDO (*see Note 12*).
14. For colonies growing on TDO, replica plate the surviving colonies to QDO media containing X- α -Gal and grow for 3–8 days at 30°C to detect activation of all four reporter genes.
15. Select candidate colonies that grow well on QDO and are intensely blue. Streak out colonies on fresh QDO plates plus X- α -Gal and grow for 2–4 days at 30°C.
16. Restreak positive colonies on SD/–Leu/–Trp/X- α -Gal to ensure clones contain only one AD/library plasmid. All resultant colonies should remain blue; a mixture of white and blue colonies indicates segregation of a noninteracting plasmid.
17. Replicate plate well-isolated colonies to QDO again to verify correct phenotype.
18. Collect restreaked and retested colonies on a QDO master plate in a grid pattern. Incubate 4–6 days at 30°C. Wrap master plate in parafilm and store at 4°C.
19. Isolate DNA from each colony using the BD Yeastmaker yeast plasmid isolation kit or similar. This DNA preparation will contain a mixture of bait and library plasmids. Library plasmids are ampicillin resistant and will be selected for after transformation into bacteria. Alternatively, primers specific to the library plasmid (AD LD insert amplimer set; Cat. #630433, Clontech) can be used to specifically amplify library inserts (*see Note 13*).

20. Transform DH5 α cells (Invitrogen) with 5 μ L plasmid isolated in **step 19**.
21. Select transformants by growth on LB-amp plates overnight at 37°C.
22. Grow 2-mL cultures in LB-amp and isolate DNA using miniprep method.
23. Determine whether positive interacting clones are duplicates by digestion with a frequent cutter enzyme such as *Hae*III or *Alu*I.
24. Further characterize one clone from each group by sequencing followed by comparison of the sequence against available databases. The protein–protein interaction should be retested by cotransformation with the bait plasmid followed by selection on SD/–Ade/–His/–Leu/–Trp/X- α -Gal.

3.3. Affinity Chromatography

In this method, the targeting sequence identified in **Subsection 3.1** is attached to a solid support and used for affinity purification of associated proteins from tissue lysates. Candidate components of the complex are identified by Western analysis with appropriate antibodies. This protocol can be adapted for use with lysates from cultured cells.

3.3.1. Tissue Lysate Preparation

1. Add 1 mL extraction buffer per mg tissue to prechilled Potter–Elvehjem tube and set on ice. Add 200–300 μ L protease inhibitor cocktail per milliliter and 1% NP-40 (*see Note 14*).
2. Add tissue to the tube with extraction buffer. Homogenize using Potter–Elvehjem tissue grinder (4–6 passes). Continue homogenization. Use Pro 200 homogenizer to remove residual fibers, keeping the tube on ice. Homogenize six times for 30 s, with a 10-s break after each homogenization.
3. Centrifuge sample at 6,000rpm using SS-34 rotor (4,302g) for 15 min at 4°C.
4. Collect supernatant and centrifuge at 48,000rpm using TLA 100.1 rotor (100,000g) for 30 min at 4°C.
5. Collect supernatant and centrifuge at 55,000rpm using TLA 100.1 rotor (132,000g) for 45 min at 4°C.
6. Collect supernatant, separate into 200- μ L aliquots, and store at –80°C.
7. Perform protein assay to determine total protein concentration (Micro BCA protein assay; Cat. #23235, Pierce, Rockford, IL).

3.3.2. Affinity Column Preparation

1. Add frit to poly-prep column and push down as far as it will go.
2. Suspend resin (enough to make 2-mL resin chamber) with prebound peptide in 6 mL bead binding buffer (BBB).
3. Add suspension to column, allow resin to settle and BBB to drain out, and then wash two times with 5 mL BBB. Do not allow the resin to dry out between washes.
4. To store column after use, add 5 mL PBS with 0.02% sodium azide, let drain, then add 5 mL PBS with 0.02% sodium azide; cap the bottom of column first, then the top, and store at 4°C wrapped in foil (*see Note 15*).

3.3.3. Loading and Eluting the Affinity Column

1. Remove top cap of column, then the bottom cap. Let the sodium azide PBS drain, then wash column two times using 5 mL BBB.
2. Load 200 μ L–1 mL clarified tissue lysate (bring total volume to 1 mL using BBB with 300 μ L protease inhibitor cocktail) to column, allow it to enter, then top off with 1 mL BBB.
3. Incubate column overnight at 4°C on a nutator.
4. Collect the flow-through into microcentrifuge tubes and save at 4°C. Wash the column with 10 column volumes of BBB, collecting 1-mL fractions until the OD₂₈₀ reaches baseline, and save washes.
5. Elute the column using 5 mL 100 mM glycine, pH 2.5. Collect 500- μ L fractions in microcentrifuge tubes that contain 25 μ L 1 M Tris-HCl, pH 9.5, and place on ice (*see Note 16*).
6. Wash column two times using 5 mL BBB containing 0.25 M NaCl, and store in sodium azide (*see Note 17*).
7. Examine fractions by SDS-PAGE (*see Note 18*).

3.3.4. SDS-PAGE

1. Assemble glass plates to form sandwich with appropriately sized spacers. Pour two companion gels: one 1.5-mm-thick gel for Western analysis and one 0.75-mm-thick gel for Coomassie staining of total protein.
2. To prepare 10% acrylamide separating gel, mix 12 mL 30% acrylamide/0.8% bisacrylamide, 9 mL separating gel buffer, 360 μ L 10% SDS, 180 μ L 10% (w/v) ammonium persulfate, 14.4 mL water, and 18 μ L TEMED for a total volume of 36 mL. This gel is for the 1.5-mm-thick gel for Western analysis. Halve the recipe for a 0.75-mm gel. Pour gel immediately and leave approximately 2 inches for the stacking gel. Overlay the separating gel with water-saturated butanol to ensure a flat interface and allow the separating gel to completely solidify (1 h).
3. To prepare the 4% acrylamide stacking gel, mix 1.34 mL 30% acrylamide/0.8% bisacrylamide, 2.5 mL stacking buffer, 100 μ L 10% SDS, 50 μ L 10% (w/v) ammonium persulfate, 6 mL water, and 4 μ L TEMED for a total volume of 10 mL. Pour gel and insert comb immediately, taking care to avoid bubbles below comb. The gel should polymerize within 30 min.
4. Once the stacking gel has set, carefully remove the comb and wash the wells with 1X SDS electrophoresis buffer.
5. Add 1X SDS electrophoresis buffer to the upper and lower chambers of the gel unit and load the samples at the bottom of each well, loading duplicate samples on both gels. Include one well for prestained molecular weight markers.
6. Complete the assembly of the gel unit and connect to a power supply.
7. Run the gel at 250 mA until the dye front reaches the bottom of the gel (4–5 h).
8. Process the 1.5-mm gel for Western blot by following steps in **Subsection 3.3.5.**, and incubate the 0.75-mm gel with Coomassie protein stain solution at least 4 h

followed by incubation in destain solution until bands are visible and background is low (overnight with gentle shaking).

3.3.5. Western Blotting

1. Prepare two sheets of 3MM paper the same size as the separating gel and one sheet of PVDF membrane slightly larger than the separating gel.
2. Before transfer, briefly immerse membrane in 100% methanol, then incubate in transfer buffer 30 min.
3. Disconnect the electrophoresis apparatus from the power supply and disassemble. Remove the stacking gel with a scalpel and discard. Soak the separating gel in transfer buffer for 30 min.
4. Pour chilled transfer buffer into a tray large enough to accommodate the transfer cassette.
5. Assemble transfer sandwich as follows: on the bottom half of a plastic transfer cassette, place sponge soaked in transfer buffer followed by a sheet of prewetted 3MM paper, then place gel on top of 3MM paper. Place the prewetted PVDF membrane directly on top of the gel. Remove all air bubbles between gel and membrane by gently rolling a test tube over surface. Place another piece of 3MM Whatman paper prewetted with transfer buffer on top of PVDF membrane. Complete the sandwich by placing the second transfer sponge on top.
6. Complete assembly by locking the top half of the transfer cassette into place.
7. Fill the transfer apparatus with chilled transfer buffer and place the cassette into the transfer tank such that the PVDF membrane is between the gel and the anode.
8. Connect leads of power supply to corresponding anode and cathode sides of electroblotting apparatus. Electrophoretically transfer proteins from the gel to the membrane at 100 V for 1 h 15 min at 4°C.
9. Turn off the power supply and disassemble the apparatus.
10. Remove cassette from blotting apparatus and partially separate membrane from gel to check for efficient transfer of prestained markers to the membrane. If transfer is incomplete, continue transfer for 15 min.
11. Remove membrane from blotting apparatus and block nonspecific sites on the membrane by incubation with blocking buffer at room temperature for 1 h with rocking. The membrane can be used directly or can be dried and used at a later time after a brief immersion in 100% methanol.
12. Dilute primary antibody in blocking buffer.
13. Place membrane in heat-sealable plastic bag with dilute primary antibody in blocking buffer.
14. Incubate membrane overnight at 4°C with rocking.
15. Open bag and collect primary antibody (it may be used several times if stored at 4°C with 0.02% sodium azide). Place the membrane in a plastic box and wash three times by agitating with 20 mL TBST for 10 min each.
16. Dilute secondary antibody in blocking buffer.
17. Incubate membrane with secondary antibody for 1 h at room temperature.
18. Wash membrane three times with TBST for 10 min each.

19. During the final wash, mix 5 mL of chemiluminescence reagents as indicated by the manufacturer. Once the final wash step is finished, the mixed reagents are immediately added to the blot.
20. Incubate the membrane at room temperature for 5 min.
21. Remove the blot from the chemiluminescence detection reagent, allow excess reagent to drain from blot, and place between sheets of plastic wrap.
22. Place the blot in an X-ray film cassette. Place film over the membrane in the dark, allow enough time for appropriate exposure, and develop.

4. Notes

1. AH108 and Y187 contain the *ade-101* auxotrophic mutation, and growth of these strains in liquid media is enhanced by the addition of 0.003% final concentration adenine hemisulfate. Because of this mutation, colonies growing on YPD plates without adenine supplementation will be slightly pink and will darken with age. White colonies that appear on these plates should be avoided, as they are the result of spontaneous mutations that eliminate mitochondrial function.
2. Drop-out media is minimal media lacking one or more amino acids that is used to select for the expression of nutritional markers that permit growth of yeast in the absence of specific amino acids. For example, the bait vector pGBKT7 contains a functional *TRP1* gene allowing yeast containing this vector to grow in media lacking tryptophan. The cDNA library is cloned into a vector containing a functional *LEU2* gene; therefore, yeast transformed with the cDNA library can grow in the absence of leucine. Most importantly for this protocol, the host strains AH109 and Y187 are engineered so that the *GAL UAS* is linked to the metabolic genes *ADE2*, *HIS3*, *LacZ*, and *MEL1*. Stable interaction between the *GAL4* DNA BD-bait and the *GAL4* AD-library fusion proteins after mating results in expression of the *ADE2* and *HIS3* genes allowing growth on media lacking adenine and histidine while expression of *MEL1* causes colonies to develop a blue color. Premixed drop-out media can be purchased from Clontech or made by the investigator according to established protocols (22).
3. The components of the blocking buffer may need to be optimized for maximal signal. Alternatives are 2%–3% BSA or 2% donkey serum.
4. PCR cycling conditions should be optimized for the individual primers and target. A good program to begin with is 94°C, 1 min; 48°–58°C (about 5° below lowest T_m of the primer pair), 1 min; 25–35 cycles; 72°C, 1–2 min (or 1 min/kb of target sequence). We typically use a “hot start” method to increase specificity of priming; this can be accomplished either manually, by adding Taq polymerase only after all other components have been preheated to 70°–80°C, or by using a commercially available Taq polymerase that is inactivated by complex with antibodies until denaturation (PrimeSTAR HS; Takara, Accuprime, Invitrogen).
5. T-vector ligations are conducted at 4°C overnight. Ligations into GFP vectors are performed overnight at 16°C.
6. To visualize fluorescence of unfixed cells, do not incubate cells in paraformaldehyde. Instead, skip to **step 9**.

7. The 50 mL culture can be grown directly from a colony instead of from an over-night culture; however, we find the procedure described here to be more reproducible.
8. It is critical to the success of the screen to determine that the bait-fusion protein does not itself activate transcription from the reporter genes. Plate yeast transformed with the fusion construct (obtained in **Subsection 3.2.3.**) on SD/–Trp/X- α -Gal, SD/–His/–Trp/X- α -Gal, and SD/–Ade/–Trp/X- α -Gal. Include empty pGBKT7 as a negative control. Colonies should be white on SD/–Trp/X- α -Gal and should not grow on the other two plates. If the colonies are blue or show background growth on –Ade and –His plates, additional constructs must be tested for transactivation. To test that the bait fusion vector is not toxic to yeast, compare the growth kinetics in culture of yeast containing the fusion to those containing empty pGBKT7. To test mating efficiency, mate AH109[bait] with Y187[pTD1-1] and AH109[pGBKT7-53] with Y187[pTD1-1] (control mating) and plate mating mixtures on SD/–Leu/X- α -Gal, SD/–Trp/X- α -Gal, and SD/–Leu/–Trp/X- α -Gal. Calculate mating efficiency as in **Note 10.**
9. It is important to determine the titer of the library each time it is used to calculate the mating efficiency and the number of clones screened. Transfer the 10 μ L reserved in **Subsection 3.2.3.** to 1 mL YPDA/Kan in a 1.5-mL microcentrifuge tube to prepare dilution #1 (dilution factor, 10^{-2}). Vortex gently and transfer 10 μ L dilution #1 to 1 mL YPDA/Kan in a second microcentrifuge tube to prepare dilution #2 (dilution factor, 10^{-4}). Mix by gentle vortexing. Add 10 μ L dilution #1 to 50 μ L YPDA/Kan and spread entire mixture on a SD/–Leu plate. Spread 50 μ L and 100 μ L for dilution #2 onto separate SD/–Leu plates. Allow liquid to soak into the agar for 10–20 min and incubate at 30°C for 3–5 days. Count the number of colonies (colony-forming units, cfu) on each plate. The titer of the cDNA library is calculated according to the following equation:

$$\frac{\text{number of colonies}}{\text{plating volume (ml)} \times \text{dilution factor}} = \text{cfu/ml}$$

10. The mating efficiency between the YH109[bait] and Y187[cDNA library] must be greater than 2% for sufficient numbers of clones to be screened. Count the number of colonies on each plate. Calculate the viable cfu/mL on each type of minimal media according to the following equation:

$$\frac{\text{cfu} \times 1000 \mu\text{l/ml}}{\text{plating volume} (\mu\text{l}) \times \text{dilution factor}} = \text{number viable/ml}$$

The cfu/mL on SD/–Leu is the viability of the library (Y187), the cfu/mL on SD/–Trp is the viability of the bait (YH109), and the cfu/mL on SD/–Leu/–Trp is the viability of the diploids. Compare the number of viable cfu/mL of the two mating partners. The strain with the lower viability is the “limiting” partner in the mating. Calculate the mating efficiency according to the following equation:

$$\frac{\text{cfu/ml diploids}}{\text{cfu/ml of limiting partner}} \times 100 = \text{mating efficiency (\%)}$$

To estimate the number of colonies screened, multiply the cfu/mL of diploids by the entire resuspended volume plated: 3×10^6 colonies is equal to or slightly greater than the number of independent clones in a typical library from Clontech.

11. To spread cells evenly on the plates, we use 5-mm sterile glass beads. To spread the colonies, place the cells on the surface of the plate, add beads (5–7 beads per 85-mm diameter plate; 7–9 beads per 150-mm plate), and shake the plate back and forth (not round and round). Pour off the beads into a waste container. If desired, the beads can be collected, soaked in ethanol, dried, and sterilized by autoclaving for reuse.
12. Because of the extended time of incubation, it is particularly important to ensure that the media is free of contamination. Autoclave media for more than 25 min and pour plates in a controlled environment such as a horizontal laminar flow hood (Baker, Sanford, ME).
13. We find that digestion of plasmid DNA after transfer of yeast plasmid into bacteria gives higher yields and more reproducible results than PCR amplification of plasmid DNA isolated directly from yeast.
14. It is important to use a variety of solubilization buffers with respect to salt and detergent composition to ensure optimal solubilization of the protein of interest.
15. To reduce air bubbles in the affinity column, always take the top cap off first, then the bottom cap. To remove air bubbles, fill the column with degassed PBS, place parafilm over the top so that there is no trapped air, invert the column, and use pressure from your thumb to gently expel any air bubbles from the tip of the column.
16. If your complex has high affinity for the peptide, you may elute the column with glycine, pH 2.5, and 0.25–0.5 M NaCl.
17. To strip the column of residual protein, use 1 bed volume of 10% acetic acid, or 1 bed volume of 5 M guanidine, until OD₂₈₀ reaches baseline, followed by two washes with 5 mL BBB.
18. To concentrate the elutions before SDS-PAGE analysis, perform TCA precipitations. Add 125 µL 50% TCA and 1.25 µL 2% deoxycholic acid to elutions, vortex, and incubate on ice for 25 min. Spin sample in microfuge for 15 min, remove supernatant, wash pellet with 500 µL acetone, incubate on ice for 5 min. Spin sample again for 5 min and repeat wash. Dry pellet for 1.5 h at room temperature and resuspend in 100 µL water for analysis by SDS-PAGE.

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***In Situ* Binding Assay to Detect Myosin-1c Interactions with Hair-Cell Proteins**

Kelli R. Phillips and Janet L. Cyr

Summary

Myosin-1c is an unconventional myosin involved in hair-cell mechanotransduction, a process that underlies our senses of hearing and balance. To study the interaction of myosin-1c with other components of the hair-cell transduction complex, we have developed an *in situ* binding assay that permits visualization of myosin-1c binding to hair-cell proteins. In this chapter we describe in detail the methods needed for the expression and purification of recombinant myosin-1c fragments and their use in the *in situ* binding assay.

Key Words: Myosin 1c; confocal immunofluorescence; baculovirus infection; Sf9 cells, protein purification; *in situ* binding assay; His₆-tag; gel-filtration chromatography; hair cell.

1. Introduction

Myosin-1c (Myo1c), an unconventional myosin of the myosin I family of ~120kDa, is composed of three domains: a globular head domain containing the ATP- and actin-binding sites; a neck domain, which contains three or four consensus sequences for calmodulin (CaM) binding; and a tail domain (*1*). Although this single-headed myosin is present in numerous tissues and has been implicated in a diverse set of functions (*1–5*), its best understood function is in hair cells, the sensory receptor cells of the inner ear, where Myo1c plays a critical role in mechanotransduction (*6,7*).

Hair cells are polarized epithelial cells with a mechanosensitive hair bundle protruding from their apical surfaces. The hair bundle is composed of actin-filled processes called stereocilia, which are arranged in rows according to height and are connected to one another through a variety of extracellular linkages (*8–10*). Deflection of a hair bundle, either by sound wave-induced pressure

changes that stimulate auditory hair cells or by head movements that stimulate vestibular hair cells, initiates transduction and results in increased neurotransmitter release and therefore increased signaling to the brain (8,11). In the hair bundle, Myo1c functions to maintain the transduction complex at its position of optimal sensitivity, allowing the transduction complex to detect minute bundle displacements (12).

Although the biophysical and electrophysiological properties of hair-cell transduction have been well characterized, the molecular identity of many of the components of the transduction apparatus has remained elusive. The paucity of hair cells in vertebrate animals and the lack of immortalized cell lines with hair-cell characteristics makes biochemical studies of hair-bundle proteins, particularly those involved in transduction, very difficult.

As the adaptation motor, Myo1c must interact with other transduction constituents. To study the interaction of Myo1c with hair-bundle proteins we have developed an in situ binding assay that allows for the visualization of interactions of recombinant Myo1c fragments to molecules located at the tips of stereocilia, the site of transduction (Fig. 1) (13,14). In this chapter, we provide methods for the expression of Myo1c fragments using baculovirus infection of Sf9 cells (15), the purification of Myo1c-T701, a Myo1c fragment containing the neck and tail regions of the motor protein (15), and the use of Myo1c-T701 in our in situ binding assay (13).

2. Materials

2.1. Myo1c Constructs and Baculovirus Stocks

1. Bac-to-Bac baculovirus expression system (Invitrogen).

2.2. Plaque Assay to Determine Baculovirus Titer

1. 3.2% solution of Seaplaque agarose (Cambrex), autoclaved and stored at room temperature.
2. Virus stocks for Myo1c-T701 construct and calmodulin (available from author).

2.3. Expression of Myo1c-T701 Construct in Sf9 cells by Baculovirus Infection

1. ES 7X cleaning solution (MP Biochemicals).
2. Grace's complete medium: Grace's insect medium (Invitrogen) supplemented with 5% fetal bovine serum and 0.1% gentamicin.

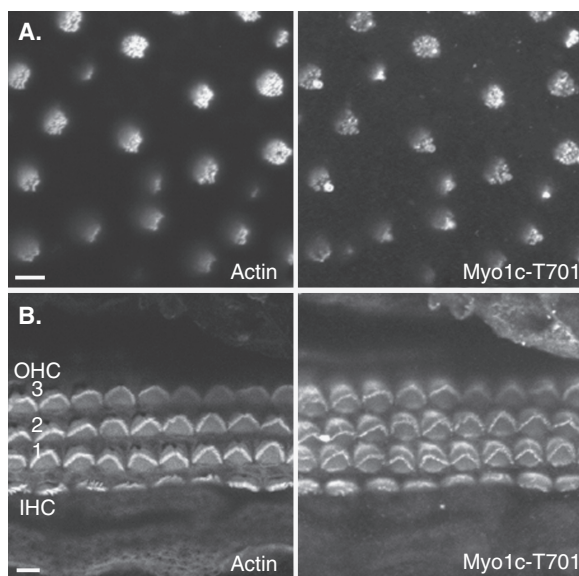


Fig. 1. Myo1c-fragment binding to hair cells using the in situ binding assay. **(A)** Binding of Myo1c-T701, a Myo1c fragment containing the neck and tail domains, to bullfrog saccular hair cells. A single confocal image is shown that contains the hair bundles of approximately 20 hair cells. Left panel shows phalloidin-labeled actin, which labels stereocilia; right panel shows Myo1c-T701 binding to the tips of stereocilia. **(B)** Myo1c-T701 binding to mouse cochlear hair cells. The cochlea contains one row of inner hair cells (IHC) and three rows of outer hair cells (OHC). Left panel shows phalloidin-labeled actin, which readily labels the V-shaped bundles of cochlear hair cells; right panel shows Myo1c-T701 binding to the stereocilia of the hair-cell bundles. Bars = 5 μ m.

2.4. Purification of Recombinant Myo1c-T701

1. Protease inhibitors: 35 mg/mL phenylmethylsulphonylfluoride (PMSF) made fresh in 100% EtOH; 0.7 mg/mL pepstatin made in 100% EtOH and stored at -20°C ; 0.5 mg/mL leupeptin stored at -20°C (see **Note 1**).
2. Lysis buffer: 25 mM Tris-HCl, pH 8, 0.5 mM MgCl_2 , 0.5 mM ethyleneglycoltetraacetic acid (EGTA). To 50 mL lysis buffer add 8.7 μ L β -mercaptoethanol, 50 μ L 35 mg/mL PMSF, 50 μ L 0.5 mg/mL leupeptin, and 50 μ L 0.7 mg/mL pepstatin immediately before use.
3. Wash buffer: 25 mM Tris-HCl, pH 8, 300 mM NaCl, 0.5 mM MgCl_2 , 0.5 mM EGTA. To 50 mL wash buffer, add 8.7 μ L β -mercaptoethanol, 50 μ L 35 mg/mL PMSF, 50 μ L 0.5 mg/mL leupeptin, and 50 μ L 0.7 g/mL pepstatin immediately before use.

4. Elution buffer: 250mM imidazole, pH 8, 25mM Tris-HCl, pH 8; 400mM NaCl, 2mM MgCl₂, 2mM EGTA. To 10mL elution buffer, add 1.7μL β-mercaptoethanol, 10μL 35mg/mL PMSF, 10μL 0.5mg/mL leupeptin, and 10μL 0.7mg/mL pepstatin immediately before use.
5. 10-mL disposable Luer-lock syringe and 22- and 25-gauge needles.
6. 100mM ATP solution. Store at -20°C.
7. Ni⁺²-NTA agarose (Qiagen, Valencia, CA).
8. Bradford reagent: 0.01% Coomassie brilliant blue G-250, 4.75% ethanol, 0.1% phosphoric acid (16).

2.5. Assessment of Aggregation State of Myo1c-T701 using Size-Exclusion Chromatography

1. Gel-filtration column buffer: 15mM HEPES-HCl (pH 7.5), 400mM KCl₂, 1mM MgCl₂, 1mM EGTA. Filter buffer to degas and remove any particulates using a 0.2-μm membrane.
2. Protein standards: Low molecular weight and high molecular weight gel filtration calibration kits (Amersham Pharmacia Biotech) or equivalent.
3. AKTA-FPLC liquid chromatography system (Amersham Pharmacia Biotech) or equivalent.
4. Superdex 200 10/300GL column (Amersham Pharmacia Biotech) or equivalent.
5. Spin-X centrifuge tube filters (0.22-μm cellulose acetate; Costar).

2.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. 4 X SDS-PAGE sample buffer: 20% (v/v) glycerol, 20mM EDTA, 250mM Tris-HCl, pH 8.0, 0.02% (v/v) bromophenol blue, 6% (w/v) sodium dodecyl sulfate. Filter using a 0.45-μm filter, dispense into 9-mL aliquots, and store at -20°C. After thawing an aliquot, but before use, add 1 mL β-mercaptoethanol and then store at room temperature.
2. Resolving gel acrylamide mixture: 30% (w/v) 150:1 acrylamide/bis (75 g acrylamide and 0.5 g bis *N,N'*-methylene-bis acrylamide; bring to a volume of 250mL with H₂O, filter through a 0.2-μm filter). Before polymerization, acrylamide is a neurotoxin: wear gloves at all times. Store at 4°C.
3. Stacking gel acrylamide mixture: 30% (w/v) 29:1 acrylamide/bis (73.7 g acrylamide and 2.5 g bis-*N,N'*-methylene-bis acrylamide; bring to a volume of 250mL with H₂O, filter through a 0.2-μm filter). Store at 4°C.
4. Gel fixative solution: 42% (v/v) ethanol, 12% (v/v) glacial acetic acid.
5. Coomassie blue stain: 0.1% (w/v) Coomassie brilliant blue R-250 (Bio-Rad), 45% (v/v) methanol, 10% (v/v) glacial acetic acid. Stir until completely in solution. Filter through a 0.2-μm filter to remove any residual particulates. Solution can be stored at room temperature and used repeatedly.
6. Gel destain solution: 30% (v/v) methanol, 5% (v/v) glacial acetic acid.
7. 10X Running buffer: 250mM Trizma-Base, 0.1% sodium dodecyl sulfate, 2M glycine, 1mM ethylenediaminetetraacetic acid (EDTA), pH to 8.0.

8. 20% sodium dodecyl sulfate.
9. *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED, Bio-Rad).
10. 200 mM EDTA.
11. 15% ammonium persulfate. Make 1 mL of this solution and replace every 2–3 weeks. Store at 4°C.

2.7. Determining Protein Concentration with the Bradford Assay

1. Bradford reagent: 0.01% Coomassie brilliant blue G-250, 4.75% ethanol, 0.1% phosphoric acid (16).
2. Bovine serum albumin (BSA) standards: 2 mg/mL stock solution in ampoules (Pierce).

2.8. In Situ Binding Assay

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.4.
2. Fixative: 16% paraformaldehyde in ampoules (EM Sciences).
3. HEN buffer: 25 mM HEPES-HCl, pH 7.5, 1 mM EGTA, and 400 mM NaCl.
4. HE buffer: 25 mM HEPES-HCl, pH 7.5, 1 mM EGTA.
5. HET buffer: 25 mM HEPES-HCl, pH 7.5, 1 mM EGTA, and 0.1% Tween-20.
6. Primary antibody: anti-Xpress antibody (Invitrogen).
7. Secondary antibody: Goat anti-mouse Alexa 488 antibody (Invitrogen).
8. Filamentous actin stain: Alexa 568 phalloidin (Invitrogen).
9. Microscope slides with two small pieces of a #2 coverslip fixed to the surface with nail polish to serve as spacers for tissue mounting.
10. Antifade: Vectashield (Vector Laboratories).

3. Methods

Myo1c is very susceptible to aggregation after prolonged or improper storage. It is critical to keep the protein at 4°C. The aggregation state of the protein is routinely checked by gel-filtration chromatography to ensure it is monomeric before use in any experiment.

3.1. Myo1c Constructs and Baculovirus Stocks

1. Myo1c constructs are generated using the polymerase chain reaction (PCR) and other standard molecular biological techniques. PCR should be performed using a high-fidelity polymerase to minimize mutations, and inserts are cloned into the pFastBac vectors. All constructs should contain a His₆ tag for purification and an Xpress tag for detection at their amino terminus. It is important to sequence all inserts to verify reading frames and to ensure that there are no mutations in the encoded protein (13,15).
2. Baculoviral stocks are generated using the Bac-to-Bac baculovirus expression system following manufacturer's protocols.

3.2. Plaque Assay to Determine Baculovirus Titer

1. In a sterile tissue culture hood, plate 10 dishes each containing 2×10^6 Sf9 cells into a 60-mm sterile tissue culture plate with a total volume of 2 mL medium. Allow cells to adhere at room temperature for 20 min.
2. Prepare a serial dilution of your virus (*see Note 2*).
3. Remove 1 mL medium from each plate and add 1 mL diluted virus stock. Do each dilution in duplicate. Allow plates to incubate in tissue culture hood for 2 h. Every 30 min, gently rock the plate by hand. Do not swirl.
4. While the virus and cells incubate, prepare fresh agarose-medium overlay mixture. For each plate you will need 4 mL agarose medium overlay mixture consisting of 1 mL sterile 3.2% Seaplaque agarose that has been melted and cooled to $\sim 60^\circ\text{C}$ (you can hold the bottle in your hand) and 3 mL Grace's complete medium pre-warmed to 39°C . Store at 39°C until use.
5. After cells and virus have incubated for 2 h, remove the media from a dish very carefully and add 4 mL 39°C agarose medium overlay mixture to the dish. Gently rock the plate to evenly coat the bottom of the plate. Work quickly: the plates must be evenly coated before the agarose hardens (*see Note 3*). Leave plates in the tissue culture hood and allow the agarose to harden for 20 min at room temperature, then move them to a humidified chamber created in a plastic container containing paper towels soaked in sterile 5 mM EDTA.
6. Incubate plates in the EDTA humidity chamber at 27°C for 7–10 days until plaques appear on the lawn of Sf9 cells. Count the plaques and multiply by the dilution factor to obtain the titer in plaque-forming units (pfu) of the stock solution (*see Note 4*).

3.3. Expression of Myo1c-T701 Construct in Sf9 cells by Baculovirus Infection

1. Wash and sterilize low-form Erlenmeyer culture flasks as follows: Stir overnight with 5% 7X cleaning solution in water, rinse out detergent, wash at least six times with hot tap water, fill with hot tap water and stir overnight; then rinse two times with distilled water, fill with distilled water, and autoclave 90 min using the wet cycle. After autoclaving, carefully discard the water, cover opening with foil, and autoclave 60 min on dry cycle.
2. Seed 1.5×10^6 Sf9 cells in a 200-mL volume of fresh complete Grace's medium in a low-form Erlenmeyer culture flask cleaned and sterilized as indicated above.
3. Inoculate cells with 6×10^6 pfu Myo1c virus stock and 3×10^6 pfu of calmodulin virus stock (*see Note 5*).
4. Incubate culture for 48 h at 27°C with 100 rpm shaking.
5. Harvest cell pellet by centrifugation at 900g. Wash pellet one time with PBS, re-sediment, remove supernatant, and store pellet at -80°C .

3.4. Purification of Recombinant Myo1c-T701

1. The method described here for purification of recombinant Myo1c-T701 is expanded from that described in Gillespie et al. (1999) (*see* **ref. 15**) for full-length Myo1c. All purification steps are done at 4°C.
2. Place centrifuge tubes on ice.
3. Remove 200-mL cell pellet from -80°C freezer. Add 9 mL cold lysis buffer to cell pellet and, as the cells thaw, resuspend them by trituration with a 10-mL pipette. Once the cells are resuspended, keep the sample on ice at all times.
4. Transfer the cell suspension to a 10-mL syringe with a 22-gauge needle. Lyse the cells by passing the cell suspension twice through the 22-gauge needle and then twice through a 25-gauge needle. Do this with force, but do not allow the sample to foam (*see* **Note 6**).
5. To the lysed sample, add 1.4 mL 5 M NaCl and 180 μ L 100 mM ATP to the solution (*see* **Note 7**); mix gently with a pipette. Adjust the volume of the sample to 24 mL with lysis buffer. Incubate the solution for 10 min on ice.
6. Centrifuge the lysate in a table-top ultracentrifuge for 30 min at 390,000g at 4°C. Prepare column while the sample is being centrifuged.
7. In the cold room or a chromatography refrigerator, prepare the affinity column by adding 1 mL 50% slurry of Ni²⁺-NTA-agarose resin to a disposable column and allow the resin to settle by gravity (*see* **Note 8**); this will result in a bed volume of 0.5 mL. Wash the column with 10 mL cold water followed by 15 mL wash buffer. Do not allow the column to dry out.
8. After centrifugation, save the high-speed supernatant containing the soluble recombinant Myo1c protein. To 20 μ L high-speed supernatant, add 80 μ L 1X SDS-PAGE sample buffer diluted in wash buffer (*see* **Note 9**) and store on ice; this is the high-speed supernatant gel sample (**Fig. 2A**, lane 1).
9. Load the remaining ~24 mL high-speed supernatant onto the column over a 30- to 60-min period using gravity flow. Save the column flow-through and apply it to the column again for a total of two passes. To 20 μ L final flow-through, add 80 μ L 1X SDS-PAGE sample buffer and store on ice as the flow-through SDS-PAGE gel sample (*see* **Fig. 2A**, lane 2).
10. Wash the column with 40–50 mL wash buffer over 45–60 min. Save the wash buffer that flows through the column and contains proteins which were washed off the column. Let the column drain completely, but do not allow it to dry out. To 20 μ L saved wash buffer, add 80 μ L 1X SDS-PAGE sample buffer and store on ice as the wash SDS-PAGE gel sample (*see* **Fig. 2A**, lane 3).
11. Carefully pipette 250 μ L elution buffer directly onto the center of the resin and collect the eluant in a 1.5-mL microfuge tube. Repeat four times for a total of five 250- μ L fractions.
12. Add 5 μ L of each fraction to a 96-well plate containing 250 μ L Bradford reagent; look for protein peak (usually fractions 2 and 3). If two fractions have significant amounts of protein, pool the two fractions.

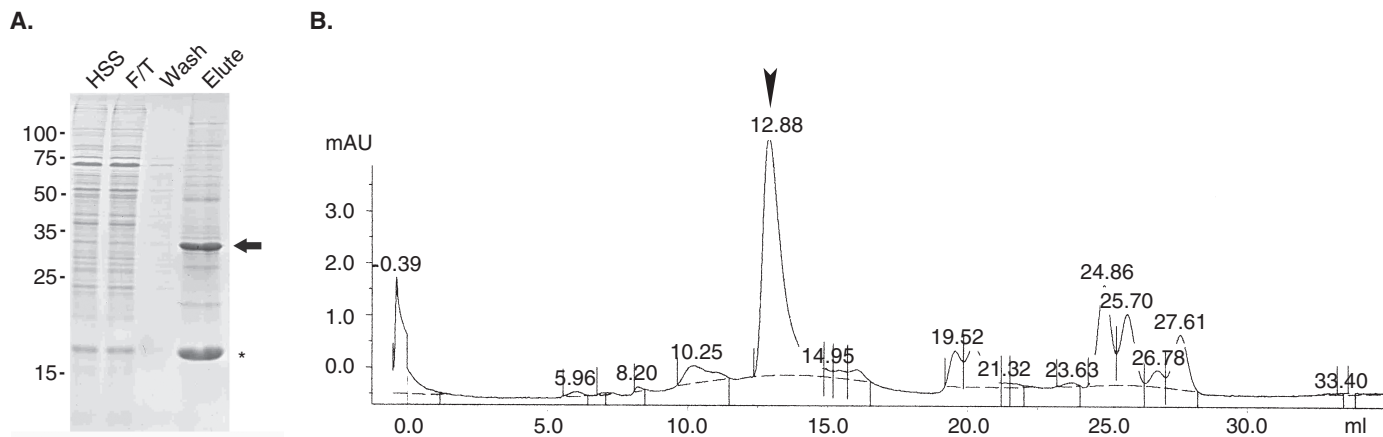


Fig. 2. Purification of Myo1c-T701 recombinant protein. **(A)** Coomassie blue-stained SDS-PAGE gel depicting Myo1c-T701 purification steps. HSS, high-speed supernatant following cell lysis; F/T, column flow-through; Wash, column wash; and Elute, protein eluted from Ni^{2+} -NTA by imidazole. Myo1c-T701 protein is indicated with an arrow and co-purifying calmodulin is indicated with an asterisk. Molecular mass markers (in kDa) are indicated at the left. **(B)** Size-exclusion chromatography elution profile of Ni^{2+} -NTA purified Myo1c-T701 preparation fractionated on a Superdex 200 10/300GL column. Elution volume (in mL) from the gel-filtration column is plotted on the x -axis and absorbance at 280 nm is indicated on the y -axis. Black arrowhead (at eluted volume ~ 12.88 mL) indicates the purified Myo1c-T701 protein complexed with co-purifying calmodulin. The small peaks that elute earlier represent large molecular weight protein, which may reflect a small amount of aggregated Myo1c-T701 protein.

13. Store the purified protein on ice at 4°C to prevent aggregation. Prepare a gel sample by combining 10µL eluate to 40µL 1X SDS-PAGE sample buffer and store on ice (*see Fig. 2A*, lane 4).
14. Assess the aggregation state of the eluted protein as outlined in **Subsection 3.5.**, the purity of the preparation by SDS-PAGE as outlined in **Subsection 3.6.**, and the concentration of the eluted protein as outlined in **Subsection 3.7.**

3.5. Assessment of Aggregation State of Myo1c-T701 using Size-Exclusion Chromatography

1. Resuspend the lyophilized proteins in the protein molecular weight standards kit in sterile water to a final concentration of 25 mg/mL.
2. Equilibrate the Superdex 200 10/300 GL column in gel-filtration column buffer at a flow rate of 0.5 mL/min.
3. Prepare three sets of protein standards: The first standard should contain only 150µg Blue Dextran 2000. The second set should contain 300µg albumin, 300µg chymotrypsin, and 200µg catalase. The third set should contain 300µg ovalbumin, 300µg aldolase, and 300µg ribonuclease A (*see Note 10*).
4. Filter the three sets of protein standards using a Spin-X centrifuge tube filter to remove any particulates.
5. Load the first protein standard on the column and run at a flow rate of 0.5 mL/min at room temperature. Monitor the elution profile by absorption at 280 nm.
6. Load the second set of protein standards on the column and run at a flow rate of 0.5 mL/min at room temperature. Monitor the elution profile by absorption at 280 nm.
7. Load the third set of protein standards on the column and run at a flow rate of 0.5 mL/min at room temperature. Monitor the elution profile by absorption at 280 nm.
8. For each individual protein standard, calculate the K_{av} as follows:

$$K_{av} = \frac{(\text{elution volume of the protein}) - (\text{elution volume for Blue Dextran 2000})}{(\text{total column volume}) - (\text{elution volume for Blue Dextran 2000})}$$

9. Create a semilogarithmic plot of the K_{av} value for each protein standard on the linear scale and the molecular weight of the standard on the logarithmic scale.
10. Using a Spin-X centrifuge tube filter, filter approximately 80µL Ni²⁺-NTA eluate.
11. Load the filtered Ni²⁺-NTA eluate onto the Superdex 200 10/300 GL column at room temperature using gel-filtration column buffer as the mobile phase. Run at a flow rate of 0.5 mL/min and monitor the eluted proteins by absorbance at 280 nm (*see Fig. 2B*; *see Note 11*).
12. Using the standard curve generated above and the elution volume of the Myo1c protein, determine the molecular weight of the Myo1c protein.
13. Preparations containing a large amount of protein aggregation should be discarded.

3.6. SDS-PAGE

1. Wearing gloves, wash plates thoroughly with 50% RBS 35 detergent solution. Rinse extensively in deionized water followed by 95% EtOH. Allow to air dry.
2. Assemble glass plates into gel pouring apparatus per manufacturer's instructions. Volumes indicated below are for one gel using the BioRad MiniProtean system.
3. Prepare a 15% resolving gel by mixing 1.1 mL 18 mOhm water, 1.88 mL 150:1 acrylamide mixture, 0.7 mL 2 M Tris-HCl, pH 8.9, 19 μ L 200 mM EDTA, and 19 μ L 20% SDS. Mix gently, then add 25 μ L 20% ammonium persulfate and 3 μ L TEMED. Mix gently and then pour 2.5 mL resolving gel solution into the sandwich created by the plates.
4. Carefully overlay the resolving gel with 300–500 μ L water-saturated isobutanol. Allow resolving gel to polymerize for ~45 min to 1 h.
5. Once polymerized, pour off isobutanol and rinse surface of resolving gel well with deionized water. Remove water and immediately pour stacking gel (*see Note 12*).
6. Prepare a 4% stacking gel by mixing 145 mL 18 mOhm water, 268 μ L 29:1 acrylamide mixture, 250 μ L 1 M Tris-HCl, pH 6.8, 10 μ L 200 mM EDTA, and 10 μ L 20% SDS. Mix gently, then add 18 μ L 20% ammonium persulfate and 2.4 μ L TEMED. Mix gently, and then pour the stacking gel using a volume that completely fills the rest of the glass plate sandwich. Immediately insert a clean comb into the stacking gel solution. Allow stacking gel to polymerize for at least 45 min.
7. Once gel is polymerized, carefully remove the comb and rinse out wells with 1X SDS-PAGE running buffer using a syringe and needle or aspirating flask.
8. Heat gel samples for 20 min at 65°C. Allow to cool; then centrifuge to sediment condensation.
9. Fill the wells of the gel with running buffer and load 10 μ L gel samples collected during the purification protocol into each well. It is important that all wells have the same buffer composition as your protein-containing gel samples. Wells not receiving a protein sample should receive 10 μ L 1X SDS-PAGE sample buffer containing the same buffer, or similar, to that which is present in the protein samples; in this case, it would be wash buffer (*see Notes 9 and 13*). At least one lane should contain commercially available molecular weight standards.
10. Assemble gel-running apparatus. Run gel in cold running buffer at 200 V (constant voltage) for ~1 h, or until the dye front of the sample buffer is at the bottom of the gel.
11. After gel has run, disassemble the apparatus, remove gel from glass plate, and fix for at least 30 min in gel fixative solution on a shaking platform.
12. Stain gel for at least 30 min in Coomassie blue staining solution on a shaking platform.
13. Destain gel for 3 h to overnight in destain solution on a shaking platform.
14. Constructs containing the Myo1c head and neck sequences migrate at ~36 kDa and co-purifying CaM migrates at ~16 kDa (*see Fig. 2A*). These bands should be readily apparent in the eluate lane.

3.7. Determining Protein Concentration with the Bradford Assay

1. Prepare protein standards by diluting BSA standards in water at the following concentrations (in $\mu\text{g/mL}$): 10, 20, 40, 80, 100, and 120.
2. To generate a standard curve, pipet 50 μL each protein standard into a well of a 96-well plate, in triplicate. The wells will contain the following amounts of BSA (in μg): 0.5, 1.0, 2.0, 4.0, 5.0, and 6.0, respectively (see **Note 14**).
3. For each standard series, pipet 50 μL sterile water to be read as the blank.
4. To each standard and blank well, add 2 μL elution buffer (see **Note 15**).
5. For the unknown, pipet 50 μL water, in duplicate. To these wells add 2 μL Ni^{2+} -NTA protein eluate.
6. Add 250 μL Bradford reagent (**16**) to each blank, standard, and sample well. Ensure wells are mixed thoroughly by pipeting up and down (see **Note 16**).
7. Read absorbances immediately using a plate reader set at 595 nm (see **Note 17**).
8. For each standard calculate the average of the three readings and create a standard curve by plotting the average absorbance at 595 nm on the y-axis and micrograms (μg) protein/well on the x-axis (see **Note 18**).
9. Determine the best-fit line for the standard data points using graphing software (see **Note 19**).
10. Use the best-fit line equation ($y = mx + b$) generated by the graphing software and absorbance values (y) of the unknown samples to solve for milligrams (mg) protein/well (x) for the unknown samples (see **Note 20**).
11. Divide resulting value by 2 to calculate the concentration of the eluted protein; the result will be the concentration of your original eluate in micrograms/microliters ($\mu\text{g}/\mu\text{L}$).

3.8. In Situ Binding Assay

1. This method is expanded from that outlined in Cyr et al. (2002) (see **ref. 13**).
2. Fix tissue with 3% paraformaldehyde diluted from 16% stock solution with cold PBS for 25 min at room temperature (see **Notes 21** and **22**).
3. Wash 1×10 min with PBS.
4. Permeabilize fixed tissue with 0.1% Sarkosyl (w/v) in PBS for 1 h at room temperature.
5. Wash the tissue 1×15 min with PBS.
6. Wash the tissue 2×5 min with 25 mM HEPES-HCl, pH 7.5.
7. Block the tissue for 1.5 h in freshly prepared 5 mg/mL BSA in 25 mM HEPES-HCl, pH 7.5 (see **Note 23**).
8. Incubate tissue overnight at room temperature with shaking in 20 $\mu\text{g/mL}$ Ni^{2+} -NTA purified Myo1c-T701 in freshly prepared 5 mg/mL BSA in HEN buffer (see **Note 24**).
9. Wash 3×10 min with HET buffer.
10. Incubate for 2 h at room temperature with shaking in 5 $\mu\text{g/mL}$ α -Xpress antibody in freshly prepared 5 mg/mL BSA in HE buffer.

11. Wash 3×10 min in HET buffer with shaking.
12. Incubate for 2 h at room temperature and protected from light with $13 \mu\text{g/mL}$ goat anti-mouse Alexa-488 secondary antibody and $33 \mu\text{M}$ phalloidin Alexa-568 in 5 mg/mL BSA in HE buffer.
13. Wash 3×10 min with HET buffer. Protect from light.
14. Wash 1×10 min in HE buffer. Protect from light.
15. Mount with Vectashield on slides containing #2 coverslip spacers for confocal imaging. Cover with a #1.5 coverslip. Seal coverslip with nail polish. Keep protected from light.
16. Image by confocal microscopy (*see Note 25*).

4. Notes

1. PMSF is toxic and should be handled with gloves. PMSF stock solution must be vortexed or sonicated to get into solution and should be added to the buffers immediately before use.
2. Prepare serial dilution as follows:
1 μL virus stock + 999 μL Grace's complete = 1 mL 10^{-3} dilution
250 μL 10^{-3} dilution + 2250 μL Grace's complete = 2.5 mL 10^{-4} dilution
250 μL 10^{-4} dilution + 2250 μL Grace's complete = 2.5 mL 10^{-5} dilution
250 μL 10^{-5} dilution + 2250 μL Grace's complete = 2.5 mL 10^{-6} dilution
250 μL 10^{-6} dilution + 2250 μL Grace's complete = 2.5 mL 10^{-7} dilution
3. To prevent agarose from hardening on the plate too soon, work with only two or three plates at a time.
4. Identifying plaques is not easy. They appear as hazy/cloudy areas on the lawn of Sf9 cells. Oftentimes it is best to tilt the plate in indirect light to visualize them.
5. For Myo1c-T701 and all other Myo1c constructs with the calmodulin-binding neck domain, you must co-express calmodulin to obtain good yields of non-aggregated Myo1c protein.
6. You need to exert enough force to adequately lyse the cells, but not so much that you cause foaming, which will denature the proteins.
7. The addition of ATP is crucial for constructs that contain the myosin head domain to dissociate the motor molecule from endogenous actin, but can be omitted if the construct does not contain the head domain.
8. Myo1c and associated calmodulin is purified by virtue of the His₆ tag present on the amino terminus of the construct.
9. To make 1X SDS-PAGE sample buffer, mix 1 part 4X SDS-PAGE sample buffer to 3 parts wash buffer.
10. Because of similarity in size of the protein standards, if you choose to run several standards at once, they need to be of sufficiently different sizes to allow you to distinguish which peak is which protein.
11. The Myo1c-T701 protein, which contains the Myo1c neck and tail sequences, routinely elutes at an elution volume of 12.88 mL (*see Fig. 2B*, arrow). Aggregated protein elutes from the column earlier.

12. If you want to run your gel the following day, do not pour the stacking gel. Instead, overlay the resolving gel with a solution containing 3 mL water, 0.7 mL 2 M Tris-HCl, pH 8.9, 19 μ L 200 mM EDTA, and 19 μ L 20% SDS, and store tightly wrapped to prevent evaporation at 4°C. Rewarm resolving gel to room temperature the next day and remove the overlay buffer before pouring stacking gel. You will lose some resolution of the resolving gel if you do not use it immediately, but for most purposes this is acceptable. Do not use gels that are more than 1–2 days old.
13. Unequal salt or detergent concentrations across the gel will result in aberrant migration in some lanes. It is important that all lanes have similar salt and detergent concentrations. In practice, a 10%–15% difference in salt concentrations between lanes is generally, but not always, tolerated.
14. Take extreme care with your pipetting technique. Pipetting errors will result in inaccurate results and can often be detected by variations in the readings between replicates.
15. It is important that the blanks and standards have exactly the same buffer composition as your unknown sample; this is accomplished by adding elution buffer to the blank and standards.
16. Take care not to generate bubbles when mixing the wells.
17. If the plate reader has appropriate software, have it subtract the average absorbance of the blanks from each reading. Do not use an auto-mixer setting on the plate reader; this can result in erroneous results from splashing between wells. If you do not have a plate reader, do the assay manually with a spectrophotometer.
18. If one of the three readings is very different from the others, this one reading can be dropped from the average. If all three readings are inconsistent, then the assay should be repeated.
19. If the curve is not linear, the standards are not accurate, and the entire assay, including the unknowns, must be repeated.
20. The absorbance of the unknown should fall within the middle of the curve. If it is at the lower end, use a more sensitive protein assay such as the BCA assay. If it is at the upper end of the curve, repeat the assay and dilute the unknown as needed.
21. Bullfrog sacculi are used following dissection and manual removal of the otolithic membrane. Removal of the tectorial membrane from mouse cochlear cultures is not necessary.
22. Fixation, permeabilization, and all washes are typically performed in a Netwell 12-well plate (Corning) or in 1.5-mL microfuge tubes in volumes of 4 mL or 1 mL, respectively.
23. To minimize the use of reagents, incubation is done in a well of a 96-well plate containing ~150 μ L solution. Seal plate with Parafilm to prevent evaporation during incubations.
24. We typically use the Myo1c Ni²⁺-NTA eluate for the in situ binding assay. If you want to use gel filtration-purified protein, you will need to dialyze it into HEN

because gel-filtration chromatography significantly reduces the concentration of the Myo1c protein and the buffer used for gel filtration reduces the binding of Myo1c in the assay. Do not use acid precipitation to concentrate gel-filtration fractions as that will denature the protein.

25. Samples must be imaged within a few hours.

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Ultrastructural Analysis of Kinesin-Related Motor Proteins During Spermatogenesis

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Summary

Kinesins are a superfamily of microtubule-based motors that power intracellular traffic and play important roles in many fundamental cellular and developmental processes. Kinesins move on microtubules from their minus to plus end (conventional kinesin) or from plus to minus end (C-terminal kinesins), carrying cargoes to different destinations. A variety of cargoes such as vesicles, proteins, lipid drops, pigments, and the nucleus are moved by kinesins along cytoplasmic microtubules. Multiple mitotic kinesins and microtubule-associated proteins (MAPs) also have direct functions in spindle formation, chromosome segregation, and cytokinesis. Spermatogenesis provides an excellent model system to study the role of kinesin motor proteins during the dramatic cytoskeletal rearrangements that take place during male germ cell development. This chapter describes how to identify the multiple functions of kinesin motors during spermatogenesis by using ultrastructural analysis. Testis perfusion is described in detail, including how to anesthetize animals and how to select seminiferous tubules under transilluminated microscopy. Practical immunocytochemical staining is also described in detail in this chapter, especially methods to enhance staining and avoid contamination.

Key Words: Kinesin; ultrastructural analysis; motor protein localization; nuclear elongation; spermiogenesis; testis.

1. Introduction

Kinesin motor proteins power the intracellular transport system and are essential for cellular function and morphology (*see* **ref. 1** for review). Numerous studies have focused on the structure and function of individual family members *in vivo* and *in vitro* (*see* **refs. 1, 2** for review). Diverse mitotic kinesin motors and microtubule-associated proteins (MAPs) also have direct functions in cytokinesis (**3,4**). Conventional kinesins carry cargoes on microtubules from their minus to plus end and C-terminal kinesins from their plus to minus end

(3,5–9). In somatic cells, a variety of cargoes such as vesicles, proteins, lipid drops, pigments, and the nucleus are moved by kinesin motors along cytoplasmic microtubules. In germ cells, the functions of kinesin motors during spermatogenesis have been the focus of investigation in the past decade (10–15).

Spermatogenesis is a special process that consists of complicated cellular change. Under light and electron microscopy, several visible changes are observed during this process: formation of acrosome, assembly and disassembly of the microtubule-based manchette, elongation and condensation of the spermatid nucleus, and appearance of sperm flagella. In the early stages of spermiogenesis, the acrosome is formed by fusion of vesicles derived from the Golgi. These vesicles move to one pole of the spermatid nucleus, where they fuse together to form the proacrosomal vesicle that then attaches and spreads along the nuclear surface. The manchette, a specialized microtubular structure unique to spermatogenesis, surrounds the spermatid nucleus during spermiogenesis and is hypothesized to assist in nuclear shaping during spermiogenesis by squeezing the nucleus via both mechanical and molecular mechanisms (16). Finally, a highly condensed and specially shaped nucleus is formed, and the sperm flagella is assembled at the distal pole of the nucleus.

The molecular mechanism of these cellular transformations has attracted much interest and likely involves multiple kinesin-related proteins. Kinesin-related motors have been identified in the mammalian testes (17). Kinesin superfamily motor proteins are candidate motors involved in acrosome biogenesis during spermiogenesis (10,13–15,17–21). The C-terminal motor KIFC1 had been demonstrated to participate in acrosome biogenesis and take part in vesicle transport directly during spermiogenesis (19). In addition to acrosome formation, the spermatid cytoskeleton undergoes dramatic change during spermatogenesis. One particularly striking phenomenon is the formation of the manchette, a transient microtubule-based structure. Kinesin is associated with the manchette and may produce force for manchette formation and/or function (22).

Numerous approaches have been developed to study the structure and function of motor proteins in somatic cells, including X-ray crystallography and cryo-electron microscopy (9,21,23–26). For germ cells, electron microscopy is an important method to study kinesin function. Immunocytochemistry has proved to be valuable to investigate the relationship between kinesins and microtubules and the functions of these motors during the morphological changes of spermatogenesis. Because spermatogenesis is a very complex biological process and the testis contains cells at different stages of their maturation, it is important to identify and isolate cells at different developmental stages.

This chapter describes how to identify the potential functions of kinesin motors during spermiogenesis by using ultrastructural analysis. A specific antibody against the C-terminal motor protein KIFC1 is used as an example; however, this method can be applied to the study of other kinesins motors in a variety of developmental processes (19). Testis perfusion method is described in detail, including how to anesthetize animals and how to select seminiferous tubules by transilluminated microscopy. Practical immunocytochemical staining is also described in detail, especially the methods to increase the staining effect and eliminate contamination.

2. Materials

2.1. Animal Anesthesia, Testis Perfusion, and Seminiferous Tubule Selection

1. Perfusion balanced salt solution (BSS): 116mM NaCl, 2mM CaCl₂, 5mM KCl, 1mM NaH₂PO₄, 2.6mM NaHCO₃, 6mM glucose (see **Note 1**).
2. 0.2M solution of monobasic sodium phosphate: to prepare this solution, add NaH₂PO₄ · H₂O, 27.58 g, in 1000 mL water.
3. 0.2M solution of dibasic sodium phosphate: to make this solution, add Na₂HPO₄, 28.38 g, in 1000 mL water.
4. Phosphate-buffered saline (PBS) buffer: 0.04M Na₂HPO₄, 0.14M NaCl, 0.01% merthiolate, pH 7.6.
5. Perfusion fixative and fixative for selected seminiferous tubules: 4% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde in 0.8M Na₂HPO₄ and 2.7M Na₂HPO₄ (see **Note 2**).

2.2. Sample Embedding and Sectioning

1. 4% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde fixative.
2. 0.1M sodium phosphate buffer, pH 7.4.
3. 70% (v/v), 95% (v/v), and 100% (v/v) ethonal series.
4. Embedding media: L.R. White (Ted Pella, Redding, CA).
5. Embedding capsule (Ted Pella).
6. Nickel grid (Ted Pella).

2.3. Immunocytochemical Staining

1. Primary antibody dilution buffer: 0.04M PBS/0.5% bovine serum albumin (BSA), stock at 4°C.
2. Probe dilution buffer: 0.5% fluorescence in situ hybridization (FISH) gelatin, 0.04M PBS, pH 7.4, stock at 4°C.
3. Washing buffer: 0.04M PBS, stock at 4°C.
4. PBS/BSA: 0.04M Na₂HPO₄, 14M NaCl, 0.01% merthiolate (w/v), 0.5% (w/v) BSA, stock at 4°C.

5. Colloidal gold: 15-nm and 30-nm colloidal gold particles coated with secondary antibodies; stock at 4°C.
6. Lead citrate: Place 30 mL glass ultrapure water into a flask and add 1.33 g lead nitrate, agitate until clear; add 1.76 g sodium citrate (dehydrate); shake for 1 min, and let stand for at least 0.5 h with intermittent shaking; add 8.0 mL 1.0 *N* NaOH (freshly prepared with glass ultrapure water) and mix gently; bring to 50 mL with boiled H₂O.
7. Uranyl acetate: Dissolve 580 mg maleic acid and 1 g uranyl acetate in 80 mL water, adjust pH to 5.2 with HCl, and bring volume to 100 mL with water.
8. 1% OsO₄ (in 0.1 *M* sodium phosphate buffer containing 0.03 *M* CaCl₂ and 0.1 *M* 3-amino-1,2,4-triazole).

3. Methods

The methods described here aim to study the function of kinesins during spermatogenesis. Kinesins are developmentally expressed during spermatogenesis. During spermiogenesis, distinctive cellular structures can be found including the acrosome, manchette, and flagella. To localize kinesins on these structures during spermiogenesis, different stages of seminiferous tubules must be identified, isolated, and fixed. The identification of different stages of spermiogenesis is the most important step; without this, no potential functions of kinesin motors can be assigned. Isolation of seminiferous tubules representing different cell associations or stages is achieved using transilluminated microscopy by identifying segments with different light absorption properties (22). The second crucial step is the choice of appropriate fixative. The mixed fixative 4% paraformaldehyde and 0.1% glutaraldehyde has been most successful in our hands compared to 2.5% glutaraldehyde (28). The third consideration is to perfuse the testis before selecting and fixing the desired segment of seminiferous tubule. Improved perfusion buffer is achieved by adding sodium nitrite as a vasodilator (29).

3.1. Animal Anesthesia, Testis Perfusion, and Seminiferous Tubule Selection

1. Anesthetize animal by injecting 1 mL pentobarbital (for 150-g rat); wait until the animal is deeply anesthetized. Use tweezers to clamp one of the toes; if the animal has no reaction, the animal is completely anesthetized. Heart should still beat at this time. Make sure not to inject more pentobarbital to void possible overdose.
2. Place the deeply anesthetized animal on its back on paraffin block in a dissecting plate.
3. Spread the forelimbs and hindlimbs and secure each paw to the paraffin block with a pin.
4. Press the abdomen to determine where the testis are if the testis is not seen. Make a cut on the scrotum to expose the testis, making sure not to destroy it. Repeat to find another testis.

5. Remove testis carefully.
6. Insert 18-gauge needle into the artery on the testis.
7. Arrange the testis and the perfusion tube so that the testis will not move during perfusion.
8. Slowly perfuse testes with Earle balanced salt solution (BSS) for 2 h, with about 50 mL room temperature perfusion BSS (*see Note 1*).
9. After perfusion, place testis into PBS buffer (on ice).
10. Isolate seminiferous tubules at appropriate stages by transillumination-assisted microdissection (**30**) (*see Note 3*).
11. Fix isolated seminiferous tubules in mixed fixative 4% paraformaldehyde and 0.1% glutaraldehyde for 1 h on ice.

3.2. Sample Embedding and Sectioning

1. Cut fixed tubules into 2-mm lengths.
2. Rinse the tubules in 0.1 M sodium phosphate buffer, pH 7.4, three times for 10 min each.
3. Rinse the tubules in distilled water, one time, 10 min.
4. Dehydrate tubules with increasing ethanol concentrations: 30 min in 70% ethanol, two times 5 min in 95% ethanol, two times 5 min in 100% ethanol.
5. Put tubules into L.R. White overnight at 4°C.
6. The next day, change L.R. White and leave it at room temperature for 1 h.
7. Embed tubules in capsules. For one capsule, put 1 or 2 tubule(s) in L.R. White.
8. Incubate the embedded sample at 55°C for 24 h.
9. Cut semi-sections for sample orientation.
10. Cut thin sections on a Reichert ultramicrotome.
11. Collect sections in silver color on nickel grids for immunocytochemical staining.

3.3. Immunocytochemical Staining

1. Block the grids by incubation in 5% egg albumin for 2 h.
2. Remove excess solution by blotting the grid on lens paper.
3. Dilute the affinity-purified primary antibodies to the appropriate final concentration with 0.04 M PBS, pH 7.4, 0.5% BSA. Measure 50 μ L diluted antibody in beam capsule, and incubate grid in the beam capsule 2–3 days at 4°C (*see Note 4*).
4. Using tweezers, hold the grid at its edge while pushing the washing buffer through a 50-mL syringe and letting the buffer flow over the grid for 15 s.
5. Remove excess solution by blotting the grid on lens paper.
6. Cut a 10 cm $\times$ 10 cm piece of Parafilm, place it on bench, and put one drop of washing buffer on the Parafilm.
7. Put the grid on the drop, shining side (where the sample resides) down. Use a culture Petri dish to cover the sample. Incubate for 10 min.
8. Remove excess solution by blotting the grid on lens paper.
9. The grids are then incubated with goat antirabbit IgG conjugated to 30-nm gold alone or with antimouse IgG conjugated to 15-nm gold particles for double

labeling; dilute the above colloid gold 1 : 20 in 0.5% fish gelatin, 0.04 *M* PBS, pH 7.4, for 1 h at room temperature.

10. Repeat **step 4**.
11. Repeat **step 7**.
12. Repeat **step 10**, using ultrapure water to replace washing buffer, for 10 s.
13. Repeat **step 11**, using ultrapure water to replace washing buffer, for 10 min.
14. Remove excess solution by blotting the grid on lens paper.
15. Air-dry the grids on a new lens paper for at least 30 min (I usually let the grids air-dry more than 1 h).
16. Measure 50 μ L 1% osmium into a 500- μ L plastic tube, place grid in osmium staining solution for 30 min at room temperature (*see Note 5*).
17. Stain the grids with 7.5% UrMgAc , 1 h at room temperature with backside of grid down or shining side up on the staining drop. The staining should be done under a cover such as in a Petri dish.
18. Pour 5 mL washing buffer into each of three 5-mL plastic beakers. Use tweezers to hold the grid at its edge, dip the grid into the washing buffer. Do this rapidly by breaking the buffer surface with each dip. Dip-wash each grid consecutively in each beaker for 10 s.
19. Remove excess solution by blotting the grid on lens paper.
20. Centrifuge the lead staining solution at 1000 *g* for 30 min, let the solution go through a filter (*see Note 6*), then place one drop on Parafilm.
21. Incubate grid in lead stain for 4.5 min at room temperature with the backside on the drop or shining side up (*see Note 7*).
22. Repeat **step 17**, replace washing buffer by ultrapure water (*see Note 8*).
23. Remove excess solution by blotting the grid on lens paper.
24. Let the grids air-dry for at least 1 h.
25. Observe samples on a JEOL, or similar, electron microscope.
26. Select appropriate areas, particularly those containing microtubules, in which signals are present, and take pictures.

4. Notes

1. Sodium nitrite may be added to the perfusion balanced salt solution (BSS) as a vasodilator to make perfusion easier. A 1% stock solution of sodium nitrite is made ahead of time and added to a final concentration of 0.00001%.
2. Paraformaldehyde powder is hard to dissolve in water if not heated. Add 40 g paraformaldehyde to 500 mL water and heat gently while stirring until the temperature reaches 55°C. Add a few drops of 5 *N* NaOH to the solution, continue heating until the temperature is about 60°C. Do not overheat. The solution should be clear when the temperature reaches 60°C. Let the paraformaldehyde solution cool down to room temperature, add appropriate amount of 0.2 *M* monobasic and 0.2 *M* dibasic sodium phosphate, and adjust the pH to 7.2 with 5 *N* HCl. Be careful; paraformaldehyde is harmful and will irritate your nose and eyes. Therefore, perform all the steps in the hood. When weighing paraformaldehyde, remember to wear a mask. Two masks may be necessary.

Table 1
Spermiogenesis Stages and the Respective Light Absorption of Seminiferous Tubule Segments

Spermiogenesis stages	VIII	IX–XII	XIII, XIV, I	II–V	VIIa-b, VI, VIIc-d
Appearance of seminiferous tubule	Dark zone	Pale zone	Weak spot zone	Strong spot zone	Strong dark zone

3. The developmental stages of spermiogenesis can be determined under transilluminated microscopy by the light absorption of seminiferous tubules (30). The density of the tubules, as visualized by transilluminated microscopy, is a direct reflection of the arrangement and types of cells (or spermatogenic stage) present in that segment, as shown in Table 1. For example, the dark zone is characterized by a very strong absorption in the center of the tubule and represents stage XII and XIII of development, containing spermiating spermatids at the lumen of the tubule. Adjacent to the dark zone is a more translucent area, displaying different patterns of density and containing round and elongating spermatids and dividing cells. The above table shows the developmental stages (I–XIV) of spermiogenesis and their respective light absorptive properties (30).
4. The most important consideration is the negative control. Omitting primary antibody is a good choice.
5. This step aims to enhance staining contrast. Do not perform osmium staining immediately after sample fixation with mixed fixative, because osmium will interfere with the recognition of antigen and antibody, thus blocking detection of the antigen.
6. The Pb^{2+} in lead citrate reacts easily with CO_3^{2-} , forming Pb_2CO_3 . Pb_2CO_3 is one of the primary causes of contamination while staining. To remove Pb_2CO_3 and have an ideal staining effect, centrifuge the lead staining solution at 1000g for 30min before staining.
7. Lead staining needs to be done in a sandwich box such as in a Petri dish. Place NaOH pellets in Petri dish, and let the pellets surround the lead staining drop. To avoid lead contamination, centrifuge the lead solution by spinning at 1000g for 10min. Do not overstain. Set the time at 4.5min. Stain the grids one by one, or stain the second one after 2min. Leave enough time for washing the grid. Be careful as NaOH pellets are harmful to your skin; handle the pellets with a spoon, never use your hand.
8. To avoid lead contamination, grid washing should be done rapidly. Boil 100mL ultrapure water in a flask 1h before lead staining. Cover the flask with foil wrap and allow to cool to room temperature. Pour the boiled water into three 5-mL plastic beakers and wash the grid by dipping it into each beaker frequently (about 30 times in 10s). Hold the grid tightly, do not drop it, and do not let the grid touch the beaker. After dip washing, remove excess water by blotting the grid on a lens paper.

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In Vitro Motility System to Study the Role of Motor Proteins in Receptor–Ligand Sorting

John W. Murray and Allan W. Wolkoff

Summary

This chapter presents fluorescence microscope assays that can be used to study microtubule (MT)-based movement and receptor–ligand sorting in vitro. The strategy is to isolate endosomes in a concentrated active form and store them in frozen aliquots for single use. Glass microchambers are then constructed and coated with fluorescent MTs, and the endosomes are thawed and bound to the MTs. Proteins of interest are then detected and quantified by immunofluorescence. For motility experiments, time-lapse movies are captured using multichannel fluorescence microscopy, and motility is initiated by the addition of ATP. Movies are later categorized and quantified for MT-based motility and other associated events such as endocytic fission. These techniques were developed to assess the role of MTs and MT motor proteins in endocytic processing within liver cells, and we have streamlined a rapid procedure for isolating abundant, highly motile endosomes from rat liver. Cultured cells and other organelles can also be examined, and many important biological questions concerning intracellular traffic and organelle composition can be studied by creative adaptation of the protocols that are presented.

Key Words: Dynein; endosomes; immunofluorescence; in vitro; kinesin; microscopy; microtubules; motor proteins.

1. Introduction

Endosomes are dynamic organelles that allow for the intracellular redistribution of various proteins. As an example, the asialoglycoprotein receptor binds to ligands such as asialoorosomucoid (ASOR) at the cell surface, and the ligand–receptor complex is internalized into endosomes where a process of sorting and redistribution is initiated. ASOR is delivered to lysosomes for degradation while the receptor recycles back to the plasma membrane (1,2). We have been investigating how the cytoskeleton functions in endocytic processing, as it has been shown that cytoskeletal depolymerizing drugs

can slow or block this process (3,4). Specifically, we have asked whether endosomes bind to and move along microtubules (MTs) during processing, what motor proteins are involved in this processing, and whether MTs are used as a mechanical structure for the sorting of protein destined for different compartments.

Despite the availability of living cell fluorophores such as green fluorescent protein (GFP), it has been difficult to quantify protein content on individual organelles. There may also be inherent difficulties interpreting results that are based on hours of protein overexpression within cells. Here we describe *in vitro* assays that allow the tracking and co-localization of multiple endogenous proteins on individual organelles using multichannel immunofluorescence. Organelles can be treated with function-blocking antibodies, exogenous protein, or other reagents and assayed for motility, fission and protein constituents (5). Single vesicle tracking studies can require large amounts of data generation, and we show how this can be accomplished using assembly line techniques and by maintaining MT-bound organelles in an active state on ice.

2. Materials

2.1. Microtubules and Endocytic Vesicle Isolation

1. MTs are polymerized from tubulin (Cytoskeleton Inc., Cat. #TL 238) and rhodamine tubulin (Cytoskeleton Inc., Cat. #TL 331) in a low-salt MT buffer [80mM K₂-PIPES, 1mM ethyleneglycoltetraacetic acid (EGTA), 1mM MgCl₂ + 3% glycerol + 1mM GTP], and Taxol (paclitaxel) is added to stabilize polymerized MTs.
2. A Beckman Airfuge at room temperature is recommended for pelleting MTs. A tabletop ultracentrifuge (e.g., Beckman TL-100) can also be used, but it does not handle very small volumes as conveniently.
3. Fluorescent asialoorosomuroid (FI-ASOR) is made from desialylated orosomuroid (alpha-1 acid glycoprotein; Sigma Cat. #G-9885) and Texas red sulfonyl chloride (Invitrogen, Carlsbad, CA).
4. Sprague–Dawley rats (Taconic Farms, Germantown, NY) are used for isolation of liver endosomes; animal care facilities and animal use protocol approval, minor animal surgery techniques, and sodium pentobarbital are required.
5. Endocytic vesicles are isolated with MEPS buffer [35mM K₂-PIPES (note the inclusion of potassium), 5mM EGTA, 5mM MgCl₂, 0.25M sucrose pH 7.2] plus 4mM dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail (Sigma Cat. #P-8340); a Sephacryl S200 gel filtration column (Pharmacia, Uppsala, Sweden) and a 1.4M, 1.2M, and 0.25M sucrose step gradient are used.
6. Cell culture reagents are required if these are to be used for the source of organelles.

2.2. Microscopy Experiments

1. Microscope chambers, 8–10 placed on a dark board, assembled from coverslips (22×40 mm, Corning #290-244), glass slides (Fisher #12-550-10), and double-stick tape (Scotch 3 M #655, 0.009 cm thick). Coverslips are coated with DEAE-Dextran (Pharmacia #17-0350-01) to attach MTs.
2. Fluorescent-labeled endocytic vesicles (or other organelles); thaw immediately before use.
3. Primary antibody to receptor [e.g., asialoorosomucoid receptor (6)].
4. Appropriate Cy2-labeled secondary antibody (e.g., Cat. #115-225-068; Jackson ImmunoResearch Laboratories).
5. 10X PMEE buffer: 350 mM K_2 -PIPES, 50 mM $MgCl_2$, 10 mM EGTA, 5 mM ethylenediaminetetraacetic acid (EDTA), 20 mg/mL bovine serum albumin (BSA), pH 7.4; made fresh from stocks and powdered BSA.
6. 10 mM ATP, neutral pH, frozen stock.
7. 200 mM DTT, made fresh.
8. 1 mM Taxol frozen stock in dimethylsulfoxide (DMSO).
9. Na-Ascorbic acid 100 mg/mL stock, made fresh (optional, as an antibleaching agent).
10. AMP-PNP and vanadate.

2.3. A Typical Microscope System (see Note 1)

1. A Lambda DG-4 xenon excitation lamp (Sutter Instruments, Novato, CA) with millisecond switching between four fluorescence filters.
2. A Sedat Quad dichroic mirror (Chroma Technologies, Rockingham, VT) with excitation and emission filters designed for DAPI, Cy2, Cy3, and Cy5 fluorescence.
3. A 60 $\times$, 1.4 n.a. Olympus objective mounted on an Olympus 1X71 inverted microscope containing a PZ-2000 (ASI, Eugene, OR) electronic and piezoelectric stage manipulator, with 25-mm circular cut-out stage plate.
4. A Lambda 10–2 emission filter wheel (Sutter Instruments, Novato, CA).
5. A Uniblitz transmitted light shutter (Vincent Associates, Rochester, NY) for transmitted light.
6. A low light CoolSnap HQ cooled CCD camera (Photometrics, Roper Scientific, Tucson, AZ).
7. All devices are controlled with Metamorph software (Molecular Devices, Sunnyvale, CA) on a PC computer with a large hard drive and DVD burner.
8. A Plexiglas chamber designed in house encloses the stage and is equipped with a hot air heater (Sy-Airthermy, World Precision Instruments).

2.4. Suggested Image Analysis Software (see Note 2)

1. ImageJ shareware (NIH Public domain, <http://rsb.info.nih.gov/ij/>; our gratitude to Wayne Rasband).
2. Adobe Photoshop (Adobe Systems, San Jose, CA).
3. Metamorph (Molecular Devices, Sunnyvale, CA).

3. Methods

3.1. Preparation of Microscope Chambers for Attaching Microtubules

1. Immerse a large coverslip (22×40 mm, Corning #290-244) in $30 \mu\text{g/mL}$ DEAE-dextran (Pharmacia #17-0350-01) in H_2O , then rinse thoroughly with nanopure H_2O and allow coverslip to dry (an aerosol dust remover can be used to blow dry). Excess DEAE-Dextran will bundle the MTs. Sigmacote, nitrocellulose, polyornithine, and polylysine (may cause bundling) are optional reagents to attach MTs.
2. Apply pieces of double-stick tape (Scotch 3 M #655, 0.09 mm thick) to the coverslip to form a channel.
3. Score a standard glass slide to form a 5-mm rectangle using a wheeled glass cutter, break, and apply the glass to the tape to create a chamber.
4. Press the glass to the double stick tape to seal and add a drop of clear nail polish to the tape-cut glass junction to maintain the seal during buffer washes.
5. Create a handle for the chamber with a small piece of rolled-up single-sided Scotch tape.

Figure 1 demonstrates the construction of the microscope chambers, which are assembled the day of the experiment. Similar chambers have been described (7–10), and their design can be tailored, for instance, to an upright versus inverted microscope or to alter the chamber volume. A black surface is helpful

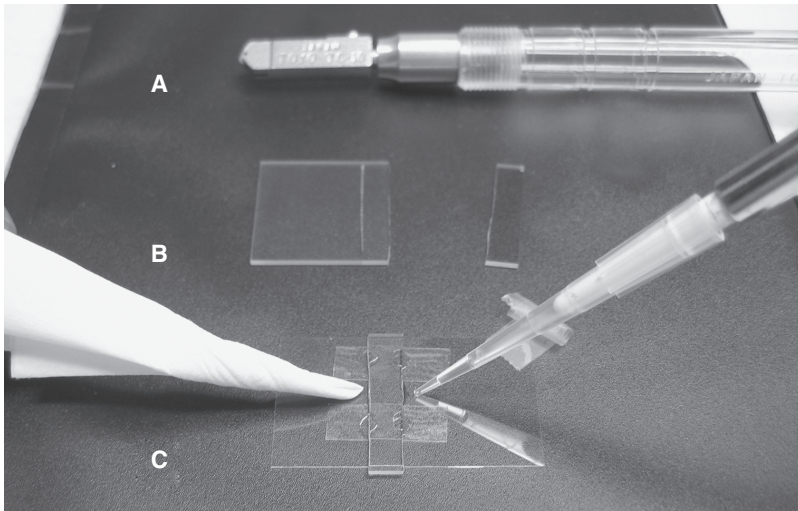


Fig. 1. Assembly of disposable microchambers for immunofluorescence and motility assays. (A) The wheeled glass cutter that is used to score glass microscope slides as shown in (B). (C) Perfusing a chamber with buffer using a pipette and Kimwipe; note the double-stick tape and large coverslip forming the sides and bottom of the chamber.

in viewing the contents of the chamber because air bubbles, dehydration, or debris can destroy its contents. Eight to ten chambers are usually laid out across the board to allow the addition of reagents by pipet. The internal volume is approximately 4 μ L. To prevent evaporation, glycerol can be added to seal the chamber, or the chamber can be placed in a humid environment.

3.2. Polymerizing Fluorescent Microtubules (see Note 3)

1. Fluorescent tubulin can be purchased [e.g., Cat. #TL 238 (unlabeled), and TL 331 (rhodamine-labeled), Cytoskeleton Inc., Denver, CO] or purified from calf brain and fluorescently labeled ([10](#)) and stored in aliquots.
2. Bring tubulin to 6 mg/mL at a 1 : 10 molar ratio of fluorescent to nonfluorescent tubulin in cold MT buffer. Clarify by centrifugation for 5 min at 20,817 *g* (14,000 rpm), 4°C. Recommended polymerization volume is 18 μ L.
3. Polymerize by warming to 37°C for 10 min, and complete (terminate) polymerization by addition of a 12-fold volume of prewarmed MT buffer plus 20 μ M Taxol.
4. Pellet Taxol-stabilized MTs in a Beckman Airfuge at 15 psi for 5 min to remove unpolymerized tubulin and resuspend to approximately 1 mg/mL in MT buffer plus 20 μ M Taxol.

3.3. Polymerizing Polarity-Marked MTs (see Note 4)

1. Polarity-marked MTs contain a mark at the minus end, and we generate these by polymerizing brightly labeled tubulin from dimly labeled tubulin “seeds,” although other schemes can be used.
2. Generate 10 mg/mL stocks of dim tubulin (at 80 : 1 unlabeled to labeled tubulin), and bright tubulin (at 6 : 1), and clarify each by centrifugation for 5 min at 20,817 *g* (14,000 rpm), 4°C.
3. Polymerize a small volume (e.g., 8 μ L) of dim tubulin into seeds at approximately 10 mg/mL in MT buffer by warming to 37°C for 5 min.
4. Shear the seeds by pipetting up and down 10 times with the pipet tip pressed into the Eppendorf tube, maintained at 37°C.
5. Rapidly dilute the seeds to 2.3 mg/mL in MT buffer and immediately add 3.5 mg/mL bright fluorescent tubulin.
6. After 6 min polymerization, add a 12-fold volume of MT buffer plus Taxol.
7. Pellet the MTs by centrifugation for 4 min at 15 psi in a Beckman Airfuge and resuspend in 500 μ L MT buffer plus Taxol.

3.4. Isolation of Fluorescent Rat Liver Endosomes

Rat liver has provided an excellent source of endocytic vesicles having robust MT-based motility and remaining active for more than 1 year when stored at -80°C. A single liver provides approximately 60 aliquots of 20 μ L each, enough for 240 experiments. A modified version of the protocol is also presented that has been used to isolate and study the motility of virus particles

grown in cell lines ([11](#)), as well as endosomes from mouse liver (unpublished data).

1. Orosomucoid (alpha-1 acid glycoprotein, Sigma Cat. #G-9885) is desialylated to asialoorosomucoid (ASOR) by exposure to 0.1 *N* H₂SO₄ for 1 h at 75°C followed by neutralization and dialysis into water or phosphate-buffered saline (PBS).
2. ASOR is labeled with an amine reactive fluorescent dye (e.g., Texas Red sulfonyl chloride or Alexa Fluor 488 succinimidyl ester; Molecular Probes, Eugene, OR) according to the manufacturer's protocols to yield a molar ratio of 2 : 1 dye to ASOR as measured spectrophotometrically [extinction coefficient for ASOR of OD₂₇₈ of 0.0375 cm⁻¹ μM⁻¹ with an estimated (from glycosylation) molecular weight of 40 kDa]; this is stored at -20°C in aliquots and retains activity for years.
3. Fluorescent ASOR, 50 μg, is injected into the portal vein of Sprague-Dawley rats. In this limited surgery, the rat is rendered unconscious with intraperitoneal injection of 50 mg/kg pentobarbital, laparotomy is performed in a similar manner as for rat liver perfusion ([12](#)), and the portal vein is injected with ASOR in 300 μL PBS.
4. Endocytic uptake and processing is allowed to proceed for 5 min, at which time the liver is perfused through the portal vein with 30 mL ice-cold PBS, and the rat is exsanguinated. Subsequent procedures are performed at 4°C.
5. The liver is removed, washed in MEPS buffer plus 4 mM DTT, and homogenized with 20 strokes in a loosed ounce homogenizer in 15 mL MEPS buffer containing 2 mM PMSF and 300 μL (a 1 : 50 dilution) of protease inhibitor cocktail (Sigma Cat. #P-8340).
6. The homogenate is centrifuged at 1,811 g (3,000 rpm) in 15-mL conical tubes for 10 min at 4°C in an Eppendorf 5810R centrifuge to form a postnuclear supernatant (PNS), and protease inhibitors and DTT are re-added at previous levels.
7. The PNS is chromatographed on a Sephacryl S200 (Pharmacia, Uppsala, Sweden) gel filtration column equilibrated in MEPS. The opaque yellow-white flow-through containing fluorescent endosomes is collected, and protease inhibitors and DTT are added again.
8. The S200 pool is brought to 1.4 *M* sucrose by addition of a 2.5 *M* sucrose-MEPS stock buffer and loaded into the bottom of a sucrose float-up step gradient consisting of 1.4 *M*, 1.2 *M*, and 0.25 *M* sucrose in MEPS buffer. The gradient is centrifuged at 200,000 g (39,000 rpm) in a Beckman SW41 Ti Rotor for 2 h.
9. Approximately 1.2 mL cloudy material is carefully collected from the 0.25 *M*/1.2 *M* interface and stored in 20-μL aliquots at -80°C.

3.5. Isolation of Fluorescent Endosomes from Cultured Cells

1. Grow 10 or more 15-cm dishes of Huh7 cells ([13](#)) to confluence.
2. Chill cells and incubate with 10 μg/mL fluorescent ASOR (ligand) on ice for 1 h (this allows for ligand binding but prevents endocytic uptake); then wash five times in culture media.

3. Allow a “single wave” of endocytic processing by warming dishes to 37°C for a specific time (e.g., 10 min).
4. Wash cells in cold MEPS buffer; scrape them off the dishes, and pellet at 240 g (1,500rpm) for 5 min. Subsequent steps are performed at 4°C.
5. Resuspend cells in MEPS plus DTT, plus protease inhibitors (as above in the whole-liver protocol), and homogenize with 10 passes through a 25-gauge needle.
6. Centrifuge at 950 g (3,000rpm) for 10 min, collect the PNS, and re-add DTT and protease inhibitors.
7. Adjust to 1.4M sucrose (as above) and load into the bottom of a sucrose step gradient of 1.4M/1.2M/0.25M using 1.2/1/0.1 mL in a Beckman TLS55 swinging bucket rotor.
8. Centrifuge in a tabletop ultracentrifuge (e.g., Beckman TL100) at 101,000 g (39,000rpm) for 2 h.
9. Collect the cloudy material at the 1.2M/0.25M interface and store frozen in 20- μ L aliquots at -80°C for single use.

3.6. Measurement of the Sorting of Receptor from Ligand Along MTs In Vitro (see Note 5)

1. Prepare assay buffer (“1X” PMEE plus 20 μ M Taxol, 4mM DTT, + 2 mg/mL Na-Ascorbic acid), MT buffer (“1X” PMEE plus 20 μ M Taxol), and blocking buffer (assay buffer plus 5mg/mL casein; undissolved casein can be removed by filtration).
2. Dilute MTs 1:50 (to ~20 μ g/mL) in MT buffer, add 5 μ L MTs to chamber, incubate 3 min at room temperature, wash three times with blocking buffer (blocking reduces vesicle binding directly to glass), and wash two times with assay buffer.
3. Add 5 μ L fluorescent-labeled vesicles; allow their binding to MTs for 10 min at room temperature in a humid chamber. Wash off unbound vesicles three times with blocking buffer (to block nonspecific antibody binding), and store chambers in a humid environment on ice to preserve motile activity.
4. Add 3 $\times$ 15 μ L primary antibody at approximately 5 μ g/mL (a 1:100 dilution), incubate 6 min, wash six times with blocking buffer (*see Note 6*).
5. Add 3 $\times$ 15 μ L Cy2-antimouse antibody (e.g., Cat. #115-225-068, Jackson ImmunoResearch Laboratories) at approximately 15 μ g/mL (a 1:100 dilution), incubate 6 min, wash six times with assay buffer.
6. At the microscope, heated to 37°C, remove a single chamber from the cold; wash in assay buffer.
7. Focus on a field containing MT-bound vesicles. Avoid excess excitation light. Initiate time-series image capture of bright field (optional) and Cy2 and Cy3 fluorescence channels, and add 50 μ M ATP. A rate of capture of 1 frame per second for 90s is recommended.
8. Repeat motility experiment for all chambers.

3.7. Quantifying the Segregation of Ligand from Receptor (see Note 7)

Time-lapse movies from the preceding experiment will contain ligand and MTs in the red (cy3) channel and receptor in the green (cy2) channel. Imaging of MTs and ligand in the same channel is convenient and allows for faster image collection. The MTs should be dim, as compared to ligand, with consistent (nonvarying) fluorescence intensity, and can therefore be subtracted out of the image.

1. Process the image data to highlight the vesicles and smooth the background noise. A spot-enhancing filter (Daniel Sage, Biomedical Imaging, Lausanne, Switzerland) for ImageJ has been helpful for this purpose.
2. Merge the images to view as a red (ligand) and green (receptor) movie (a “.tif” file stack is preferred if the movie is not too large).
3. Print out the first in-focus image of the sequence and note all visible vesicles. Noting vesicles during movie viewing will bias the results toward increased motility, as the movement itself makes a vesicle easier to distinguish.
4. Play the movie and score each vesicle as “motile,” “nonmotile,” or “unscorable” (see below: scoring vesicle motility). Also note any vesicles that undergo fission. We have found that nonmotile vesicles undergo limited or no fission (6).
5. For all fission events, determine how the protein distributes to the daughter vesicles:
 - a. Crop the movie to include only the fission event for ease of analysis.
 - b. Separate the fluorescence channels
 - c. In frames immediately following fission, measure the average intensity of three background areas adjacent to each daughter vesicle. Include the MT itself in the background signal if the MT and protein signal are imaged in the same channel.
 - d. Trace (e.g., using a drawing tool) the vesicle pixel area and measure the number of pixels and average pixel intensity of each daughter vesicle. Fluorescence signal = area $\times$ (average pixel intensity – background). Larger areas generally give proportionally greater signals despite background subtraction; therefore, the user needs to be as consistent as possible.
6. Tally the daughter vesicle intensities to reveal how the proteins sort during fission events.

Figure 2 demonstrates fission of a MT-bound endocytic vesicle. A single vesicle is seen (circled) that divides into two vesicles after 23s exposure to ATP. One of the daughter vesicles (also circled) continues to move to the right through 50s. Interestingly, this daughter vesicle appears at least partially subdivided into areas of distinct fluorescence. Using these techniques, we have quantified the segregation of the asialoglycoprotein receptor from its ligand in vitro (14). On average, 90% of the asialoglycoprotein receptor segregates with 60% of ASOR during a fission event, whereas 45% of fission events lead to

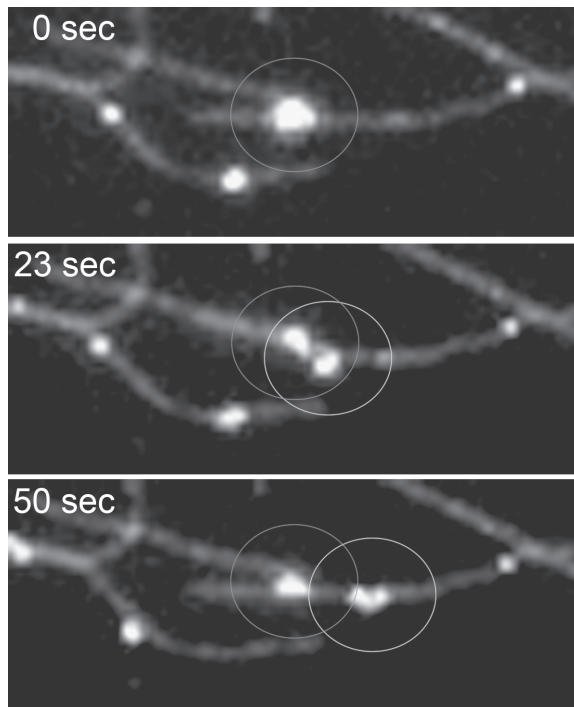


Fig. 2. A microtubule (MT)-based endocytic fission event observed in vitro. Rat liver endocytic vesicles were bound to MTs and stained for the transferrin receptor using the protocols given (*see Subsection 3.6.*). ATP was added to the microscope chamber to initiate motility, and images were captured in red (MT and ASOR) and green (transferrin receptor) channels at 1 frame per second. Images were converted to black and white for publication, MTs are the visible filaments, transferrin receptor and ASOR both appear as bright spots. Analysis of color merged time-lapse movies revealed significant numbers of fission events, and the degree to which the transferrin receptor partitioned to daughter vesicles can be quantified as indicated in **Subsection 3.7. (14)**.

complete segregation of the receptor from ligand. These studies demonstrated that endocytic contents will segregate along MTs and that in vitro assays could be used to determine the amount of segregating protein during fission events.

3.8. Measurement of Motility Parameters

Following is a list of parameters that can be used to quantify the MT-associated activities of organelles in vitro as captured in time-lapse movies.

1. **Binding to MTs:** Incubate a MT-coated chamber with vesicles for 10 min at room temperature, wash $5 \times 15\mu\text{L}$, and collect multiple images. For each image trace

the length of the MT and count the number of vesicles bound per micrometer of MT.

2. **Number of moving vesicles:** As described above (*see Subsection 3.7.*), time-lapse movies of vesicles responding to the addition of ATP can be quantified for the number of motile events per number of MT-bound vesicles. Fluorescence channels are merged, and visible vesicles are noted from the first (or most in focus) frame and manually scored. Movement is scored relative to the position of the MT, as some gliding (moving of the MT itself) can occur. Motor-based movement will only be seen when ATP (or other nucleotides) are added, and buffer-alone controls should be performed for comparison. ATP addition can also lead to vesicle release from MTs, and this can be scored as an additional parameter.
3. **Polarity of MT movement:** The direction of movement with respect to MT polarity can indicate which motors are responsible for the movement and whether the movement is expected to bias the location of the organelle within a cell. These motility experiments are performed with chambers coated with polarity-marked MTs (*see Subsection 3.3.*). Only unambiguously marked MTs should be counted, and therefore many MT-bound vesicles may need to be eliminated from the scoring.
4. **Velocity:** The movement of vesicles can be tracked over time, and we have favored manual tracking software in ImageJ for this purpose. Endosomes display a stop-and-start movement that is difficult to classify into absolute velocity and may therefore be broken down into “non-zero velocity” (where consecutive frames of zero velocity are eliminated) and the pause time (the number of consecutive frames that are eliminated) (15). The rate of image capture will affect these parameters.
5. **Run length:** Measurement of the run length, the total distance that a vesicle travels before arresting or detaching from a MT, can indicate how different motors cooperate to produce movement. It has been shown that kinesin-1 has greater run length than dynein and that multiple motors acting on a single bead or vesicle will produce greater run length than single motor molecules (16,17).

3.9. Co-Localization of Motor Protein with Specific Organelles

1. Endocytic vesicles (or other organelles) are allowed to bind directly to the glass surface of untreated microscope chambers (18), or vesicles are bound to MTs that have been attached to microscope chambers, as described above.
2. Immunofluorescence staining (as above) is performed using an array of primary antibodies to motor proteins as well as the protein or organelle marker of interest.
3. Images for the organelle marker, the motor protein, and bright field (or phase contrast or similar) are captured. The bright-field image serves as a reference to indicate that the microscope field contains phase-dense vesicles and does not contain air bubbles or debris.
4. The “percent co-localization” of the markers is scored by counting the number of fluorescent spots in channel A (the organelle) that contain significant fluorescence

in channel B (the motor protein). With bright, uniform fluorescence, this can be done automatically. Typically, a two-frame movie (.tif file) is created to aid in manual scoring (see **Note 7**).

3.10. Antibodies as Inhibitors of Microtubule-Based Motility (see Note 8)

Some antibodies have been shown to block cytoskeletal based movement, and it is believed that cross-linking or interfering with the motion of the motor protein can inhibit motility (19–21). The effect of the primary antibody can be examined by addition of this antibody to MT-bound vesicles for 5 min, followed by washing. ATP is then added to the chamber under time-lapse imaging, and movies are scored for the level of motor-based motility. As an example, **Fig. 3**

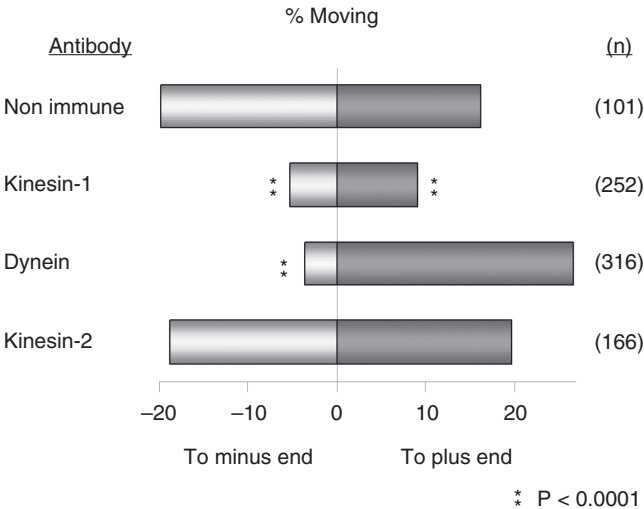


Fig. 3. The use of motor protein antibodies to inhibit MT-based motility. Polarity-marked MTs were attached to microscope chambers, and vesicles were bound to MTs and stained for the sodium taurocholate cotransporting polypeptide (ntcp), a hepatocyte-specific bile acid transporter. Chambers were treated with nonimmune IgG (control), kinesin-1, dynein, or kinesin-2 primary antibodies in separate experiments with 10 to 30 chambers for each condition. On addition of ATP, 40% of the vesicles were motile after treatment with nonimmune IgG (control), and this movement was toward the plus and minus ends of the MT with equal frequency as indicated. Kinesin-1 antibody reduced motility toward both the plus and minus ends of MTs, dynein antibody reduced motility toward the minus ends, and kinesin-2 antibody had no effect. These results demonstrate that antibodies have specific effects on MT-based motility and that these assays can be used as a tool to understand the regulation of vesicle transport.

demonstrates the effect of three motor protein antibodies on the motility of vesicles that contain the bile acid transporter, *ntcp* (15). Nonimmune IgG is used as a control. Antidynein antibody was found to inhibit minus-end-directed motility, whereas kinesin-1 antibody was found to inhibit both plus- and minus-end motility, and kinesin-2 antibody had no effect on motility. Previous studies showed that this kinesin-2 antibody inhibited motility of late endocytic vesicles (6). This example shows that antibodies have specific effects, and the results of these experiments can be compared to the immunofluorescence staining of the antibody.

3.11. Nucleotides and Chemical Inhibitors

The nucleotide AMP-PNP has been shown to inhibit kinesin-based motility, and inclusion of 1 mM AMP-PNP with 50 μ M ATP blocks kinesin-based motility of endocytic vesicle movement *in vitro*. Likewise, low concentrations of vanadate can specifically inhibit dynein, and inclusion of 5 μ M vanadate blocks dynein-based motility of endocytic vesicles (14,22,23). It has also been shown that kinesins, but not dyneins, can hydrolyze GTP to produce MT-based movement, and we have found that endocytic vesicles will move slowly across MTs when incubated with 1 mM GTP instead of ATP (unpublished observations).

3.12. Factors That Contribute to Reliable *In Vitro* Motility

1. A high concentration of organelles: Ideally, 20 or more MT-bound vesicles should be visible in each microscopy field, which is generally a higher concentration than is needed for Western blot or enzyme assays.
2. DTT, protease inhibitors: We assiduously add DTT and protease inhibitors and have found that a temporary exposure to nonreducing conditions can inhibit motor activity.
3. Fast organelle isolation: The time between liver homogenization and freezing of endosomes in aliquots for the foregoing protocol is 4–5 h.
4. Freezing in aliquots: Although freezing can decrease motor activity, it has been even more vital to have a reproducible stock of organelles.
5. Removal of ATPases and other inhibitors: For liver, we have found that including a gel filtration step in the isolation removed contaminating ATPases and potentially other inhibitors (24). Such was not required for isolation of motile virus particles from cultured cells (11), but other inhibitors may be present, and could include cross-linking agents, ADP, Pi, high or low pH, high salt, or other ions. Buffer reagents can go bad and lead to lowered motility.
6. Familiarity and speed of the hands-on microscopy technique will dramatically improve results.
7. Control experiments should be performed each day of experiments to ensure that motility is active.

4. Notes

1. Many microscope platforms can be used. Low light detection, limited intensity excitation, temperature control, rapid switching between multiple fluorescence channels, a full field rate of image capture of 1 s or less, and automated X-Y-Z stage movement are all recommended. Thermal gradients should be avoided as these cause stage movement. We prefer stage plates with a simple 25-mm-diameter hole and modeling clay to hold slides to the stage. Excitation light is damaging to motor proteins and must be standardized within the experimental conditions and kept to a minimum. Analog (videotape) recording can work well, although documentation is more difficult and image intensity information may be less accurate. Laser scanning confocal systems can inhibit motility because of damage from the laser light. However, spinning disk confocal, which captures images on a CCD and requires less excitation illumination, appears ideal.
2. It is critical to understand that image data are normally stored as either 8-bit or 16-bit files; this means that every pixel contains up to $2^8 = 256$ or $2^{16} = 65,536$ levels of gray-scale intensity, respectively. Most high-end cameras capture images at 12 or 16 bits and store them as 16-bit files. However, computers display images as 8-bit, therefore must choose a range for which to display the image, and usually “normalize” the image to squash 65,000 levels into 256. Thus, the displayed image does not necessarily reflect what has been captured on the camera, and careful attention must be paid to how the image is presented and quantified.
3. The MT stock should be good for 2–3 weeks in the dark and is normally diluted 1 : 50 (to 20 $\mu\text{g}/\text{mL}$) before addition to microscope chambers. The MTs need to be long (e.g., 20 μm), free of background haze (denatured/unpolymerized tubulin), and not bundled. Haze or fluorescent “junk” can be removed by repelleting in the Airfuge through 30% glycerol (in MT buffer plus Taxol); shorter centrifugation times may be helpful. “Flaky” MTs indicate Taxol-induced polymerization. Tubulin may vary from lot to lot, and polymerization concentrations or brightness may need to be adjusted. Bundled MTs indicate that a buffer component has gone bad or is incorrect. Tubulin polymerizes upon warming and must be kept cold.
4. If MT fluorescence appears too dim or too bright compared with other fluorescence signals, then the concentration of labeled tubulin should be adjusted. Polarity-marked MTs should be used the same day because annealing and breaking over time will result in loss in accuracy of the polarity mark. Performing MT gliding assays with a single species of motor protein can be used to check the accuracy of polarity marks. We have used a recombinant kinesin motor protein (KR01; Cytoskeleton Inc.) for this purpose, although its motility is slow and light sensitive. Polarity marks are considered accurate when 80%–90% of the scorable MTs move in the proper direction. Note that loss of accuracy will skew data toward equal plus- and minus-end movement. Ambiguous MTs must be eliminated when scoring directionality, and therefore many more experiments are required as compared to using nonmarked MTs. Substituting GMPCPP for GTP may increase the accuracy of this procedure (8), and freezing the marked MTs may allow their use at later dates.

5. In our hands, vesicle motility is sustained for 2 min or less, and large data sets are generated using multiple chambers (e.g., 30 or more). Oxygen-scavenging systems have been used (25), but these can cause MT bundling. ATP regeneration systems have not aided our assays. We have found the most consistent motility with low ATP concentration for rat liver endosome movement, but other systems may require higher levels of ATP. Low ATP concentration should limit extraneous ATPase activity that can produce ADP and free phosphate.
6. Specificity of antibodies should be validated regardless of statements from commercial sources. Some antibodies will yield “nonspecific” staining of all organelles, MTs, or to the glass, or they may aggregate or denature. Western blot should be performed, and antibodies that yield signal that is not attributable to the antigen should be avoided. We have found that most antibodies that function in Western blot also function for immunofluorescence of unfixed material. Fixatives that are used in whole-cell immunofluorescence such as formaldehyde can reduce antigenicity. In addition, light scattering and autofluorescence within whole cells can cover up weak immunofluorescence staining that will be visible in *in vitro* experiments. Buffer conditions, detergents, and other factors can also affect immunofluorescence detection. Controls for antibody specificity include the following:
 - Lack of immunofluorescence in the absence of primary antibody (a control for the secondary antibody).
 - Competitive absorption of the immunofluorescent signal by inclusion of the antigen during the primary antibody incubation step.
 - Usage of multiple antibodies to the same antigen.
7. Image processing and analytical methods are rapidly evolving. Automated methods are desirable but difficult to employ successfully for reasons of background signal fluctuation, discontinuous vesicle movement, movement of the stage, and other technical issues.
8. The effect of an antibody should be tested at different concentrations. In some experiments, motility assays can be performed in the presence of both primary and secondary antibody to vesicle-associated proteins [e.g., the asialoglycoprotein receptor or ntcp (14,15)].

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Enrichment and Disassembly of Ectoplasmic Specializations in the Rat Testis

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Summary

Ectoplasmic specializations are testis specific intercellular adhesion junctions found in Sertoli cells. They are tripartite structures consisting of the plasma membrane of the Sertoli cell, a submembrane layer of actin filaments and an attached cistern of endoplasmic reticulum. Ectoplasmic specializations occur in areas of attachment to spermatids and as part of the basal junction complex between neighboring Sertoli cells. They are functionally related to a number of fundamental events that occur during spermatogenesis, including attachment and then release of developing sperm cells and the translocation of spermatocytes through the blood–testis barrier. The structures may contain viable molecular targets for the development of contraceptives. Here we describe techniques for obtaining, from rat testes, testicular fractions enriched for spermatids with attached ectoplasmic specializations and for disassembling the complexes with gelsolin to obtain supernatants enriched for plaque components. The techniques involve stripping the epithelium from tubule walls, mechanically fragmenting the epithelium, using step sucrose gradients to enrich for spermatids with attached junction plaques, and then incubating with exogenous gelsolin to release plaque components into solution.

Key Words: Ectoplasmic specializations; adhesion junctions; Sertoli cells; Testis.

1. Introduction

Ectoplasmic specializations are unique adhesion junctions that develop in Sertoli cells at certain sites of attachment to adjacent cells in the seminiferous epithelium of the testis. These sites occur apically where Sertoli cells are adherent to elongate spermatids, and basally where elaborate junction complexes attach neighboring Sertoli cells to each other. Ectoplasmic specializations are tripartite structures composed of the Sertoli cell plasma membrane, a layer of hexagonally packed actin filaments and a cistern of endoplasmic reticulum. Because ectoplasmic specializations occur only in Sertoli cells, heterotypic

adhesion junctions between Sertoli cells and spermatids have ectoplasmic specializations only on the Sertoli cell side of the junction, whereas homotypic basal adhesion junctions between adjacent Sertoli cells have ectoplasmic specializations on both sides of the junction. Adhesion molecules conclusively shown to be present in the plasma membrane of Sertoli cells both at apical and at basal ectoplasmic specializations include $\alpha_6\beta_1$ integrins (1,2), nectin-2 (3), and junction adhesion molecules (JAMs) (4). In contrast to the situation at adherens junctions in other epithelia, cadherins are not a major component of ectoplasmic specializations (5,6), although N-cadherin is reportedly present (7). At apical sites, the ligand for nectin-2 is nectin-3 in the plasma membrane of the spermatid, and at basal sites the ligand is another nectin-2 molecule in the plasma membrane of the adjacent Sertoli cell (3). Although the ligand(s) for $\alpha_6\beta_1$ integrin is(are) unclear, γ_3 laminin (8) and ADAM proteins are possible candidates.

In addition to the integral membrane adhesion proteins and actin filaments, other elements present at ectoplasmic specializations include the adaptor proteins vinculin (9) and afadin (3), and the actin-associated proteins α -actinin (10), fimbrin (9), espin (11), Keap 1 (12), and cortactin (13). Signaling elements localized at the sites include ILK [integrin-linked kinase] (14), Rac 1 (15), and Fyn tyrosine kinase (Src family member) (16). PIP₂, PLC, and gelsolin also have been reported as present (17); however, we recently have re-evaluated the presence of gelsolin at the sites and believe our initial findings may largely have been caused by secondary binding to the sites of exogenous gelsolin present in serum (18). In addition, myosin VIIa is present in the structures (19), but myosin II is not (20). A number of studies have focused on the role of signaling cascades and of proteases and protease inhibitors in regulating ectoplasmic specializations (8,21,22). There currently is much interest in these unique adhesion junctions because the structures may contain molecular targets for contraceptives (7,23–27).

To obtain material to facilitate the identification of components present in ectoplasmic specializations and to isolate the structures to explore function, we have developed a technique to acquire testicular fractions enriched for spermatids with attached ectoplasmic specializations from rat testes (17,28–30). The technique involves manually extracting epithelium from seminiferous tubules, mechanically fragmenting the epithelium, then centrifuging the fragments through step sucrose gradients to enrich for spermatids with attached ectoplasmic specializations (Fig. 1). This material can then be used as starting material for functional and other studies. For example, we have used these preparations as starting material for doing binding and motility assays to test for the association of microtubule-based motor proteins with the junction plaques (29–31). In addition, we have taken advantage of the actin-severing

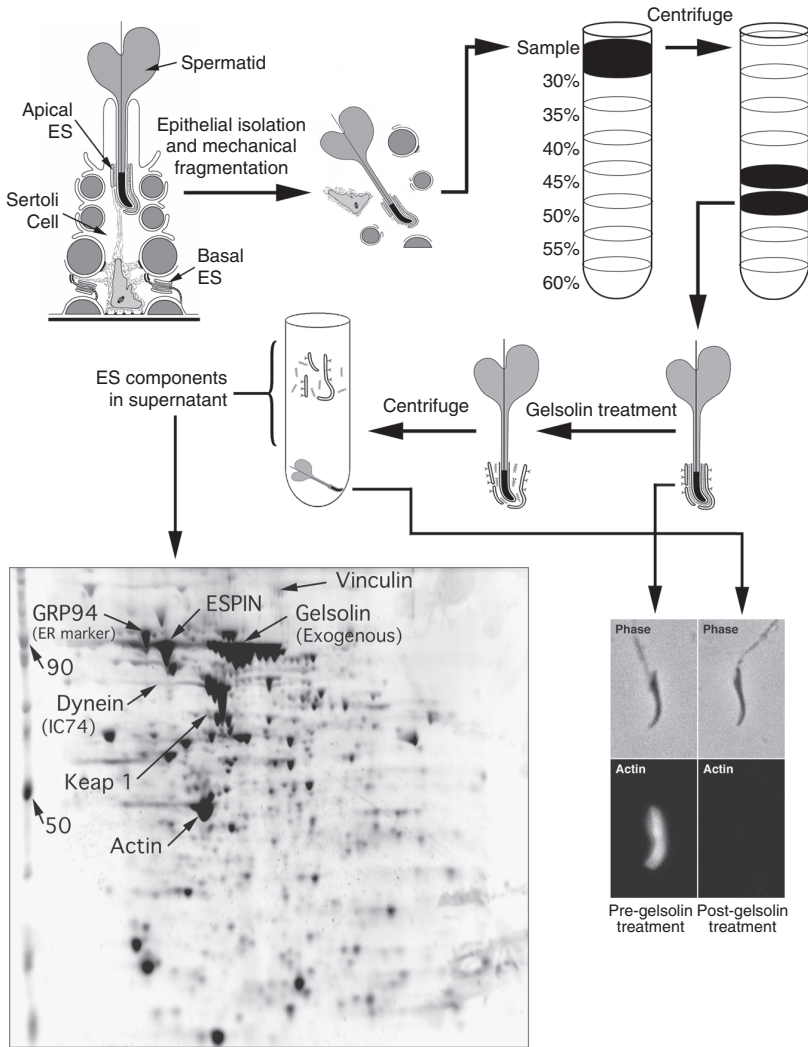


Fig. 1. Summary of the technique used to obtain testicular fractions enriched for ectoplasmic specialization components and a two-dimensional (2D) gel of supernatants from gelsolin-treated spermatid/ectoplasmic specialization complexes. On the 2D gel, the positions of some of the known components of ectoplasmic specializations are indicated. These positions were determined using replicate 2D immunoblots.

capacity of exogenous gelsolin to disassemble the plaques *ex vivo* and obtain supernatants enriched for plaque components for analysis of plaque-associated proteins by electrophoresis (30). These supernatants also can be used as starting material for doing proteomics.

2. Materials

2.1. Preparation of the Gradients Columns

2.1.1. Stock Materials

1. 100 mL 0.2 M PIPES, pH 6.8. Store at 4°C.
2. 100 mL 0.1 M ethyleneglycoltetraacetic acid (EGTA), pH 6.8. Store at 4°C.
3. 100 mL 0.1 M MgCl₂. Store at 4°C.
4. Sucrose (dry).
5. Protease inhibitors:
 - a. 1.0 mg/mL soybean trypsin inhibitor. Make up in distilled water and store in 100 μ L aliquots at -20°C.
 - b. 1.0 mg/mL leupeptin. Make up in distilled water and store in 50 μ L aliquots at -20°C.
 - c. 1.0 mg/mL pepstatin A. Make up in 100% ethanol. Store for up to 1 week at 4°C.
 - d. 0.1 M phenylmethylsulfonyl fluoride (PMSF). Make up in 100% ethanol. Store at -20°C.

2.1.2. Working Solutions

1. 50 mL PEM buffer: 20 mL 0.2 M PIPES stock, 0.5 mL 0.1 M EGTA stock, 0.5 mL 0.1 M MgCl₂ stock. Bring to approximately 40 mL, adjust pH to 6.8. Transfer to a volumetric flask, and add 50 μ L soybean trypsin inhibitor stock, 25 μ L leupeptin stock, 25 μ L pepstatin A stock, and 50 μ L PMSF stock. Bring volume to 50 mL.
2. 50 mL PEM/60 buffer: 20 mL 0.2 M PIPES stock, 0.5 mL 0.1 M EGTA stock, 0.5 mL 0.1 M MgCl₂ stock, 30 g sucrose. Add the liquid stocks to a 100 mL beaker with a stirring bar and put on a magnetic stirrer. Slowly add the sucrose adjusting the stirring bar speed so that it keeps the solution moving. Let stir until the sucrose is dissolved (an hour or more). Check and adjust pH to 6.8. Add 50 μ L soybean trypsin inhibitor stock, 25 μ L leupeptin stock, 25 μ L pepstatin A stock, and 50 μ L PMSF stock. Transfer to a volumetric flask and bring to 50 mL.

2.1.3. Equipment

1. Ultraclear 5 $\times$ 41 mm centrifuge tubes (Beckman).

2.2. Isolation and Fragmentation of Seminiferous Epithelium

2.2.1. Working Solutions (Using Stock Solutions in Subsection 2.1.1.)

1. 100 mL PEM/250 buffer: 40 mL 0.2 M PIPES stock, 1.0 mL 0.1 M EGTA stock, 0.1 M MgCl₂ stock, 250 mM sucrose. Bring to near volume, adjust pH to 6.8, then transfer to volumetric flask. Add 100 μ L soybean trypsin inhibitor stock, 50 μ L leupeptin stock, 50 μ L pepstatin A stock, and 100 μ L PMSF stock. Bring volume to 50 mL.

2.2.2. Equipment

1. Microdissecting probe with straight end.
2. Microdissecting probe with bent tip.

2.3. Centrifugation of Step Sucrose Gradients and Collection of Fractions

1. Beckman Coulter Optima XL-100K ultracentrifuge and a SW 55 Ti rotor fitted with adaptors for 5×41 mm Ultra-Clear centrifuge tubes.
2. 1 mL syringes with 22- to 23-gauge needles.

2.4 Gelsolin Digestion of Ectoplasmic Specializations

1. 100 mL 0.1 M CaCl_2 . Store at 4°C.
2. 100 mL 0.2 M MgCl_2 . Store at 4°C.
3. 1.0 L 50 mM MES buffer: 11.67 g MES, 0.771 g DTT, 10 mL 0.2 M MgCl_2 stock, 1 mL 0.1 M CaCl_2 stock. Adjust pH to 6.3 with KOH and bring to volume.
4. Gelsolin: Resuspend 0.2 mg gelsolin (Sigma) in 500 μL MES buffer and dialyze for 60 min in the cold in 500 mL MES buffer. Flash freeze 100 μL aliquots and store at -70°C . (Final concentration is approximately 0.4 mg/mL.)

2.5. Staining for Actin in Pre- and Postgelsolin-Treated Material

Alexa Fluor 488 phalloidin or Alexa Fluor 568 phalloidin (Invitrogen) made up in PEM/250 buffer according to the manufacturer's recommendations.

3. Methods

3.1. Preparation of the Gradient Columns

1. The following seven solutions of different sucrose concentrations are prepared before removing tissue from animals (*see Note 1*).
30% sucrose/PEM: 5.0 mL PEM/60 (stock) + 5.0 mL PEM (stock)
35% sucrose/PEM: 5.85 mL PEM/60 (stock) + 4.15 mL PEM (stock)
40% sucrose/PEM: 6.65 mL PEM/60 (stock) + 3.35 mL PEM (stock)
45% sucrose/PEM: 7.5 mL PEM/60 (stock) + 2.5 mL PEM (stock)
50% sucrose/PEM: 8.35 mL PEM/60 (stock) + 1.65 mL PEM (stock)
55% sucrose/PEM: 9.15 mL PEM/60 (stock) + 0.85 mL PEM (stock)
60% sucrose (PEM/60 stock)
Mix each solution well on a vortex mixer and keep solutions at 4°C until the step gradients are made.
2. To each of three Ultra-clear 5×41 mm centrifuge tubes (Beckman) add 93 mL of each of the sucrose solutions (30%, 35%, 40%, 45%, 50%, 55%, 60%) starting first with 60% (*see Note 2*). Use a new 1–200 μL gel-loading tip to add each solution. Place the pipet tip along the side of the tube and lower to a position just above the last solution added. As the solution is slowly added to the gradient,

gradually move the pipet tip upward so that it stays just above the rising level of the solution in the tube. A sharp interface initially is visible between steps of the gradient. Mark on the outside of the tube the position of each interface as the gradient is poured. If there is a delay between pouring the gradients and loading the columns, keep the columns at 4°C.

3.2. Isolation and Mechanical Fragmentation of Seminiferous Epithelia

1. Anesthetize a mature male Sprague–Dawley rat (at least 250 g).
2. When the animal is under deep anesthesia, open the right and left sides of the scrotum and expose the testes. Cut the mesentery holding the caudal part and body of the epididymis to each testis and then clamp the contents of the spermatic cord that connects each testis to the rat. Cut through the material distal to the hemostat between each testis and the head of the epididymis.
3. Kill the rat while the animal is under deep anesthesia.
4. Place the testes in a plastic Petri dish and decapsulate each organ by making a longitudinal cut with a scalpel through the capsule and then squeezing the seminiferous tubule mass through the large cut thereby separating the mass from the capsule.
5. Transfer the seminiferous tubule masses to a new plastic Petri dish containing ice-cold PEM/250. Using two scalpels in a scissors-like fashion, repeatedly cut through the masses freeing individual seminiferous tubules into solution (*see Note 3*). Using a transfer pipet, collect isolated tubules and place them into a new plastic Petri dish containing fresh ice-cold PEM/250.
6. Transfer the dish containing the isolated seminiferous tubules to the stage of a good dissecting microscope fitted with a dark-field condenser illuminated with fiber optics. Seminiferous epithelia are separated from tubule walls mechanically by using two microprobes, one with a straight pointed end and the other with a bent tip. Fix one end of the seminiferous tubule to the Petri dish with the tip of the straight probe and then place part of the other probe distal to the bend against the tubule near the first probe. Keeping the first probe fixed in position, move the bent probe away from the first while applying a gentle force to keep the tubule squeezed between this probe and the Petri dish (**Fig. 2**). The result is that the seminiferous epithelium is squeezed out the free end of the seminiferous tubule like “sausage meat” being squeezed out of its skin.
7. Collect the separated epithelium with a 1–200 μ L gel-loading pipet tip and transfer to a 15 mL conical clear plastic centrifuge tube on ice containing 1 mL PEM/250. Collect epithelium over a period of approximately 30 min.
8. Concentrate the epithelium by centrifuging on a tabletop clinical centrifuge and remove the supernatant. Add 100–150 μ L fresh ice-cold PEM/250.
9. Mechanically fragment the epithelium by gently aspirating the material seven times through a 1–200 μ L gel-loading pipet tip (*see Note 4*). This material contains dissociated spermatids with attached ectoplasmic specializations, together with all other components of the epithelium (*see Note 5*).

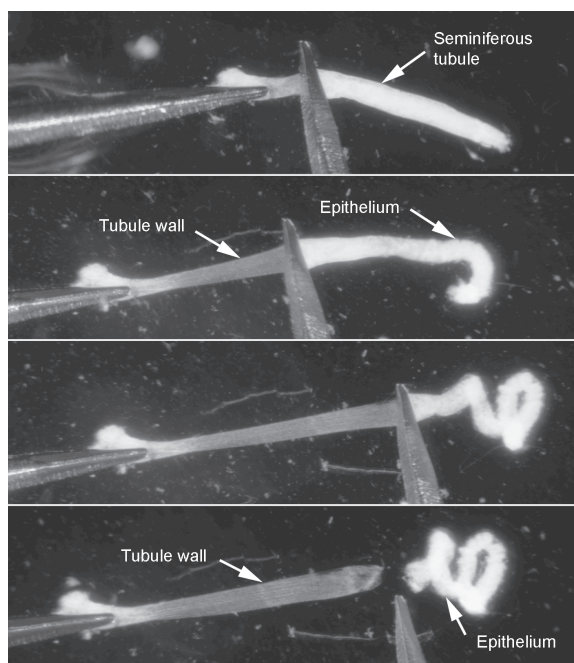


Fig. 2. Micrographs showing the method of mechanically stripping the epithelium out of the seminiferous tubules.

3.3. Centrifugation of Step Sucrose Gradients and Collection of Fractions Enriched for Spermatids with Attached Ectoplasmic Specializations

1. Pre-cool the rotor and the centrifuge chamber to 4°C.
2. Load equal amounts (approximately 50 μ L) of fragmented epithelia onto the tops of the three gradients. If there is more sample than room on the gradient, carefully remove some of the top layer of the gradient in each tube.
3. Put the three tubes into balanced positions in the rotor and spin at 2370 *g* for 6 min at 4°C.
4. Extract the upper of two large bands, usually at or just below the 40%/45% interface, using a 1 mL syringe fitted with a 22- to 23-gauge needle. Gently pass the needle through the wall of the tube and extract the layer (*see Note 6*).
5. Pool the layers from all three tubes into 1 ml PEM/250 in a 1.5 mL Eppendorf tube on ice. Gently mix the contents of the tube to dilute the sucrose.
6. Concentrate the sample, now enriched for spermatids with attached ectoplasmic specializations, by centrifuging the material for 2 min at setting 6 on a desktop Eppendorf centrifuge. Rotate the tube 180° and centrifuge again.
7. Place the pellet in the tube on ice until epithelia are isolated, fragmented, and similar fractions are obtained from each of the two additional animals.

3.4. Gelsolin Digestion of Ectoplasmic Specializations

1. Wash the pellets of fractions enriched for spermatids with attached ectoplasmic specializations by resuspending each of the three pellets (one from each of the three rats) in 100 μ L ice-cold MES buffer by gently “flicking” the side of the tube.
2. Pool the three fractions into one tube and add 700 μ L cold MES buffer. Allow this to sit on ice for 10 min (*see Note 7*).
3. Pellet the pooled material in a desktop Eppendorf centrifuge for 2 min at setting 6. Rotate the tube 180° and repeat the centrifugation. Discard the supernatant.
4. To the pellet, add 300 μ L MES buffer containing 0.4 mg/mL gelsolin previously warmed to room temperature (*see Note 8*).
5. Resuspend the pellet by gently “flicking” the base of the tube. Incubate this for 90 min at room temperature on a LabQuake rotator.
6. Following the incubation, gently aspirate the material through a 1–200 μ L gel-loading pipet tip. Collect a small amount of this material (20 μ L) to monitor actin filament loss from ectoplasmic specializations (*see Note 9*).
7. Centrifuge the material on a desktop Eppendorf centrifuge for 2 min at setting 10, rotate the tube 180°, and repeat the centrifugation. Keep the supernatant that is enriched for ectoplasmic specialization components.

3.5. Staining for Actin in Pre- and Postgelsolin-Treated Material

1. Load 10–20 μ L of sample containing the spermatids into acid-washed Perfusion chambers (Cytoskeleton, Inc.). Let sit for 10 min to allow cells to attach to the coverslip. Do this in a closed Petri dish containing a wet block of cotton to prevent drying.
2. Add 10 μ L fluorescent phallo toxin to one end of the chamber and draw into the chamber with a piece of filter paper placed at the opposite end of the chamber. Let the slide incubate at room temperature in the humidity chamber for 20–30 min.
3. Wash with buffer in the same fashion as the stain was applied.
4. Evaluate the actin staining using a fluorescence microscope fitted with the appropriate filter sets.

4. Notes

1. The protocol described here is for three reproductively active Sprague–Dawley rats (>250 g). Epithelia are separated from one animal at a time, fragmented, and loaded onto three-step sucrose gradients. The fractions enriched for spermatids with attached ectoplasmic specializations are collected, concentrated by centrifugation, and then kept on ice until similar fractions are collected from the additional two animals. The samples are pooled and then treated with gelsolin to free plaque components into solution. Spermatids are separated from the material by centrifugation.

2. Gradient columns should be prepared while epithelia are being isolated from seminiferous tubules or just before loading the columns with the epithelial fragments. If possible, one person prepares the gradients while another isolates and fragments the epithelium.
3. Another technique that is useful is to hold the mass of tubules with one scalpel and then pull tubules away from the mass with the other scalpel.
4. When mechanically fragmenting the epithelial isolates, gently aspirate through the pipette tip. Being too rigorous removes progressively more of the plaque.
5. Fractions isolated from sucrose gradients are enriched for elongate spermatids and are not pure samples. In fully mature animals, late-stage spermatids also are present in the sample. These cells carry with them actin-related tubulobulbar complexes in addition to ectoplasmic specializations; therefore, supernatants from gelsolin-digested samples from this material would contain material from tubulobulbar complexes as well as ectoplasmic specializations. These contaminants could be reduced by using testes from animals at an age before the first cohort of late spermatids is present in the epithelium, or by collecting epithelium only from those tubules containing elongate spermatids (32).
6. Be careful not to accidentally remove the needle from the tube before collecting the entire layer or the remaining material will leak out through the hole in the wall.
7. Collect a small aliquot of this material (20 μ L) to use for actin filament staining with fluorescent phalloxin to verify the presence of ectoplasmic specializations attached to spermatids.
8. The actin-severing property of exogenously added gelsolin is used to disassemble the actin layer of ectoplasmic specializations in epithelial fractions enriched for spermatids with attached junction plaques, thereby releasing the actin and endoplasmic reticulum components of the ectoplasmic specialization into solution. Other material is pelleted by centrifugation and removed.
9. The status of junction plaques after gelsolin treatment can be monitored by staining samples with fluorescent phalloxins. This sample can be compared with a similar sample collected from spermatids before incubation with gelsolin to verify that the ectoplasmic specialization has been disassembled.

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Single-Molecule Observation of Rotation of F_1 -ATPase Through Microbeads

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Summary

F_0F_1 -ATP synthase catalyzes the synthesis of ATP using proton-motive force across a membrane. When isolated, the F_1 sector, composed of five polypeptide chains with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$, solely hydrolyzes ATP into ADP and phosphate, and is thus called F_1 -ATPase. Rotation of a shaft domain in F_0F_1 -ATP synthase has been hypothesized by Paul Boyer, and ultimately was confirmed by direct observation as rotation of the γ -subunit in an isolated $\alpha_3\beta_3\gamma$ subcomplex. Unitary turnover of ATP induces 120° steps, consistent with the configuration of three catalytic sites arranged 120° apart around γ . We have shown the relationships between chemical and mechanical events by imaging individual F_1 molecules under an optical microscope. A new scheme emerges: as soon as a catalytic site binds ATP, the γ -subunit always turns the same face (interaction surface) to the β hosting that site; $\sim 80^\circ$ rotation is driven by ATP binding; $\sim 40^\circ$ rotation is induced by completion of hydrolysis [and/or phosphate release] in the site that bound ATP one step earlier.

Key Words: F_1 -ATPase; motor protein; single molecule biophysics; rotary molecular motor; streptavidin bead.

1. Introduction

Motor proteins convert the chemical energy of ATP hydrolysis to mechanical energy and thus induce large structural changes to move. With recent developments in optical microscopy, how motor proteins work has been elucidated at the single molecular level to establish the precise correspondence between chemical reactions and mechanical movement. For the mechanical part, unitary steps, which are directly related to the mechanism, were identified in the case of kinesin (**1**), myosin V (**2**), and myosin IV (**3**) as displacements of microbeads bound to motors. F_1 -ATPase has also been studied as one of

the most important subjects in single molecule biophysics; rotation has ultimately been confirmed by direct observation (4), and the unitary 120° step was identified (5) through fluorescently labeled actin filament attached to the γ -shaft.

Recently, Yasuda et al. have applied gold colloidal beads to the F₁ rotation assay and ultimately resolved substeps (6), perhaps for the first time in studies of motor proteins, indicating the appearance of metastable structure(s) during unitary steps. We also visualized rotation by attaching a polystyrene bead duplex to the γ -shaft, while watching simultaneously which of the three sites bound and released a fluorescent ATP analog (7). Bead rotation assays are quite useful not only for linear molecular motors but also for rotary molecular motors because the accuracy of determining displacement increases and its applications are thus broad. Through a 0.2- μ m bead duplex with dark-field microscopy, a precision of $\sim 0.3^\circ$ with 0.25-ms time resolution is achieved (Shibano et al., manuscript in preparation).

In this chapter, we describe two types of rotation assay with a polystyrene bead duplex: one is streptavidin coated and the other is bovine serum albumin (BSA) coated. In general, in rotation assays by our group the streptavidin-bead assay is common, whereas the BSA-bead assay has never been described in our previous papers, because we have used the BSA-bead assay only for a broad check of the activity of samples; high-quality data are acquired with the streptavidin bead after biotinylation of the γ -shaft. Although principal properties of rotation observed by the two methods appear to be identical, the probability to find molecules showing clear-stepping is different. In spite of such a disadvantage, the BSA-bead assay is still useful as chemical modification of the protein is unnecessary in this assay system. Additionally, the basic outline of optical setup, video capturing, and analyses of bead tracking are described.

2. Materials

2.1. Purification of $\alpha_3\beta_3\gamma$ Subcomplex

1. *Escherichia coli* cells in which the α -, β -, and γ -subunits of F₁-ATPase from thermophilic *Bacillus* strain PS3 are expressed simultaneously (8). We mostly use α -C193S, γ -S109C, γ -I212C, and β -His₁₀ at the amino terminus (6,7), which we refer to hereafter as “wild-type” F₁. The GT mutant (7), which has β -G181A (9), β -T165S (10), α -W463F, and β -Y341W (11–13), is also used.
2. Ni-NTA resin matrices (Ni-NTA Superflow; Qiagen, Valencia, CA).
3. Empty disposable column (PD-10; GE Healthcare Bio-Sciences, Piscataway, NJ).

4. Prepacked column for desalting (PD-10 columns containing Sephadex G-25; GE Healthcare).
5. Prepacked column for gel filtration (Supdex 200 HR 10/30; GE Healthcare).
6. Imidazole buffers with high ionic strength and phosphate (IHB): 100 mM potassium chloride, 100 mM potassium phosphate, and desired amount (50 mM, 100 mM, 300 mM, and 500 mM) of imidazole-HCl, pH 7.0.
7. Buffer with phosphate and ethyleneglycoltetraacetic acid (EDTA) (PEB): 2 mM EDTA and 100 mM potassium phosphate, pH 7.0.

2.2. Streptavidin-Bead Rotation Assay

1. Polystyrene microspheres with amino-modified surface (0.2 μ m in diameter; Polysciences, Warrington, PA). Typically, 250 μ L 2.8% stock is coupled to proteins; this volume could be scaled to smaller volumes if necessary.
2. Biotin-(AC₅)₂-Sulfo-Osu (Dojindo, Kumamoto, Japan); water-soluble biotinylation reagent for amines with longest spacer, 30.5 Å.
3. Biotin-PEAC₅-maleimide (Dojindo); reagent for sulfhydryl groups.
4. Adenosine triphosphate (ATP; Roche-Diagnostics, Basel, Switzerland).
5. Creatine phosphate (Sigma-Aldrich, St. Louis, MO).
6. Creatine kinase (Sigma-Aldrich).
7. Potassium oxide (Fluka and Riedel-de Haën, Seelze, Germany) for cleaning glass coverslip. Grade “pro analysis” is chosen.
8. Glass coverslip: 24 × 32 and 24 × 24 mm with 0.12–0.17 mm thickness (Matsunami, Tokyo, Japan).
9. 3-Glycidyloxypropyl-trimethoxysilane (HMDS; Fluka) to coat top side of a flow chamber.
10. Water-resistant paper as a spacer. We prefer to use a coated paper of Parafilm.
11. White petrolatum as a glue to fix the spacer between glasses.
12. Washing buffer with pH 8.5 HEPES-KOH (HWB1): 10 mM HEPES-KOH, pH 8.5 (see **Note 1**).
13. Washing buffer with pH 8.0 HEPES-KOH (HWB2): 10 mM HEPES-KOH, pH 8.0.
14. Washing buffer with phosphate (PWB): 20 mM potassium phosphate, pH 8.0.
15. Rotation buffer with phosphate (PRB): 20 mM potassium phosphate, pH 8.0, 2 mM MgCl₂, and desired amount of ATP.
16. Rotation buffer with HEPES-KOH (HRB): 10 mM HEPES-KOH, pH 8.0, 2 mM MgCl₂, and desired amount of ATP.
17. Buffer with (*N*-2-morpholino)propane sulfonic acid (MOPS) and magnesium (MB): 10 mM MOPS, pH 7.0, 50 mM KCl, 2 mM MgCl₂.

2.3. BSA-Bead Rotation Assay

1. Bovine serum albumin (BSA; Sigma-Aldrich).
2. Polystyrene microspheres with amino-modified surface (0.2 μ m in diameter; Polysciences).
3. MB buffer with 10 mg/mL BSA (MB/BSA).

2.4. Microscope and Video Imaging

1. Olympus IX-71 microscope (Olympus, Tokyo, Japan) equipped with a dark-field condenser with high numerical aperture (NA), 1.4–1.2.
2. 60× Olympus objective with 1.45 NA.
3. Optical table (RS-2000; Newport, Irvine, CA) with M6 taps 25 mm apart. A microscope was fixed on the table.
4. Customized sample-stage with XY translator. In our case, 240 × 210 × 17.5 mm board made of stainless steel is fixed on the microscope instead of an accessory stage. Above the ~100-mm hole at the center of the board, a commercial XY translator (Sigma-Koki, Hidaka, Japan), made of steel and equipped with micrometers, is tightly fixed with an M5 screw (*see Note 2*).
5. Charge-coupled device (CCD) camera based on NTSC video signal (CCD-300RC; Dage-MTI, Michigan, IN). Video output should be selected not to AC coupling output but to DC coupling, to ensure linearity of signal and thus precision of image analyses.
6. PC and video capture board. Real-time capturing, 29.98 frames per second, is required for NTSC. Two types are applicable (*see Note 3*); HD recording (Meteor-II; Matrox Electronic Systems, Dorval, Canada) equipped with video recording software (StreamPix; NorPix, Quebec, Canada); or RAM storage (LG-3; Scion, Frederick, MD).

2.5. Analyses

1. Image processing program. ImageJ, developed at NIH, is generally recommended as it runs on many types of PCs and is perhaps fastest. It opens the TIFF file that contains multiple images (stack).
2. Data analysis software. If you are not familiar with programming language but need automated data processing, Igor Pro (WaveMetrics, Lake Oswego, OR) is strongly recommended.

3. Methods

3.1. Purification of $\alpha_3\beta_3\gamma$ Subcomplex

1. Thaw and triturate ~10 g stock of *Escherichia coli* expressing F_1 -ATPase (kept at -80°C) with 50 mM IHB.
2. Sonicate cells using a probe sonicator for 15 min. Keep the tube containing cells immersed in an ice bath during sonication.
3. Sediment cell lysate at 20,000 *g* for 30 min at 4°C .
4. Apply supernatant to a Ni-NTA column prepared in an empty PD-10, which has been washed with 50 mL MQ and 30 mL 500 mM IHB and equilibrated with 50 mL 50 mM IHB.
5. Wash the column with 50 mL 50 mM IHB and 100 mL 100 mM IHB.
6. Elute F_1 with 300 mM IHB. Collect 1.5-mL fractions.
7. Determine protein peak by measuring absorbance using spectrophotometer. Either two or three fraction tubes around the peak are pooled and applied to PD-10 column containing G-25, which has been equilibrated with PEB.

8. Elute F_1 with PEB and collect 1-mL fractions.
9. If removal of endogenously bound nucleotides is required, apply sample to butyl-Toyopearl column (Tosoh, Tokyo, Japan) instead of G-25 (14,15).
10. Apply protein peak to the Superdex column set in high-pressure chromatograph with the column equilibrated with PEB. Elute F_1 with PEB and check samples around peak by SDS-PAGE.

3.2. Streptavidin-Bead Rotation Assay

3.2.1. Preparation and Storage of F_1

1. Couple biotin (biotin-PEAC₅-maleimide; Dojindo) to two cysteines in the γ -subunit of F_1 by 30 min incubation at room temperature at 1–8 μ M F_1 . The molar ratio of F_1 : biotin is set to be the number of introduced cysteines in γ : 1 : 2 for the mutant with both γ -S109C and γ -I212C.
2. Arrange sample tubes resistant to -200°C in a plastic holder fixed in Styrofoam bucket. Pour liquid N_2 into the Styrofoam so that the bottoms of the tubes are completely immersed in liquid N_2 .
3. Drop 15 μ L solution containing F_1 into the tubes using a pipet so that it freezes instantly. If you need more protein for a day's experiments, drop additional 15- μ L aliquots of protein solution per tube.
4. Store tubes containing protein at -80°C . Make sure to use gloves resistant to liquid N_2 to transfer tubes. Wild-type F_1 is stable for several years; however, mutations may interfere with the stability of an assembly of subunits when stored below 0°C .

3.2.2. Streptavidin-Bead Preparation

1. Clean beads by centrifugation to eliminate the surfactant and NaN_3 in the aqueous solution (see **Note 4**). Dilute amino-modified beads to a final concentration of 1.4% with 500 μ L HWB1 and centrifuge. Remove and discard supernatant and triturate the pellet with HWB1. Repeat these washes six times.
2. After the last step, resuspend the pellet in HWB1 to a final concentration of 2.8%. Add 0.5 mg biotin-(AC₅)₂-Sulfo-Osu, and incubate the mixture for 4 h at room temperature with continuous mixing with an end-to-end mixer.
3. Clean biotin-modified beads by centrifugation with 500 μ L HWB1 three times, then 500 μ L HWB2 three times.
4. Add 0.1 mg or more streptavidin (see **Note 5**). The final concentration of the beads here is 1.4% = 5.7 nM (see **Note 6**).
5. Before use, clean 20 μ L streptavidin-bead stock with 200 μ L PWB by centrifugation three times (see **Note 7**). The concentration of the beads is 0.14% = $\sim$ 0.1%.

3.2.3. Experimental Chamber

1. Prepare two beakers of saturated KOH: one is for the first treatment, and the other for overnight treatment (see **Note 8**). Place glass coverslips setting on a stand into the first beaker and soak for 30 min (see **Note 9**). Wash the coverslips with distilled water, and then store in the second beaker overnight.

2. Wash the coverslips by soaking in distilled water in a clean beaker. Replace the water seven times or more. Keep coverslips in water until just before use, then dry on a clean bench.
3. Coat top coverslip (24×24 mm) with HMDS to avoid binding of the protein.
4. Use thin waterproof paper as spacers between a top coverslip and a bottom coverslip. We prefer to use a guard paper of laboratory film (Parafilm, Menasha, WI). The paper is cut about 2 mm wide and 20 mm in length.
5. Sandwich the cut paper between parafilms filled with white petrolatum. Carefully pull out the parafilm to extend the petrolatum as thin as possible.
6. Place two papers onto a dried coverslip (24×36 mm) about 10 mm apart, and a coverslip (24×24 mm) coated with HMDS is placed onto it. Press the top coverslip down with forceps. The resulting flow chamber has an internal volume of 5–10 μ L.

3.2.4. Rotation Assay

1. Thaw the stock of F_1 quickly. F_1 originated in thermophile is kept at room temperature (*see Note 10*).
2. Infuse $\sim 5 \mu$ L F_1 into the flow chamber using a pipettor. Typical concentration of F_1 applied is 50 pM that is diluted into MB just before infusion (*see Note 11*). If the solution does not fill in the chamber completely, use additional F_1 solution.
3. After 2 min, infuse $\sim 20 \mu$ L (~ 3 – 4 times internal volume of the chamber) of PWB to wash unbound F_1 . Introduce solution slowly from one side until fluid appears at the other side. Apply filter paper to the fluid to remove the fluid drop that forms on the side. Do not allow a suction to form in the chamber.
4. Infuse $\sim 10 \mu$ L streptavidin beads into the chamber. The chamber should show uniform white turbidity with the beads.
5. After 10 min, infuse $\sim 10 \mu$ L PWB into the chamber to wash the beads of unbound F_1 .
6. Infuse $\sim 20 \mu$ L of either PRB or HRB. Seal both sides using a mound of white petrolatum.
7. Set the flow chamber on a microscope objective and observe (*see Note 12*). Temperature of room is between 20° and 24°C (*see Note 13*).

3.3. BSA-Bead Rotation Assay

3.3.1 BSA-Bead Preparation

1. Clean 30 μ L amino-modified beads by centrifugation with MB three times.
2. Mix washed beads with an equal amount of 10 mg/mL BSA in MB (*see Note 14*).
3. Centrifuge mixture to remove excess BSA. The pellet is resuspended with 120 μ L MB.

3.3.2. Rotation Assay

1. Prepare flow chamber as for streptavidin-bead rotation assay as in **Subsection 3.2.3**.

2. Infuse $\sim 5\mu\text{L}$ F_1 -ATPase diluted in MB into the flow chamber.
3. After 2 min, infuse $\sim 20\mu\text{L}$ MB/BSA to cover the part of glass that is sticky to proteins.
4. Infuse $\sim 10\mu\text{L}$ BSA beads into chamber.
5. After 10 min, add $\sim 20\mu\text{L}$ MB/BSA with desired amount of ATP.

3.5. Microscope and Video Imaging

1. Center and collimate the halogen lamp. The position of the condenser lens is also collimated to realize Köhler illumination by viewing 1.0- to $0.5\mu\text{m}$ beads or a grid using the eyepiece.
2. Set $10\mu\text{m}$ -scale grid onto the objective and record to determine the magnification of the microscope and camera.
3. Place the flow chamber with $0.2\mu\text{m}$ beads attached to F_1 onto the objective, and focus on the beads using a PC monitor.
4. Adjust gain and black level of the CCD.
5. Collect images with CCD camera that is directly connected to the video capturing board. Images are digitized as 8-bit format and stored in the PC without compression (see **Note 15**).
6. After data acquisition, convert the video sequence to the format of multipaged TIFF as a single file, which is called a stack.
7. Check the minimum and maximum pixels values in a stack; the value should be between 0 (or 1) and 255 in 8-bit format. If some pixels show either less than 2 or more than 254, either the level of the CCD or the light intensity is adjusted. Saturated pixels may interfere with the precision of measurements.

3.5. Analyses

1. Open a stack file using the image analysis software. Because $0.2\mu\text{m}$ beads appear as a black particle, as shown in **Fig. 1A**, invert image contrast before analysis.
2. Determine the position of the bead (**Fig. 1B,C**, inset). Either two-dimensional (2D) Gaussian fitting or calculation of the centroid is recommended (see **Note 16**). Igor Pro software has an algorithm for 2D Gaussian fitting from the first and thus is quite useful (see **Note 17**). Various types of “plug-ins” are released at the ImageJ Website that might also be applicable. To calculate the centroid, the appropriate threshold should be set before calculation to avoid underestimation of bead displacement.
3. The angle of γ is calculated by data analysis software (**Fig. 1B,C**). The x and y positions from certain points are simply converted into the angle θ by the calculation of $\theta = \arctan(y/x)$ in each frame.
4. To convert the angle into revolution number, the angle is accumulated every time γ exceeds 0° . Note that the value increases when γ rotates counterclockwise.

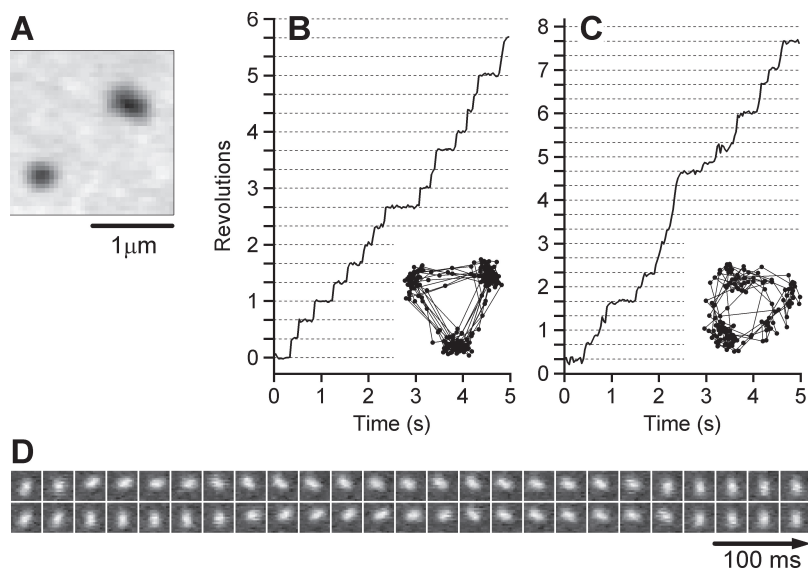


Fig. 1. (A) Micrograph of a single 0.2- μm bead (left bottom) and dual beads (right top) as bright-field image. Dual beads are bound to F_1 immobilized onto the glass surface. (B) An example of rotation and XY trace (inset) of dual 0.2- μm beads in streptavidin-bead rotation assay. Revolutions are calculated from trace, and data are not filtered. F_1 , GT-mutant; [ATP] = 2.1 μM ; buffer, PRB. (C) An example in bovine serum albumin (BSA)-bead assay. Note that the stepping is dimmer as compared with streptavidin-bead although it is one of the best measurements in our observation. [ATP] = 1.0 μM . (D) Sequential micrographs of bead stepping of the dual beads. Intensity profile is digitally inverted to calculate the position of the beads. This molecule is the same in the micrograph (in A) and analyses (B).

4. Notes

1. pH adjustment of HEPES is done between 10 and 100 mM. Dilution of HEPES from higher concentrations changes pH, especially around 8.5.
2. To avoid creeping drift of the sample, the micrometer should not push the edge but instead on the center of the layers of XY translator. The edge type saves space around the stage but is not recommended.
3. In the case of general fast video-capturing, contiguous video images are temporarily stored into RAM in PC, and transferred to a hard disk (HD) when saved. The upper limit for one recording sequence thus depends on the size of RAM; if you have 1 GB, limit is ~ 18 min without image compression for a black-and-white image with 640×480 pixels. In contrast, HD recording systems store images directly into HD; therefore, the limit depends on the size of HD, which is commonly ~ 100 times larger than RAM.

4. Unless stated, all procedures of bead preparation are done on ice. Microtubes are centrifuged at 10,000 g for 20 min at 4°C.
5. Streptavidin powder is dissolved just before use. Repeated cycles of freezing and thawing of the stock of small aliquots is not recommended.
6. Streptavidin-coated beads are stable at 4°C for at least a month. Excess streptavidin in the stock is washed by centrifugation just before use; the washed stock gives us repeatable results for only a few days.
7. For washing streptavidin beads, pH is the important factor. If the pH of a solution is less than ~8, the bead pellet becomes firm and is difficult to dissolve without BSA. However, if BSA is added to the streptavidin beads, the beads may work as BSA beads; careful experimental design is needed.
8. Saturated KOH solution can be used for a month. If the solution in the beaker becomes slightly turbid, the solution is discarded or used only for first treatment.
9. To transport glass coverslips from a beaker to another beaker, titanium forceps resistant to alkali are used to grasp a coverslip stand.
10. The stability of F_1 depends on mutation. The wild-type is stored at room temperature for a few days, and GT, generally on ice within a day.
11. If beads showing rotation are low in number, the concentration of F_1 applied into the flow chamber should be increased, possibly up to 1 μM . Frequency of rotated beads depends on a stability after mutation or labeling probability with biotin in γ , and also the cleanliness of the glass surface.
12. The bright-field image of beads 0.2 μm in diameter is difficult to see through the eyepiece. In most CCD cameras, contrast of bead images should be enhanced by reducing the black level and increasing halogen illumination.
13. Control of temperature is important to realize nanometer measurement. In general, we turn off the air conditioner before measurement and incubate at least 24 h to equilibrate the temperature of the whole room and all apparatus.
14. In the BSA-bead method, BSA is assumed to adsorb onto polystyrene readily and permanently (16).
15. A potential problem for using video capturing is that dropping of frames may happen in the case when either other software is running on the same PC, or PC is accessed by other PCs through a network. In HD recording, fragmentation of files in HD, which can occur by repetition of overwriting and erasure of data, may also cause frame dropping. Unless you stamp a time code in each frame, dropping is not detectable because the resulting sequence is apparently contiguous even though it was constructed from segmentalized images.
16. For the single bead in Fig. 1, accuracy estimated by standard deviation in 100 points was 5.3 nm for both X and Y in the case of 2D Gaussian fitting. In contrast, accuracy was slightly lower in the case of centroid calculation; 6.1 nm standard deviation for X and 6.3 nm standard deviation for Y.
17. If you work with Igor Pro software for data analysis, loading a stack and 2D Gaussian fitting can be done with the following command lines. In these command lines, "image" and "TempImage" are arbitrary names of an image sequence and

a temporal single image, respectively. Italic signature represents variable. “*n*” indicates the order of frame when (*n* + 1)th frame in a stack is destination for analysis. Ordinate (*x*₀, *y*₀) and (*x*_{*i*}, *y*_{*i*}) indicate left top and right bottom, respectively, of region of interest (ROI) for analysis. To complete calculation of all frames, repeat third and fourth lines with flow control by Macro programmed in “Procedure Window” in Igor.

ImageLoad/T = tiff/O/C = -1 /N = image

Make/O/I/U/N = (DimSize(image,0),DimSize(image,1)) TempImage

TempImage[][] = image[p][q][*n*]

CurveFit Gauss2D TempImage [*x*₀, *x*₁] [*y*₀, *y*₁]

18. In **refs. 6 and 7**, the description regarding mutations in the γ -subunit contains erratum; γ -S107C and γ -I210C. Correction is “ γ -S109C and γ -I212C.” Similarly, “ γ -S109C” is correct instead of “ γ -S107C” in **refs. 4, 5, 14, and 15**.

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The Use of FRET in the Analysis of Motor Protein Structure

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Summary

Fluorescence resonance energy transfer (FRET) is a spectroscopic phenomenon that consists of long-range dipole–dipole interaction between two chromophores. This method can be employed to gain quantitative distance information on macromolecules. FRET is particularly useful to characterize structural states of motor proteins, because the spatial relationship between various mechanical elements of the motor undergoing its mechanical cycle is essential to understand how force and movement are generated. In this chapter, we describe the technique, including the equations, methods of introducing fluorescence probes in specific loci of the protein, and data analysis. Practical guidelines and hints are also provided for protein preparation, labeling, and measuring FRET efficiency. The protocol is presented for interhead distance measurements in the dimeric kinesin-like motor, Ncd. However, it can easily be adapted to many other motor proteins.

Key Words: Fluorescence resonance energy transfer (FRET); motor proteins; the Förster equation; myosin; kinesin; fluorescence probes.

1. Introduction

Molecular motors use energy derived from ATP hydrolysis to move unidirectionally along cytoskeletal “tracks”; myosins use actin filaments, kinesins, and dyneins–microtubules. Although the mechanisms by which various motors generate force may be dissimilar, one feature is common for all motors: these are conformational changes in the motor domain. Even if we assume that the force-producing mechanism utilizes the thermal motion of the motor or the environment (the Brownian ratchet model), conformational changes induced by the chemical reaction at the active site are required to rectify the movement to restrict it to only one direction.

Fluorescence resonance energy transfer (FRET) has frequently been used to obtain information about distances between selected residues of a protein, therefore to monitor conformational changes occurring in the motor molecule and to provide multiple constraints on the motor structure. FRET distances can be determined under physiologically relevant conditions, including the aqueous environment and neutral pH. These measurements can be performed, in most cases, in the presence of the polymer tracks: actin or microtubules. A substantial part of our understanding of the mechanism by which motor proteins function comes from FRET-based studies. FRET, among other techniques, has been used to verify the rotation of the lever arm during the power stroke exercised by myosin (1–4). Using this technique, Vale and co-workers (5) were able to determine the mobility of the kinesin neck linker in the presence of nucleotides; this has led to a widely accepted molecular model of kinesin walking (5).

In the classic approach, motor protein is covalently labeled with a pair of chromophores named “donor” and “acceptor” that possess special spectral characteristics (Fig. 1). When the donor molecule is excited, there occurs partial transfer of the excitation energy to the acceptor via a nonradiative dipole–dipole coupling between the chromophores. The distance, typically 20–80 Å, is obtained from steady-state or time-resolved fluorescence measurements of the extent of donor quenching resulting from the presence of the acceptor. Here, we describe the protocol for this kind of FRET measurement. However, it is noteworthy to mention three relatively new methodologies in this field: single pair (molecule) FRET (spFRET), luminescence resonance energy transfer (LRET), and transient FRET measurements.

Laser and detector technologies enable visualization of a single chromophore; therefore, it is possible to measure FRET, and distances on single macromolecules. spFRET has several important advantages over the classic approach, especially when applied to molecular motors. Using spFRET, there is no need to synchronize the entire population of motors to study changes in their conformations as the motor is undergoing its mechanochemical cycle. In the conventional approach, intermediate states are simulated using nucleotide analogs. Derivatives of vanadate, beryllium fluoride, and aluminum fluoride in complexes with ADP have often been used with myosin and kinesin. However, it is not completely clear that each of the analogs generates a structural state of the motor that mimics an intermediate state of the enzyme reaction. For example, ADP-BeF₃ can produce both “open” and “closed” states in myosin (6). Another advantage of spFRET is the ability to detect low concentration of intermediates that behave differently from the rest. Finally, when the amount of biological material is limiting, with

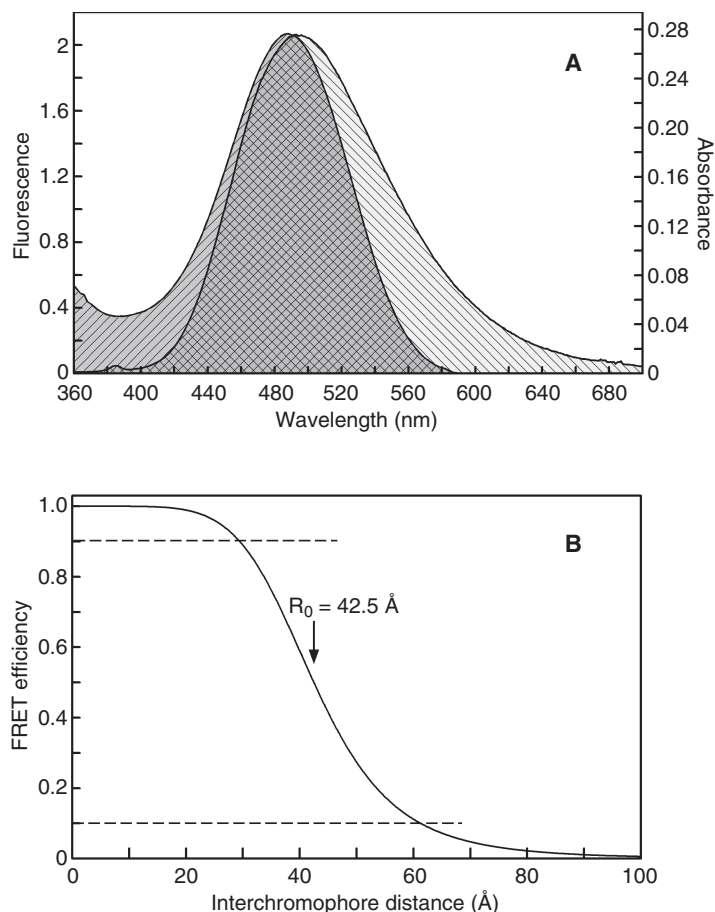


Fig. 1. (A) Spectral overlap of Ncd-C253S-labeled 1,5-IAEDANS (light gray) and QSY-35 (medium gray). The cross-hatched area is proportional to the overlap integral in the Förster equation. In this case, $J = 8.53 \times 10^{14} \text{ nm}^4 \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$. (B) It is generally assumed that measured fluorescence resonance energy transfer (FRET) efficiency should be higher than 0.1 (10%) and lower than 0.9 (90%), which limits the range of distances that can be determined by FRET; with R_0 equal to 42.5 Å, this range is 30–60 Å.

spFRET distance information can be obtained with minute quantities of the protein.

The other variant of FRET, LRET, has been developed by P.R. Selvin (7) and T. Heyduk (8,9). LRET uses Tb^{3+} or Eu^{3+} cations as long-lifetime (~1 ms) luminescence probes attached to a reactive chelate that can be employed to modify thiol groups of proteins. The advantage of this method lies

in overcoming or reducing the uncertainty in the orientation factor, κ^2 , in the Förster equation (see **Subsection 1.1.**, below), a considerable increase of the maximum measurable distance to about 100–110 Å, and a large improvement of the signal-to-background ratio. Recently, this technique has been employed for molecular motors (8,10).

Transient FRET measurements are used to monitor the dynamics of the movement of one structural element of the motor molecule relative to the other or to the microtubule or actin. Many of the structural transitions in motor proteins are slow enough to be quantified by commercially available stopped-flow instruments or even regular fluorometers (11,12). These kinds of measurements provide rate constants of the FRET-associated processes rather than a distance between chromophores. Generally speaking, FRET is better suited to detect changes in distances rather than absolute values of the distance. However, reconstructions of protein complexes and actin filament have also been successful (13,14).

Despite the remarkable similarity of the motor domains of conventional kinesin and the minus-end-directed kinesin-like protein, Ncd (*non-claret disjunctional*), the mechanisms by which they generate movement may be entirely different. For kinesin, docking of the flexible neck-linker against the head appears to be the force-generating event. In contrast, there is limited evidence that Ncd generates movement by pivoting the stalk segment and one of the heads about a residue in the neck-head junction of the other head. In this chapter, we describe FRET measurements in dimeric Ncd that can provide experimental evidence for the existence of such a power stroke.

1.1. The Equations

When attached, a macromolecule donor and acceptor can exchange their excitation energy by the dipole–dipole mechanism, the efficiency (E) of which is given by Eq. 1:

$$(1) \quad E = \frac{R_0^6}{R_0^6 + r_i^6}$$

where R_0 denotes the critical Förster distance and r_i is the interchromophore distance. The critical distance is given by Eq. 2:

$$(2) \quad R_0^6 = 8.79 \times 10^5 \cdot \kappa^2 \cdot n^{-4} \cdot \varphi_D \cdot J$$

n is the refractive index of the medium between the donor and the acceptor; φ_D is the quantum yield of fluorescence of the donor in the absence of the acceptor; and κ^2 is the orientation factor for dipole–dipole interaction. The fluorescence

spectrum of the donor must overlap the absorption spectrum of the acceptor. J , the overlap integral, is given by:

$$(3) \quad J = \frac{\int_{\lambda}^{\infty} F_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 \cdot d\lambda}{\int_{\lambda}^{\infty} F_D(\lambda) \cdot d\lambda}$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor at wavelength λ , and $\varepsilon_A(\lambda)$ denotes the extinction coefficient of the acceptor at wavelength λ . The efficiency of FRET can be obtained by measuring the quantum yield of the donor in the presence (φ_{DA}) and in the absence of the acceptor (φ_D):

$$(4) \quad E = 1 - \frac{\varphi_{DA}}{\varphi_D}$$

The same information can be obtained from time-resolved fluorescence measurements:

$$(5) \quad E = 1 - \frac{\tau_{DA}}{\tau_D}$$

where τ_{DA} and τ_D refer to the lifetime of the donor in the presence and in the absence of the acceptor, respectively. Knowing E , the distance r_i is calculated from Eq. 6:

$$(6) \quad r_i = R_0 \cdot \left(\frac{1-E}{E} \right)^{1/6}$$

The uncertainty of the measured distance can be computed using standard formulae for error propagation (15):

$$(7) \quad \sigma_{r_i} = \left[\sigma_{R_0}^2 \left(\frac{r_i}{R_0} \right)^2 + \sigma_E^2 \left(\frac{-R_0^6}{6r_i^5 E^2} \right)^2 \right]^{1/2}$$

where σ_{r_i} , σ_{R_0} , σ_E denote standard deviations of the measured distance, R_0 , and E , respectively. σ_{R_0} is obtained from Eq. 8:

$$(8) \quad \sigma_{R_0} = \frac{R_0}{6} \left[\left(\frac{\sigma_{\varphi_D}}{\varphi_D} \right)^2 + \left(\frac{\sigma_{\kappa^2}}{\kappa^2} \right)^2 \right]^{1/2}$$

where σ_{φ_D} and σ_{κ^2} mean standard deviations for the quantum yield and orientation factor, respectively.

The orientation factor κ^2 in Eq. 2 cannot be measured directly. Theoretically, its value, which depends on the relative orientation of the donor and acceptor, can change from 0 to 4. It is usually assumed that $\kappa^2 = \frac{2}{3}$. Using fluorescence anisotropy measurements, one can set lower and upper limits on the value of κ^2 , thus reducing the uncertainty in R_0 (16).

2. Materials

2.1. Ncd-250–700

1. Ncd constructs can be expressed in the *Escherichia coli* strain BL21 (DE3). A single colony with Ncd expression plasmid is grown overnight at 37°C in Luria-Bertani (LB) medium with 30 µg/mL kanamycin and is used to inoculate 2.5 L of the same medium. A large-scale culture is shaken at 250 rpm to OD₆₀₀ of 0.8–1.0.
2. The expression of Ncd is initiated by the addition of 1 mM isopropyl β-D-thiogalactoside with parallel reduction of the temperature to 25°C. After 3 h, the cells are harvested by centrifugation at 3,000 g for 10 min and resuspended in buffer PB [10 mM HEPES, pH 7.2, 80 mM NaCl, 1 mM MgCl₂, 1 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM DL-dithiothreitol]. Next, the cells are centrifuged at 6,000 g for 20 min, the supernatant is discarded, and the pellet is frozen.
3. Frozen bacterial cells are thawed, resuspended in buffer PB, and disrupted in a French pressure cell (Thermo-Spectronic) in the presence of 1 mM phenylmethanesulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 2 µg/mL aprotinin. Insoluble material is removed by centrifugation (Sorval SS-34 rotor; 18,000 rpm, 20 min).
4. The supernatant is transferred onto an SP-Sepharose (Amersham-Pharmacia) column equilibrated with buffer PB. Ncd is eluted with a linear gradient of 0.1–0.3 M NaCl in PB and frozen in the presence of 10% sucrose. Only peak fractions are collected. Occasionally, a second column (Q-Sepharose, Amersham-Pharmacia) is used, although, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), at high overexpression of the protein, an additional chromatography step does not improve the purity of the preparation (see Note 1). The collected fractions are frozen and kept at –70°C.

2.2. Paclitaxel-Stabilized Microtubules

Porcine brain tubulin is purified through two cycles of polymerization–depolymerization, followed by a microtubule-associated protein (MAP)-depleting step (17); it is then kept at –70°C. Tubulin is polymerized and guanine nucleotides removed according to the following procedure:

1. On the day of the experiment, tubulin is thawed and centrifuged (20,000 g, 4°C, 20 min). The supernatant is supplemented with an equal volume of 1 M PIPES, pH 6.9, <1/5> volume of dimethylsulfoxide, 1 mM MgCl₂, 1 mM GTP.
2. After 20 min incubation at 37°C, 10 μM paclitaxel (Taxol; *see Note 2*) is added. Assembled microtubules are centrifuged (226,000 g, 37°C, 30 min) in a prewarmed tube in a buffer containing 80 mM PIPES, pH 6.8, 1 mM MgCl₂, 10 μM paclitaxel, and 60% glycerol (*see Note 3*); the pellet is resuspended in the labeling buffer (described below) containing 10 μM paclitaxel (*see Notes 4 and 5*).

2.3. Spectrophotometer and Scanning Fluorescence Spectrometer

Use a spectrophotometer (SPEX, PTI, SLM, ISS) equipped with a circulating water bath. Set quartz 0.4 × 1 cm cuvettes.

3. Methods

3.1. Introducing a Donor–Acceptor Pair

It is likely that this is the most difficult part of the FRET experiment. Most often thiol groups are used to attach dyes to proteins, although the number of commercially available reagents to modify other groups, such as Lys or His, is really impressive. Frequently, however, the residue that must be conjugated is not reactive, and, in addition, the protein contains reactive Cys residues in other locations. In such cases, site-specific mutagenesis must be used to replace native thiols with Ser or Ala residues and/or add a Cys residue in the desired position. Few proteins contain a single reactive thiol residue; therefore, the replacement of the thiol groups is sometimes quite extensive, leading to Cys-light proteins ([1,5,10,11](#)). One should also consider changes in the motor structure and its motile function caused by the mutations. However, even a protein with a single reactive cysteine may not entirely circumvent the problem: if the second site, for example, for the acceptor, is also a thiol after sequential labeling of the protein first with the donor then with the acceptor, the labeled protein will contain a mixture of donor–donor, acceptor–acceptor, and donor–acceptor species. This, of course, must be avoided, so the second molecule of the label must be incorporated differently, although in some cases chromatography can be used to separate mixtures of protein derivatives ([18](#)). Many ingenious labeling strategies were invented: for example, noncovalently attached light chains can be modified separately and subsequently exchanged with unlabeled light chains ([19](#)); this works for myosin II. Surface glutamine residues can be labeled with primary amines using the enzyme transglutaminase ([20](#)). All molecular motors possess a nucleotide-binding site that can be used to bind fluorescent

nucleotide derivatives (mant-ATP, ethenoadenoside-ADP, etc.) (21–23). A unique method of introducing a donor–acceptor pair was employed by Suzuki et al. (24); they engineered blue fluorescence protein (BFP) on the C-terminus and green fluorescence protein (GFP) on the C-terminus and N-terminus of *Dictyostelium* myosin S1, respectively. Nucleotide-induced changes in FRET were observed between these probes, in agreement with the swinging lever-arm hypothesis.

A general method of measuring FRET between two amino acid residues that exhibit identical or similar reactivity is the “trace incorporation” method (25), which consists of modifying a small fraction of the subunits with a donor and saturating the unmodified sites on other subunits with an acceptor. In this case, if the fraction of donor-labeled subunits is small and the FRET efficiency high, the error in the calculated distance resulting from the presence of some molecules with both subunits labeled with the donor is negligible. For example, with R_0 equal to 42 Å, FRET efficiency of approximately 0.70, and 5% of donor-labeled heads, the estimated error in the distance is about 1.2 Å. This method has, however, certain limitations. To calculate FRET efficiency, one must assure that the spectra of the donor in the presence and absence of the acceptor are compared at the same donor concentration. This concentration cannot be estimated from the absorbance because the OD values are generally below 0.01. To address this issue, papain digestion can be used to standardize samples used for FRET measurements.

3.2. Labeling Cys-670 of Ncd

1. Ncd is modified at 5°C in 20 mM HEPES, pH 7.2, adjusted with KOH, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM EGTA, 5 mM magnesium acetate, 50 mM potassium acetate, and 5% sucrose. The protein is first labeled with the donor (5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid, 1,5-IAEDANS) at a molar ratio of 1:0.1 or 1:0.15 for experiments with microtubules. The actual incorporation of the donor is usually 0.04–0.05 mol of 1,5-IAEDANS per head of Ncd.
2. After labeling for 1 h, the sample is divided into two parts: in one part, the reaction was stopped by the addition of dithiothreitol or β -mercaptoethanol to 1 mM, whereas the second is treated with a threefold molar excess of the acceptor QSY-35-iodoacetamide (QSY-35, Molecular Probes; see **Notes 6 and 7**).
3. An additional sample of Ncd is incubated only with a threefold molar excess of QSY-35.
4. In both samples labeled with the acceptor, the reaction is stopped with dithiothreitol or β -mercaptoethanol after 4.5 h incubation with QSY-35.
5. The excess of the dye is removed using NAP-10 or PD-10 columns (Amersham-Pharmacia) equilibrated with the labeling buffer.

6. The extent of Ncd labeling with QSY-35 is determined spectrophotometrically using ϵ_{280} of $27,960 M^{-1} cm^{-1}$ for Ncd (calculated from amino acid composition) and ϵ_{490} of $24,000 M^{-1} cm^{-1}$ (Molecular Probes) for QSY-35, and employing the relation $A_{280(Ncd)} = A_{280} - A_{490} \times 0.23$, where 0.23 is the contribution of QSY-35 to the absorbance at 280 nm, obtained from a spectrum of *N*-acetyl-cysteine-QSY-35 adduct. The labeling ratio for all dimeric forms of Ncd was generally 1.0 ± 0.1 (see Note 8).

3.3. Measuring FRET Efficiency

Theoretically, the steady-state FRET experiment consists of obtaining two spectra of the donor: in the presence and absence of the acceptor (Fig. 2). The method looks deceptively straightforward and simple. In practice, there are many pitfalls and dangers. The most important disadvantage of this steady-state approach is that it requires comparing two separate samples, one containing the donor-acceptor pair and the other only the donor, which means that we have to know exactly the concentration of the donor in both samples. It is rather difficult to estimate the donor concentration from the absorbance, especially if the absorption spectra of the donor and acceptor overlap.

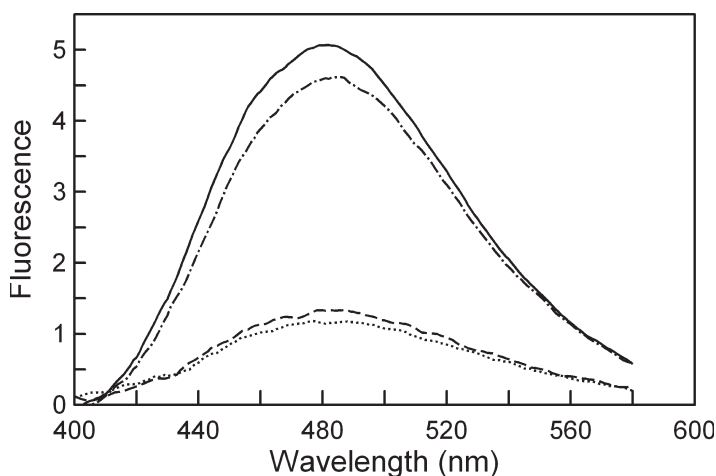


Fig. 2. FRET between heads of dimeric Ncd (see ref. 25). Fluorescence spectra of 1,5-IAEDANS attached to Cys670 of Ncd-C253S bound to microtubules in the absence and in the presence of the acceptor (QSY-35). Solid line, no acceptor, no nucleotide; dashed-dotted line, no acceptor, 1 mM ADP; dashed line, acceptor present, no ADP; dotted line, acceptor present, 1 mM ADP. The efficiency of FRET in this graph is equal to 0.75 and the computed distance is 35 Å.

The efficiency of FRET can also be determined from lifetime measurements. However, Eq. 5 is applicable if the fluorescence of the donor decays in the strictly monoexponential fashion; this is rare for large and complex macromolecules. For multiexponential fluorophores, a method based on integrated fluorescence decay was developed (26). Because the lifetime of a chromophore does not depend on its concentration, the concentration of the donor in samples used for FRET does not have to be closely matched. Below we describe a steady-state FRET experiment that additionally tries to circumvent the problem of low absorbance of the donor.

1. Four samples are prepared in the buffer used for labeling supplemented with 1 mM dithiothreitol: (a) Ncd containing only the donor, (b) Ncd doubly labeled with the donor and acceptor, (c) Ncd with the acceptor, and (d) unlabeled Ncd. The concentration of the protein in these samples should be about 1–3 μM . If high concentration of the protein is used, and/or the absorption at the excitation wavelength is not negligible (>0.05), a correction for the inner-filter effect is necessary (27).
2. Fluorescence spectra of these samples are obtained. The spectra of Ncd labeled only with the donor and those doubly labeled are corrected for background fluorescence using samples (d) and (c), respectively. As already mentioned, QSY-35 is a nonfluorescent acceptor. However, when conjugated to Ncd, it shows a very low level of background fluorescence. Therefore, the emission spectra of 1,5-IAEDANS were corrected for this effect by using Ncd labeled only with QSY-35. If spectra (a) and (b) were obtained at the same donor concentration, we could them use directly in Eq. 6. However, the donor concentration cannot be estimated from its absorbance because the OD values are generally below 0.01. We use papain digestion to standardize samples used for FRET measurements. Papain (Sigma P-3125; 0.12 mg/mL for 20 min) cuts Ncd into polypeptides smaller than can be resolved on a 15% SDS gel. These peptides do not precipitate. Obviously, there is no FRET in the digested samples. By comparing the fluorescence intensity of samples containing the donor to those containing the donor and the acceptor, a normalization factor is computed and used to compensate for unequal donor concentration in samples (a) and (b) (see ref. 25).
3. When microtubules are present, the spectra are corrected for scattered light using microtubule complexes with unlabeled or QSY-labeled Ncd.

3.4. Computing the Distance

Knowing the Förster critical distance, R_0 , and FRET efficiency, E , one can compute the distance from Eq. 6. To compute R_0 , the values of κ^2 and n must be known; they are usually assumed to be equal to $\frac{2}{3}$ and 1.4, respectively. J , the overlap integral, is most often computed using a computer program

provided by the maker of the fluorometer. Quantum yield of the donor can be determined with the comparative method (27) using quinine sulfate in 0.1 M H_2SO_4 as a standard. The fluorescence spectrum used to compute J or ϕ_D must be corrected for wavelength dependence of the photomultiplier and the emission optics. Files containing such correction factors are usually supplied with the fluorometer software. It is noteworthy that ϕ_D does not have a dramatic influence on the values of R_0 and r_i . For example, a 20% error in measuring ϕ_D results in 3%–4% error in R_0 .

If FRET efficiency is estimated from steady-state fluorescence, there is no information regarding the distribution of the measured distances or the presence of multiple lifetime components in the decay. Often, such parameters are of crucial importance for testing the agreement of the motor behavior with a particular force-generation model. Therefore, in these cases, it is essential that both the steady-state and lifetime data be collected. A good example of such an approach is the analysis of the lifetime data obtained with *Dictyostelium* myosin II head, providing the evidence that there exist two distinct lever-arm orientations that precede the power stroke (1).

Finally, we note that FRET provides the value of the separation between chromophores and not between amino acids to which these chromophores are attached. The size of the probe itself and the length of the linker that connects the chromophore with the protein may introduce a considerable error in the distance. To test models of motor proteins at the atomic resolution, the conformation and orientation of fluorescence probes are required, which can be obtained by applying conformational searching algorithms (28). This kind of study can be pursued if a high-resolution three-dimensional structure of the protein is available.

In the example of FRET measurements in dimeric Ncd, as already described, the objective was to detect possible alterations in the interhead separation that resulted from interaction of the motor with nucleotides and microtubules (Fig. 3). However, nucleotides (ADP, ATP, AMP-PNP) in the presence and absence of microtubules had only small effects on the distance between Cys-670s (Fig. 3A). Computer simulation indicated that rotation of one of the heads about Gly-347 would produce only small changes in the interhead distance. At the same time, a high-resolution structure for the Ncd-Asn600Lys mutant became available (Fig. 3B). In this mutant, a 75° rotation of the stalk and one of the heads about Gly-347 was seen. This observation led to the conclusion that Ncd-Asn600Lys represents an intermediate, force-generating conformation of the Ncd mechanochemical cycle (29), in excellent agreement with the explanation hinted by FRET measurements and confirmed by a recent cryo-electron microscopic (cryo-EM) study (30).

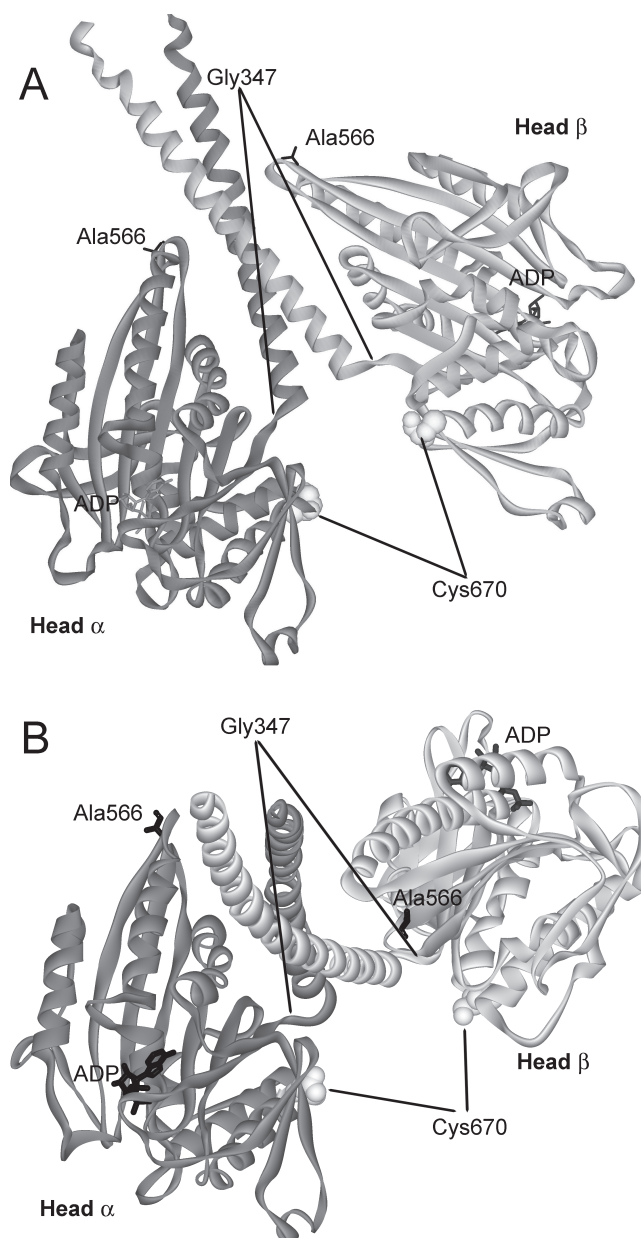


Fig. 3. (A) Structure of wild-type Ncd in dimeric form comprising residues 281–700 (33). (B) Structure of Asn600Lys mutant of Ncd (residues 292–700) (29). Mutation Asn600Lys has been suggested to simulate one of the conformations of the Ncd mechanochemical cycle. The orientation of head α (dark gray) is the same as in (A). Comparing to wild-type Ncd, head β (light gray), the neck, and stalk residues are rotated by $\sim 75^\circ$ about Gly-347 in head α , while holding this head in place. Note position of Ala-566 after rotation. The last residue visible in the crystal structure of head β is Asn-668, which is shown instead of Cys-670. The coordinates were obtained from Protein Data Bank (file 2NCD for A and 1NM6 for B) and rendered with ViewerLight (Accelrys Corp.).

4. Notes

1. Both the yield and the purity of Ncd improve if the isolation procedure is carried out expeditiously.
2. Guanylyl-(α,β)-methylene-diphosphonate (GMP-CPP) can be used instead of paclitaxel. This GTP analog binds to the tubulin exchangeable nucleotide-binding site, located in the β -subunit, and promotes polymerization of normal and stable microtubules (31).
3. Protein concentration is estimated by the Read (32) modification of the Bradford method, using bovine serum albumin as a standard.
4. Assembled microtubules are kept for no more than a few hours at room temperature.
5. Microtubule concentration is usually given as the concentration of α,β -tubulin heterodimer.
6. QSY-35 can be dissolved in anhydrous dimethylformamide.
7. We used a nonfluorescent acceptor QSY-35, because when the emission spectra of the donor and of the acceptor overlap and acceptor fluorescence exceeds that of the donor 10 times or more, it is usually difficult to unambiguously extract donor fluorescence from the spectrum.
8. Labeled Ncd must be kept on ice and used within a few hours.

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Structure Determination of the Motor Domain of Yeast Kinesin Kar3 by X-Ray Crystallography

Hee-Won Park

Summary

Kinesins are molecular motors that share a common structural core with myosins and G proteins and play diverse roles in organelle transport and cell division and movement. Kinesin motors use the chemical energy derived from ATP hydrolysis to generate force for moving on the microtubule track. The mechanism by which kinesin motors capture the energy from ATP hydrolysis and convert it to a force is not completely known. Structural elements that undergo movement and the force-producing conformational changes of the motor must be identified to elucidate this mechanism. X-ray crystallography is the method of choice for elucidating the structural changes of kinesin motors during ATP hydrolysis.

Key Words: X-ray crystallography; kinesin; molecular motor; ATPase; vesicle transport; mitosis/meiosis.

1. Introduction

Kinesins are molecular motors that transport organelles, vesicles, or chromosomes on microtubules using the chemical energy derived from ATP hydrolysis as a power source ([1](#)). The mechanisms of force generation and movement by molecular motors are not well characterized. Kinesin motors contain three domains: the motor domain containing the nucleotide-binding and microtubule-binding sites, the coiled-coil stalk domain involved in dimerization, and the basic tail domain that may bind to cargo ([2,3](#)). Kinesin motor inhibitors may have potential therapeutic values since cell-cycle arrest by disrupting motor function provides a new target for development of antimitotic drugs that are different from the microtubule-targeted cancer chemotherapy ([4,5](#)).

Kinesin superfamily includes more than 600 proteins from a variety of species ([6](#)), but only a handful of kinesin structures have been reported. Here,

we describe a procedure for solving the structure of the motor domain of Kar3 from *Saccharomyces cerevisiae* using X-ray crystallography. Because the kinesin superfamily members contain the conserved motor domain, structure determination procedure of the Kar3 motor domain described in this chapter can be used as a standard structure determination procedure for the motor domains of any kinesin proteins.

2. Materials

2.1. Buffer Preparation

1. Lysis/purification buffer for the motor domain of Kar3 (LPK): 10 mM HEPES-HCl, pH 7.2, 1 mM β -mercaptoethanol, 1 mM $MgCl_2$, 100 mM NaCl.
2. Crystallization well solution for the motor domain of Kar3 (CWSK): 20%–26% PEG2000ME, 200 mM NaCl, 50 mM HEPES-HCl, pH 7.0–8.0.

2.2. Plasmid Construction

Motor domain of Kar3 is amplified using the polymerase chain reaction from yeast genomic DNA and cloned into a bacterial expression vector. For example, the Kar3 motor domain (residues 373–729) is cloned into pMW172 within the *Bam*H1/*Nde*I restriction sites. The plasmid pMW172 is a derivative of the T7 promoter-based pET vectors (7) and has been used for expressing the motor domains of various kinesins including Kar3, Ncd, and KHC of kinesin-1 (8) (see Note 1).

2.3. Bacterial Strains and Protein Purification Columns

1. Use bacterial strains that contain an inducible T7 RNA polymerase. All DE3 strains contain a T7 RNA polymerase, which is inducible by isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma). BL21(DE3) strain used for pMW172/Kar3 expression is available from Novagen.
2. SP-Sepharose column can be purchased from Amersham Biosciences. This cation-exchange column separates proteins based on their positive charges.
3. Superose 12 column (Amersham Biosciences) is used for high-resolution purification of proteins based on molecular weight.

2.4. Crystallography

1. Screening kits are commercially available: Hampton Research Crystal Screen 1 and 2 (<http://www.hamptonresearch.com/>), Emerald Wizard Crystal Screen 1 and 2 (<http://www.emeraldbiosystems.com/>), and Molecular Dimensions Structure Screen 1 and 2 (<http://www.moleculardimensions.com/>). Detailed information on the composition of each screening kit, is available on the company's Web site. Coverslips (e.g., siliconized or plastic), Libro-style plates, and various sizes of cryo-loops with magnetic caps (e.g., loop diameters from 0.05 to 1.0 mm) can be purchased from the same companies that sell the screening kits.

2. Extremely intense and highly collimated synchrotron radiation is recommended for solving the atomic and crystal structures of proteins. Submit a proposal for allocation of beam time at any synchrotron sources. There are four synchrotron facilities available in the United States, and many more are available throughout the world (http://www-als.lbl.gov/als/synchrotron_sources.html).
3. The HKL2000 program suite is software for processing diffraction data, and installed at most synchrotron beamlines. Installation of HKL2000 at a home computer needs substantial licensing fees, and thus it is advised to finish processing diffraction data at the synchrotron beamline.
4. The program O is required for model building and the CCP4 program suite is used for phasing and crystallographic refinement. These programs are free to academic and nonprofit institutions. In order to obtain O, go to the home page of Alwyn Jones (http://alpha2.bmc.uu.se/~alwyn/Essential_O/essentials_frameset.html) and download an appropriate version for your computer operating system (e.g., Windows XP, Mac OS, Linux, or Unix). The CCP4 program suite can be downloaded from the CCP4 main Web page (<http://www.ccp4.ac.uk/main.html>). It is also advised to download the CCP4 Graphical User Interface (CCP4i) program package, Tcl/Tk/Blt, which simplifies running CCP4 programs.
5. The automatic model building program ARP/wARP can be obtained from the Web site (<http://www.arp-warp.org>).

3. Methods

3.1. Protein Expression and Purification

1. Transform the plasmid into competent BL21(DE3) host cells (*see Note 2*) and plate the cells on an LB/amp agar plate [Luria-Bertani (LB) agar with ampicillin added to medium].
2. Prepare the seed culture by inoculating a single colony of the transformed *Escherichia coli* cells into 80 mL LB media and grow overnight at 37°C with shaking.
3. After overnight growth, inoculate the entire seed culture into 800 mL LB media in the presence of 50 µg/mL ampicillin at 37°C until the culture OD₆₀₀ reaches 0.5.
4. Induce the expression of proteins by adding of 0.4 mM IPTG, reduce the temperature to 18°C and continue to grow overnight in a shaker incubator system.
5. Harvest cells by centrifugation at 5,000 *g* for 30 min and store pellets at -80°C.
6. Thaw the cell pellets and resuspend them in 100 mL LPK. Disrupt cells by sonication (e.g., Versonic Sonicator). For example, we use four 30-s pulses at 150 W with a 30-s chilling period between each pulse. The cell lysis step must be done on ice to avoid heating the sample.
7. Centrifuge the lysate at 27,000 *g* for 30 min and use the supernatant for the purification steps.
8. Two-step purification strategy is applied. As a first step, load the supernatant on a SP-Sepharose column (Amersham Biosciences) equilibrated with

LPK buffer and elute the protein with a gradient of 100 to 500 mM NaCl in LPK buffer. For example, the Kar3 motor domain elutes at 200 mM NaCl (9). After that, protein is loaded on a Superose 12 column (Amersham Biosciences) and eluted with LPK buffer + 200 mM NaCl.

9. Concentrate the purified protein by centrifugation at 2,800 g using an Amicon Ultra centrifugal filter (Millipore) to the final volume of 0.5 mL. Use the filter membrane with a 10,000 molecular weight cutoff. Store the purified proteins frozen at -80°C .
10. Estimate the protein concentration by Bradford assay (Bio-Rad) (10). It is recommended to use protein as needed. Warm up the spectrophotometer (Eppendorf BioPhotometer) before use. Prepare standards containing a range of 1 to 20 μg of protein (e.g., albumin or gamma globulin is recommended) in 200 μL volume. Dilute the purified protein to estimated amounts of 1–20 μg of protein per tube in 200 μL volume. Add 800 μL dye reagent and incubate 5 min. Measure the absorbance at 595 nm. Prepare a standard curve of the absorbance at 595 nm versus the concentration of protein for the standards. To determine the concentration of the purified protein from its absorbance, use the standard curve to find the concentration of standard that would have the same absorbance as the sample. Typical concentration of the purified Kar3 motor domain is 20 mg/mL. Purification yield is about 10 mg purified protein from 800 mL cell culture.

3.2. Crystallization

Commercially available kits are used to screen for the growth of protein crystals for X-ray diffraction analysis. These commercial screening kits are based on the sparse matrix method for protein crystallization (11). The reservoir solution and precipitant conditions of the screening kits are empirically derived based on published crystallization conditions of various proteins in the past, so as to sample as large a range of buffer, pH, additive, and precipitant variables as possible, using small amounts of proteins (11). Hanging drop vapor diffusion is the most popular crystallization method which uses the vapor phase to bring about equilibration between a drop of mother liquor and the reservoir solution in a closed cell (12) (see **Note 3**).

1. Place a drop of mother liquor—a mixture of protein solution with a precipitant (e.g., 2 μL protein and 2 μL precipitant)—on either a siliconized or a plastic coverslip that is then inverted over a reservoir which contains 1 mL of the precipitating solution.
2. Seal the reservoir with high-vacuum grease applied around the circumference of the reservoir. The initial concentration of precipitant in the drop is such that the protein is fully soluble. As water in the drop diffuses into the concentrated solution of the reservoir, less water is available in the drop to keep the protein in solution. The protein in the drop gradually becomes supersaturated, a thermodynamically metastable state. The degree of supersaturation is defined as the ratio of the protein concentration to the concentration at the solubility limit of the protein (13). From

the thermodynamically unstable state of supersaturation, the drop solution can achieve the thermodynamically stable state by forming either crystals or amorphous precipitates. When an amorphous precipitate appears, the protein usually remains in that state. Occasionally, however, given sufficient time, crystals may grow from the amorphous precipitate (12). The strategy used to induce crystallization of protein is to bring a protein solution slowly to supersaturation. A large number of conditions have to be explored in the hope of finding conditions that yield crystals. In this sense, the hanging drop method is useful because it requires only a small amount of protein (e.g., 0.5–1 μL) to screen a large number of conditions. Disposable plastic tissue culture plates (Linbro) can be used to supply a large number of reservoirs. For example, the crystals of Kar3 protein are grown at 18°C in a hanging drop consisting of 2 μL protein (15 mg/mL) + 2 μL of well solution containing CWSK (14) (see Note 4).

3. To prevent the protein crystals from X-ray radiation damage during data collection, they are flash-frozen and maintained at 100 K under a stream of nitrogen. Ironically, this flash-freezing process can also damage the protein crystals, and thus cryoprotectants should be used to protect them during the freezing process. The cryoprotectants are small organic molecules such as glycerol, ethylene glycol, polyethylene glycol-400, methyl-pentenediol, or monomeric or dimeric sugars. Cryoprotectant solution usually contains the crystal-growing reservoir solution mixed with one of the cryoprotectants. The type of cryoprotectant that is right for the crystals and a sufficient concentration which prevents the crystals from ice formation during freezing is determined empirically (see Note 5). To soak the crystals in the cryoprotectant solution, use an appropriate diameter of cryo-loop that matches with the width of the crystal. Transfer the crystal from the drop of mother liquor to the cryoprotectant solution, leave it for at least 2 min, and then flash-freeze it by dipping the loop containing the crystal in liquid nitrogen.

3.3. Data Collection

The most common method used for collecting diffraction data in X-ray crystallography is by rotating the crystal about a single axis. We assume that the data collection is performed at a synchrotron beamline.

1. Mount the cryo-loop containing a crystal onto the goniometer and maintain the crystal under a cryo-condition of 100 K. The magnetic mounting system gives a convenient way for rapidly attaching the crystal to the goniometer. The ferromagnetic base of the cryo-loop allows the crystal to be placed on the goniometer head with the magnet in one motion.
2. Adjust the crystal-to-detector distance to match the maximum resolution of the diffraction.
3. Set an adequate exposure time per frame to avoid saturation of the detector. Typical exposure times vary from 5 to 120 s. A set of aluminum and silver foils of varying thickness are installed in the filter arrays and, instead of varying the

exposure times, can be used to attenuate the X-ray beam to prevent saturation of the detector.

4. Choose a radiation wavelength (*see* **Note 6**).
5. Decide a rotation angle for each frame. Typical rotation angles are either 0.5° or 1.0° . The optimal rotation angle can be decided by interpreting two frames exposed 90° apart. Most data integration programs such as HKL2000 (**15**) allow the user rapidly to index and interpret frames. The diffraction pattern can then be predicted for other crystal orientations to check for overlap of reflection profiles (**16**).
6. Select the total rotation angle that is appropriate for the crystal symmetry. Collecting 180° of data is recommended, which will always ensure maximum completeness (**16**).

3.4. Data Setup by HKL2000

Various data integration and scaling programs are available for use by those collecting data on synchrotron beamlines. The following procedure is based on the HKL2000 suite (**15**). The HKL2000 suite contains four programs: XDISP for frame display and measurement, DENZO for autoindexing and integration, SCALEPACK for data merging and scaling, and HKL2000 for bringing together these programs using a graphical interface. For additional information about these programs, please refer to the HKL manual, available online as a PDF file (http://www.hkl-xray.com/hkl_web1/hkl/manual_online.pdf).

1. Run the program by typing 'HKL2000' and selecting an appropriate detector type for the synchrotron beamline. A main HKL2000 window is displayed.
2. In the main HKL2000 window, click the 'Data' tab and indicate the directory names where the frames are and the output files will be written.
3. Click the 'Set Up Data Files' button and select all frames you want to process.
4. Click the 'Display' button to display the first frame on a display window.
5. In this display window, click the 'Peak Sear' button to start the peak search routine. The number of peaks will appear on the right-hand side. The picked peaks are displayed as small red circles over the image. These peaks are used for autoindexing in the next step (*see* **Subsection 3.5.**).

3.5. Data Autoindexing and Integration by HKL2000

The autoindexing step deduces the orientation of the crystal and refines the parameters for the frame you have displayed whereas the integration step processes all the frames and gives the intensities of reflections for each frame. The procedure is as follows.

1. In the main HKL2000 window, click the 'Index' tab and then click the 'Index' button to start the autoindex routine. If autoindexing works, the predicted reflections will be displayed to line up with the peaks.
2. Click the 'Bravais Lattice' button to display the Bravais lattice table. This table shows the 14 possible Bravais lattices with a percent value that represents the

amount of distortion the unit cell parameters would accommodate to fit the lattice. Next to this percentage are the distorted-to-fit unit cell parameters, and below are the undistorted unit cell parameters, for comparison. Choose the highest symmetry lattice that fits the data with minimal distortion.

3. Click the 'Fit All' button and then click the 'Refine' button to refine all the parameters—crystal orientation, crystal-to-detector distance, unit cell dimensions, etc.—simultaneously.
4. In the display window, click the 'Zoom wind' button to display a zoom window. On the zoom window, click the 'Int. box' button to show an integration box. In the zoom window, zoom in on an area of the image and on the main HKL2000 window, clicking the 'Spot Size' button to adjust the size of the expected reflection area by matching with that of the actual peak.
5. Process the entire series of frames by clicking the 'Integrate' button, which runs DENZO.

3.6. Data Scaling by HKL2000

The data scaling step merges the reflection intensities from each frame into a set of unique reflections after removing outliers.

1. To scale diffraction data, click the 'Scale' tab on the main HKL2000 window.
2. Choose space group for the crystal by clicking the 'Space Group' button (*see Note 7*).
3. Run SCALEPACK by clicking the 'Scale Sets' button. The outliers that have inconsistent intensities to the averaged intensity of the symmetrically related reflections will be written into a reject file.
4. Checkmark the 'Use the rejections on next run' button, which disallow the rejected outliers being included in scaling, and re-run SCALEPACK by clicking the 'Scale Sets' button.
5. The program writes scaled reflections into a file (σ_{ca}) and the corresponding log file (λ_{og}).

3.7. Phasing and Electron Density Calculation

Two components are needed in order to calculate electron density maps for a protein structure: structure factor amplitudes and the corresponding phase angles. The amplitudes are proportional to the square root of the intensities of the reflections, so that these amplitudes are determined from the intensity measurements of each reflection (e.g., the reflections from a Kar3 crystal). However, the phase angles cannot be directly measured. We solve this phase problem by the molecular replacement method as described next. This method works well when the sequence similarity of the known structure and unknown structure is higher than 50%.

1. Use the motor domain (residues 350–670) from an Ncd dimer structure (PDB 2NCD) as a search model.

2. Convert the scaled reflections (σ_{ca}) into MTZ format using the program SCALEPACK2MTZ (17) running on the CCP4i interface. SCALEPACK2MTZ also converts the reflection intensities to structure factor amplitudes.
3. Perform the molecular replacement by using the program MOLREP (18) running on the CCP4i interface. MOLREP requires the MTZ-formatted reflection file and the search model as inputs. A successful molecular replacement search finds the orientation and position of model(s) that match with the observed amplitudes of the reflections from the Kar3 crystal. The output model is reoriented by applying the appropriate rotation and translation parameters to the input search model of Ncd.
4. Inspect crystallographic packing of this reoriented Ncd model using the O program (19). Detailed information related to the O program can be found on the Web site http://alpha2.bmc.uu.se/~alwyn/Essential_O/essentials_frameset.html. First, run O by typing 'ono' (19). The main O window will be displayed. Second, read the reoriented Ncd model coordinates into the program by typing a command 'sam_atom_in'. Third, display the reoriented Ncd model by typing a series of commands 'Molecule_name, Object_name, Zone, semicolon (;), and end'. Finally, display the symmetry mates of the reoriented Ncd model by typing two commands 'Symmetry_setup, Symmetry_object'. The packing of symmetry mates of the reoriented Ncd model is considered to be good if they do not interpenetrate to a significant degree.
5. Carry out rigid body and restrained refinements by using the program REFMAC running on the CCP4i interface (20). REFMAC requires the MTZ-formatted reflection file and the reoriented Ncd model. REFMAC refines the reoriented Ncd model against the Kar3 reflections and writes the refined model as an output.

3.8. Automated Model Building

The following procedure is based on the ARP/wARP program (21) running on the CCP4i interface. ARP/wARP requires the MTZ-formatted file, which contains the amplitudes and phases (e.g., the Kar3 reflection file with the phases calculated from the reoriented Ncd model) and the protein sequence file (e.g., the sequence of the Kar3 motor domain).

1. Run the ARP/wARP main module for 'automated model building starting from existing model'. When successful, the program writes a coordinate file with the Kar3 model (see Notes 8 and 9).
2. Run the ARP/wARP main module for 'build solvent atoms' (see Note 10). When successful, the program writes a coordinate file with water molecules.
3. Run the ARP/wARP LigandBuild module (see Note 11). When successful, the program writes a coordinate file with correctly positioned ligand molecule(s).

3.9. Crystallographic Refinement

1. Use the program REFMAC (20) for crystallographic refinement. REFMAC requires a MTZ-formatted reflection data file and a model coordinate. For

example, the reflection data file contains the Kar3 reflections in MTZ format, and the coordinate file includes a complete model of Kar3, Mg-ADP, and water molecules.

2. Before starting the crystallographic refinement, the unique set of the Kar3 reflections is partitioned into a test set, consisting of 5% of reflections, and a working set comprising all remaining reflections. The test set is omitted from the refinement process and used only to calculate the free R value (R_{free}). The R_{free} value gives more objective criteria to follow the refinement and allows to avoid overfitting the reflection data (22).
3. The outputs of REFMAC are the refined model and the MTZ file containing weighted coefficients for weighted 2Fo-Fc and Fo-Fc maps where Fo is the measured amplitudes of Kar3 crystal, whereas Fc is the calculated amplitudes of the refined Kar3 model.
4. Calculate the weighted 2Fo-Fc and Fo-Fc maps using the program FFT (Fast Fourier Transform) running on the CCP4i interface (23). The Fo-Fc difference map locates the correct atom positions of Kar3 protein, ligand, and water molecules. Positive peaks appear where the data “expect” more atom density and negative peaks where the data “want” less atom density.
5. Manually correct the model coordinates using the 2Fo-Fc and Fo-Fc maps in O.
6. Perform a few iterations of model building in O and refinement in REFMAC until no more corrections can be made.

3.10. Final Model Evaluation

The final model of Kar3 should have an R_{free} value below 30.0%. The 2Fo-Fc map calculated with the final model should show continuous main chain density and well-defined density for most of the side chains. If side chains have poor density and high temperature factors (e.g., the values larger than 60.0 Å), they are flexible or adopt more than one conformation in the crystal lattice. In general, the residues with poor side chain density are exposed to solvent and thus make few interactions with the rest of the structure.

1. Examine the stereochemistry of the main chain with a Ramachandran plot using the program PROCHECK (24) running on the CCP4i interface. The Ramachandran plot shows the main chain phi and psi torsion angles for residues, except glycine, which is unique in that it lacks a side chain. Ideally, none of the residues should be found in the disallowed region. The percentage of residues in the disallowed region is an excellent guide to stereochemical quality of the final model. For example, no residues of the final Kar3 model are in the disallowed regions (see Note 12).
2. A low deviation of the final model from ideal geometry indicates the excellent quality of the model. REFMAC checks if bond lengths and bond angles of the final model differ from an ideal geometry and write deviations in a log file. In

general, the root mean square deviation (rmsd) of bond lengths should be distributed less than $0.02 \text{ \AA}$ from an ideal geometry and the rmsd of bond angles about 2° (25). For example, for the Kar3 model the rmsd of bond lengths is $0.006 \text{ \AA}$ and the rmsd of bond angles is 1.2° (14).

4. Notes

1. The pET plasmids that express a hexa-histidine tag at the N-terminus have been also used for expressing the motor domains of kinesin proteins (14,26). The pET plasmids can be purchased from Novagen, whereas the pMW plasmids are not available commercially.
2. If a kinesin gene uses “rare” codons that are infrequently used by *E. coli*, the expression of kinesin protein in *E. coli* may be severely diminished. There are many Web-based programs which determine the number of rare *E. coli* codons in a DNA sequence. For the gene encoding the motor domain of Kar3, the number of rare Arg codons is 14. Rosetta(DE3) cells (Novagen) may be a useful alternative to enhance the expression of Kar3 protein as they are designed to supply tRNAs for the rare *E. coli* codons on a compatible chloramphenicol-resistant plasmid.
3. Sitting drop vapor diffusion is the alternative to the hanging drop vapor diffusion method. Experience shows that sitting drops tend to yield larger crystals since they reach equilibrium more slowly than hanging drops. An optimal crystallization condition obtained with the hanging drop method can be used with the sitting drop method to attain supersaturation. In this method, drops of mother liquor are placed in the depressions of disposable glass cups (Fisher Scientific) or microbridges (Hampton Research). The samples are then sealed in the reservoir, which contains 1 mL of the precipitating solution. Through the vapor phase, the concentration of salt or organic solvent in the reservoir equilibrates with that in the sample as described in the hanging drop method.
4. Inclusion of ligands (e.g., MgATP, MgADP) in the mother liquor drop helps crystallization of the kinesin proteins (14,27).
5. Finding the right cryoprotectant with a sufficient concentration is tedious and sometimes impossible. Paratone-N (Hampton Research) becomes a popular cryoprotectant, which can be used for protein crystals regardless of their growing conditions. A mixture of Paratone-N and mineral oil in a 1 : 1 ratio should be sufficient for cryo-data collection.
6. The wavelength of X-ray radiation produced by a rotating anode source at home is fixed at the value characteristic for the anode metal, usually copper with wavelength $1.54 \text{ \AA}$ (16). In contrast, the radiation wavelength of a synchrotron beam is adjustable. Wavelengths below $1.00 \text{ \AA}$ are used at most synchrotrons, which minimizes absorption of radiation by the crystal and its mother liquor and the air scatter (16).
7. The space group of protein crystals needs to be determined before scaling the diffraction data.

8. When the automatic model building procedure fails, a manual model-building procedure is available by using O (19). First, run O by typing 'ono'. A main O window will be displayed. Read the 2Fo-Fc and Fo-Fc maps using a command 'Fm_file'. In the main O window, select the 2Fo-Fc map from the pull-down menu 'Density' and display it contoured at a level of 1.0σ (electron density). Select the Fo-Fc map from the pull-down menu 'Density' and display the positive density contoured at a level of 3.0σ (electron density). Read the refined Ncd model by typing 'Sam_atom_in' command and display the model by typing a series of commands 'Molecule_name, Object_name, Zone, semi-colon (;), and end'. Build the main chain by using a command 'Baton_build'. This command requires a dipeptide object, DI, which indicates that the distance between two consecutive C α atoms should be 3.8 Å. Another requirement is to have an object called CA, which is a set of C α atoms of the Ncd model. Because the maps are based on the molecular replacement solution and refinement, rebuilding of the model is minimal and localized to connecting loops. Start by placing C α atoms into the electron density of the loop region. Continue these until the complete assignment of a loop. Use a command 'Lego_ca' to extract the coordinates for the other main chain atoms of the loop from a protein database consisting of well-refined protein structures (28) by choosing the best matching main chain to fit the C α trace of the loop.
9. For building the side chains into an electron density map, use various commands 'Mutate_repla, Mutate_inser, Mutate_delet, and Lego_Side_Ch'. The 'Mutate-Repla' command is used for replacing Ncd residues to Kar3 residues, the 'Mutate_Inser' command for adding Kar3 residues to the structure, and the 'Mutate_Delet' command for removing Ncd residues from the structure. These Mutate commands convert the sequence of Ncd to that of Kar3. Build the side chains into the densities using the 'Lego_Side_Ch' command and a side-chain database of the rotamers (29). Achieve the best fits of side chains with the corresponding densities by changing torsional angles (the command 'Tor_Residue'). Since changing the main chain torsional angles disrupts the geometry of the molecule, restore the geometry with the 'Refi_Zone' command, which enforces standard bond lengths and angles and fixes dihedral angles.
10. For building water molecules, select peaks from the positive difference map by using the program PEAKMAX (written by Ian Clifton) in the CCP4 suite. Peaks ($>3\sigma$) are chosen if the distance from any peak to H-bonding atoms of the protein molecule is between 2.0 Å and 3.5 Å. Include these water molecules in the crystallographic refinement (see below) after verification of their positions by visual inspection of the local electron density in O.
11. For building ligands, download the coordinates of ligands from the Hetero-compound Information Centre Uppsala (HIC-UP) Web site. For example, the motor domain of Kar3 is bound to Mg-ADP, and their coordinates can be downloaded from the HIC-UP Web site. Read the Mg-ADP model coordinates by using

the 'Sam_atom_in' command and display the Mg-ADP model by using a series of commands 'Molecule_name, Object_name, Zone, semi-colon (;), and end'. Identify electron density for Mg-ADP and fit the Mg-ADP model into this density by using the 'Move_zone' command and changing the torsional angles of the Mg-ADP model.

12. Sometimes residues (i.e., 0.4% of all nonglycine residues) occur in the disallowed region of the Ramachandran plot (30). Disallowed residues are usually located at the surface of the molecule and in short loops, being in many cases at the junction of two secondary structural elements (30).

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High-Resolution Structural Analysis of the Kinesin–Microtubule Complex by Electron Cryo-Microscopy

Keiko Hirose and Linda A. Amos

Summary

To understand the interaction of kinesin and microtubules, it is necessary to study the three-dimensional (3D) structures of the kinesin–microtubule complex at a high enough resolution to identify structural components such as α -helices and β -sheets. Electron cryo-microscopy combined with computer image analysis is the most common method to study such complexes that cannot be crystallized. By selecting microtubules that have a helical symmetry, 3D structures of the complex can be calculated using the helical 3D reconstruction method. Details of the interaction are studied by docking the individual crystal structures of the kinesin motor domains and tubulin heterodimer into the 3D maps of the complex. To study the structural changes during ATP hydrolysis, structures of the complexes in the presence and absence of different nucleotides are compared.

Key Words: Kinesin; microtubule; molecular motors; electron microscopy; three-dimensional reconstruction.

1. Introduction

Microtubules (MTs) are essential components of the eukaryotic cytoskeleton, the system of fibers that maintains the structure of the cell. Some of the most important roles of MTs involve cooperation with motor proteins such as dynein and the numerous members of the kinesin family. For example, the interactions of both kinds of microtubule motors with MTs are essential in driving axonal transport and in the formation and operation of the mitotic spindle, which separates chromosomes during cell division. These interactions that produce directional movement along MTs require cycles of ATP hydrolysis, taking each motor domain through a series of nucleotide-bound states. Using electron microscopy of frozen-hydrated specimens (cryo-EM) and image analysis, we

are able to study the structural conformations of some of these nucleotide states.

Such studies are most easily carried out on highly purified proteins. Tubulin can be purified to a high level of purity from the large amount present in mammalian brain tissue. Under suitable conditions, this pure protein will reassemble into MTs. Kinesin is most conveniently studied in the form of truncated, tail-less motor domains (heads), which can be produced using widely available recombinant methods. When excess amounts of the kinesin heads are added to MTs, one head binds to each tubulin dimer. The state in which it binds depends on the nucleotides present in excess in the solution. Then, if we have chosen MTs that have a helical symmetry, the 3D structures of the kinesin–MT complex can be studied by helical 3D reconstruction methods ([1–4](#)) (see **Note 1**).

Unfortunately, most of the MTs polymerized from purified tubulin do not have a true helical symmetry. Reassembled MTs vary in structure and usually include specimens with 12–16 longitudinal protofilaments of tubulin subunits, with the peak at 13 or 14, depending on the condition used for polymerization ([5,6](#)). MTs of 13 protofilaments or 14 protofilaments do not have perfect helical symmetry because, after they have assembled first as 2D sheets, when they close to form a tube there is a mismatch between the heterodimers at the seam, despite the fact that the tubulin monomers form a perfect helical lattice ([7–10](#)) (**Fig. 1**). In 13-protofilament MTs, protofilaments run parallel to the axis of the MT. With the addition of a 14th protofilament, rotation of the lattice by a few degrees allows the tubulin monomers to match, but the dimers still mismatch as in the straight 13-protofilament MTs. However, when a 15th protofilament is added, the lattice can most easily match by rotating in the other direction and, in this case, the heterodimers do match. These rotations mean that the protofilaments do not run straight, when the number of protofilaments is more (or less) than 13, but instead rotate gradually around the axis as left-handed (most 14-protofilament MTs) or right-handed (most 15-protofilament MTs) helices. Not all the 15-protofilament MTs have a helical symmetry, but we can select those that do from the images and calculate the 3D structure using helical 3D reconstruction methods ([11](#)). Many of the 16-protofilament MTs also have a helical structure, but the proportion of 16-protofilament MTs in the *in vitro* polymerized MTs is usually less than that of 15-protofilament MTs.

Thanks to favorable specimens, improvements in electron microscopy, and increased care in the analysis of helical images, the resolutions being achieved in studies of this sort are gradually moving closer to allowing interpretation at near-atomic level ([12,13](#)). By docking the crystal structures into the 3D maps in different nucleotide states, we aim to detect which of the secondary structural

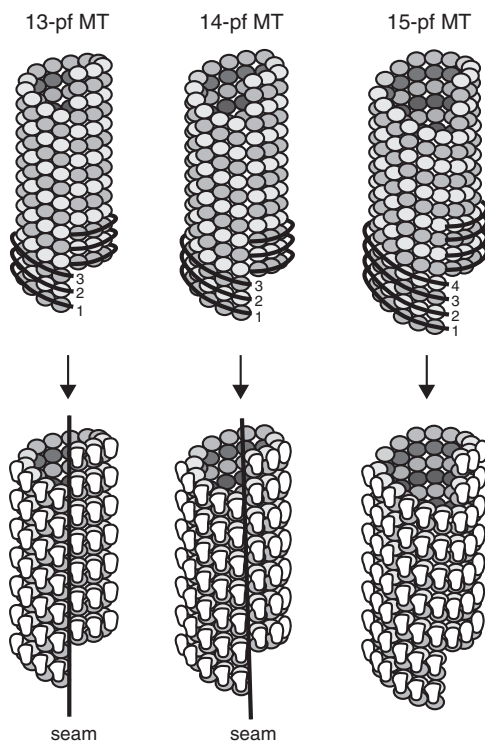


Fig. 1. Microtubules (MTs) with 13, 14, and 15 protofilaments (pf), undecorated (top) and decorated (bottom), with kinesin heads. Each $\alpha\beta$ -tubulin heterodimer is represented as a dark and a light sphere and is 8 nm long. Each column of heterodimers (protofilament) is 5 nm wide. Protofilaments in the 13-protofilament MT are straight; those in the 14-protofilament and 15-protofilament MTs follow a left-handed or right-handed family of helices, respectively, owing to small rotations of the surface lattice (7,8). The lattice of monomer subunits also forms a family of three or four shallow left-handed helices; in the 15-protofilament MT, where there is a 4-start monomer family, the heterodimers form a 2-start family. Thus, when decorated with kinesin heads, the kinesin-decorated MT has a 2-start helical structure. The 13-protofilament and 14-protofilament dimer lattices each have a step at the “seam” (9,10).

elements change their structure, assuming that the 3D maps have enough resolution. In determining details of the differences in conformation between different stages in the ATPase cycle, we hope to understand the mechanisms used to transmit the complex sequence of signals back and forth between the extensive MT-binding site of kinesin, the nucleotide-binding pocket, and the “neck” that ultimately connects the motor domain to the cargo.

2. Materials

2.1. Microtubules

2.1.1. Tubulin

Tubulin is purified from pig brain tissue. In general, fresh brain tissue (within 2 h after slaughter) is homogenized and centrifuged. Tubulin is purified from the supernatant by two cycles of polymerization and depolymerization. The resulting tubulin preparation contains a fair amount of microtubule-associated proteins (MAPs). For further purification, a PC column (Whatman P10 phosphocellulose) or DEAE Sephacel column is used ([14,15](#)). Alternatively, the higher-salt method of Castoldi and Popov ([16](#)) is very efficient. Purified tubulin, before or after column purification, can be stored in liquid nitrogen. For storing, it is better to keep the concentration of tubulin high. Tubulin is also commercially available (e.g., from Cytoskeleton).

2.1.2. Buffers and Chemicals for MT Polymerization

1. PEM buffer: 80 mM PIPES, pH 6.8, 1 mM ethyleneglycoltetraacetic acid (EGTA), 2–5 mM MgCl_2 , 1 mM dithiothreitol (DTT).
2. GTP, pH adjusted to ~ 7.0 by KOH. We usually make a 0.1 M stock solution and store at -20°C .
3. Dimethylsulfoxide (DMSO)
4. Taxol dissolved in water-free DMSO. We usually make a 10 mM stock solution and store at -20°C .

2.2. Kinesin

Motor domain constructs of kinesin-family motors can usually be expressed in *Escherichia coli* and purified either with the help of a tag (e.g., $3 \times \text{His}$) or without one ([17,18](#)). Either a monomeric or dimeric construct is used. Purified kinesin is usually in a solution containing a high concentration of salt (e.g., 0.2 M NaCl), which decreases affinity of kinesin to MTs. To fully decorate MTs with kinesin, the purified kinesin should be concentrated as much as possible (usually 50–100 μM). Kinesin can be stored in liquid nitrogen for years.

2.3. Preparation of the Electron Microscopy Specimens

2.3.1. Carbon Films

1. Holey carbon films. EM grids coated with holey carbon film are used for cryo-EM of MTs. One way to prepare holey films is described by Fukami and Adachi ([19](#)). Briefly, a slide glass treated with water repellent is cooled down in a refrigerator, and then taken out to form minute dew droplets on the surface. Immediately, 0.5% cellulose acetobutyrate (in ethyl acetate) is poured on the surface. After drying, the size of the holes is checked under a light microscope. Films that have many

holes with a 1- to 2- μm diameter are selected. Selected slide glasses are immersed in a rehydrophilic agent, rinsed in water, and then inserted in water to float the film. EM grids (e.g., 300-mesh, copper grids) are placed on top of the film and scooped up with a piece of paper or Parafilm. The grids can be kept for several months, preferably in a desiccator. Before use, the grids are carbon coated (requires an evaporator), and the plastic is dissolved with ethyl acetate. Grids coated with holey films are also commercially available. Some contain a regular array of holes in a choice of sizes (www.QUANTIFOIL.COM). In either case, it is better to use a freshly carbon-coated film. During storage, the carbon becomes hydrophobic, which affects formation of the layer of solution over the holes. We usually carbon coat the grids on the day of rapid freezing, and glow-discharge the grids just before applying the sample (see below).

2. Grids coated with a carbon film without holes, used for negative staining. A plain carbon film can be prepared by evaporating carbon on a mica sheet. The carbon-coated grids for negative staining can be stored for weeks, but the film becomes gradually hydrophobic, and few MTs remain on the surface after blotting; this problem can be reduced by glow-discharging old carbon-coated grids.

2.3.2. Decoration of MTs with Kinesin

1. Decoration buffer. Buffers suited for kinesin decoration vary depending on the kinesin construct (see **Note 2**). In general, a solution with a slightly low pH increases affinity of kinesin to MTs, but a solution with a higher pH helps to prevent aggregation and nonspecific binding of kinesin. For example, MEM solution: 20 mM MES, pH 6.5–6.8, 5 mM MgCl_2 , 1 mM EGTA, 10–20 μM taxol or HEM solution: 20 mM HEPES, pH 7.2, 5 mM MgCl_2 , 1 mM EGTA, 10–20 μM taxol are used as the decoration buffer. Taxol should be added to these solutions just before use.
2. Nucleotides [e.g., ADP, AMPPNP (adenylylimidodiphosphate)], depending on the nucleotide state that is to be studied. To obtain a nucleotide-free state, apyrase (buy a preparation with high activity for hydrolysis of ADP to AMP) is used.
3. 1%–2% w/v uranyl acetate to check the sample by negative staining.

2.3.3. Rapid Freezing

1. Freezing apparatus. Freezing is achieved, as for most specimens, by plunging the grid plus sample into a small container of liquid ethane that is cold enough to be close to solidification, being kept cold within a dewar of liquid nitrogen (**Fig. 2A**). The freezing apparatus may be driven by gravity or by some form of electrical motor that can be turned on and off quickly, for example, one that is driven by a solenoid. The apparatus has tended to be homemade. A robot for automated specimen preparation (www.Vitrobot.com) has become available recently and allows users to perform cryo-fixation under constant conditions (temperature, relative humidity, blotting conditions, and freezing velocity) more easily, but it is expensive. **Subsection 3.3.** describes the manual version of the process.

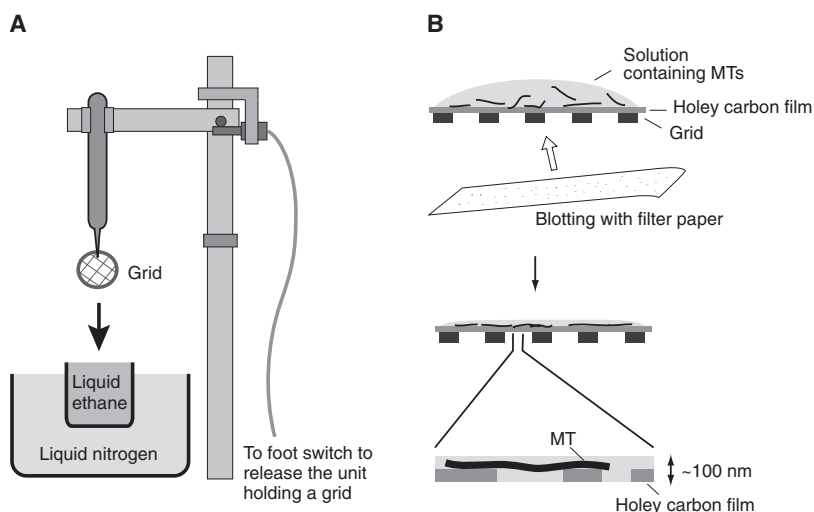


Fig. 2. Schematic drawing of a rapid freezing apparatus (**A**) and a method to make a thin layer of MT solution over a hole (**B**). When the vertical arm holding the forceps is released by the foot switch, the arm slides down by gravity, and the grid is plunged into ethane slush. Just before freezing, the solution containing MTs and kinesins is applied onto an electron microscopy (EM) grid coated with holey carbon. The excess solution is blotted from the back side. Ideally, a thin layer of solution (~ 100 nm) forms over the holes.

2. Grid boxes designed to store frozen grids in liquid nitrogen.
3. Liquid nitrogen container to store frozen grids.

2.4. Electron Microscopy

1. An electron microscope equipped with anticontamination blades and a low-dose system.
2. Cryo-specimen holder with a grid-mounting station (e.g., GATAN).
3. EM films (e.g., Kodak SO163) or a slow-scan charge-coupled device (CCD) camera attached to the EM.

2.5. Image Analysis

1. If films are used to record images, a negative film scanner with the step size of $\sim 10 \mu\text{m}$ (which means a $2\text{-}\text{\AA}$ step when the image was taken at $\times 50,000$) and with a high precision is required.
2. Several versions of software packages are available for 3D reconstruction [e.g., the MRC package (20,21) and PHOELIX (22)]. For the work described in this chapter, we used a set of programs provided by Dr. C. Toyoshima (23,24), which was developed to obtain 3D maps of helical structures at a higher resolution.

3. Methods

3.1. Preparation of Microtubules

1. When frozen-stored tubulin is used, leave tubulin on ice for ~0.5 h and spin at the top speed using a tabletop centrifuge to remove denatured protein.
2. Add PEM buffer, GTP, and DMSO so that the final concentrations of tubulin, GTP, and DMSO are 5–10 mg/mL, 0.5 mM, and 10% (or 15%; *see Notes 3 and 4*), respectively.
3. Incubate at 37°C for 20–30 min.
4. Add taxol to 50–100 μ M (about the same concentration as the tubulin dimer) to stabilize MTs.
5. Centrifuge at ~35,000g for 15 min at room temperature (*see Note 5*).
6. Add a PEM buffer supplemented with taxol and without GTP or DMSO to the pellet. Leave for a few hours or overnight at room temperature.
7. Gently resuspend the pellet. Be careful not to break the MTs.
8. Measure the tubulin concentration. Taxol-stabilized MTs at a high concentration (4 mg/mL or higher) can be stored in liquid nitrogen.

3.2. Decoration of Microtubules with Kinesin

To find a good condition for decoration (*see Note 2*), kinesin–MT mixtures are checked by negative staining before preparation of the specimen for cryo-EM. To prevent bundling of MTs by kinesin, we decorate MTs already partially attached to the carbon film on an EM grid.

1. If frozen-stored MTs are used, thaw the MTs by leaving them at room temperature for ~1 h.
2. Dilute MTs to ~1 mg/mL with a decoration buffer (*see Subsection 2.3.2.*) containing taxol. MTs should be kept at room temperature to prevent depolymerization.
3. Dilute MTs further to ~0.1 mg/mL for negative staining or 0.01–0.05 mg/mL for cryo-EM (*see Notes 6 and 7*).
4. Dilute kinesin to 25–50 μ M using the decoration buffer. Diluted kinesin should be used as soon as possible.
5. Glow-discharge the grids (coated with a holey film for cryo-EM or with a plain carbon film for negative staining).
6. Apply ~3 μ L diluted MT solution onto the grid and leave for ~30 s in a humid chamber to allow the MTs to settle down on the carbon film.
7. Add 1 μ L diluted kinesin solution and 1 μ L nucleotide or apyrase (adjust the concentrations of the stock solutions using the MEM or HEM solution plus taxol so that the desired final concentration can be achieved) to produce the desired state. The final concentration of kinesin we usually use is 5–10 μ M.
8. For negative staining, the grid is stained with uranyl acetate, blotted, and air-dried.

3.3. Preparing Cryo-EM Specimens

1. Set up the freezing apparatus. Fill the dewar of the freezing apparatus with liquid nitrogen to cool down the inner, metal cup.
2. Condense ethane gas into the metal cup (*see Note 8*).
3. Precool a grid box in a different container (e.g., a styrol foam box, with a lid to minimize water condensation) with liquid nitrogen (*see Note 9*).
4. Prepare a grid as described in **Steps 5–7** of **Subsection 3.2**.
5. Check liquid ethane. It should be cold enough to be close to solidification, but not completely solid. If it is already solidified, mix to thaw it (e.g., using a metal stick).
6. Hold the grid with a pair of forceps and attach to the freezing apparatus (*see Fig. 2A*) so that the sample side of the grid is facing away from you.
7. Blot the excess solution with a slip of filter paper from the side facing you (*Fig. 2B*; *see Note 10*), and immediately plunge the sample into liquid ethane.
8. Transfer the frozen grid into a liquid nitrogen-filled container (*see Note 11*).
9. Place the grid in the grid box submerged in a bath of liquid nitrogen. All the tools that contact the grid should be precooled with liquid nitrogen.
10. Store the grid box in a liquid nitrogen storage tank. Although the grid can be stored for several months, contamination of ice crystals gradually accumulates on the surfaces.

3.4. Cryo-EM

1. Set up the low-dose system. The low-dose settings we usually use (for imaging on EM films) are the following:
 1. ‘Search mode’: $\times 3,500$ – $5,000$; use a minimum spot size; a high degree of defocus helps to enhance the contrast and makes it easier to identify MTs.
 2. ‘Focus mode’: $\times 250,000$.
 3. ‘Exposure mode’: $\times 50,000$; exposure time = 1 s.
2. Mount the frozen grid on a cryo-specimen holder using a grid-mounting station. Remember to precool the tools that contact the grid with liquid nitrogen. Also, be very careful to avoid condensation of water into liquid nitrogen that contacts the grid (*see Note 9*).
3. Transfer the holder to the EM. Wait for about 30 min so that the temperature of the cryo-holder stabilizes.
4. View the whole grid at a very low magnification (e.g., $\times 50$) and a small spot size to check for the thickness of ice. If the EM has a function to record the stage positions, the positions of the grid squares where you want to take pictures can be recorded. During the whole process, care should be taken to minimize the dose of electrons.
5. Go to ‘Search mode’ and select the area to be imaged. If the microscope is equipped with a CCD camera, it is convenient to take a CCD picture in ‘Search mode’ (*Fig. 3A*). You can then take your time looking for good MTs in the CCD image without worrying about the electron dose.

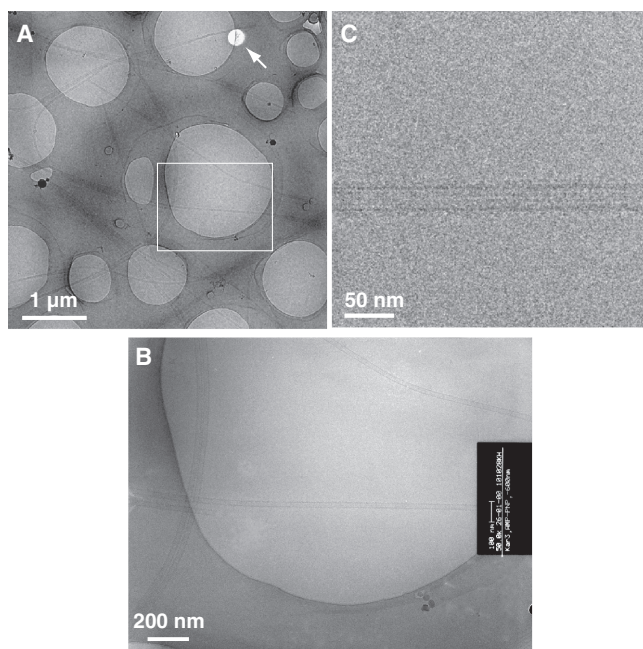


Fig. 3. Procedure for low-dose imaging. For deciding the area to be imaged, a CCD image is recorded in ‘Search mode’ (A). (B) The ‘Exposure-mode’ image recorded on a film, corresponding to the boxed area in A. After recording the image on film, the same area is checked using the CCD (C). (C) Decoration with kinesin heads is clearly seen. A white round spot in A (white arrow) is a hole made by irradiation of electron beam in the focus mode.

6. In ‘Focus mode,’ adjust the focus, and correct astigmatism. The defocus range we usually use is between -1.2 and $-2.4\mu\text{m}$.
7. Record the image of the selected area on an EM film (Fig. 3B). CCD can also be used instead of a film.
8. The same area can be checked by taking a CCD image (Fig. 3C).

3.5. 3D Image Reconstruction

3.5.1. Selecting and Digitizing Images

1. Select the MTs with 15 (or 16) protofilaments by checking the moiré patterns (5,7,25,26) on the negatives (Fig. 4A). Also check the focus, astigmatism, and regularity of the kinesin decoration. Ideally, the MT should be long and straight, fully decorated, and show a regular moiré pattern typical of a 15- (or 16-) protofilament MT. If the contrast of the negative is low, it helps to print out the picture on paper.

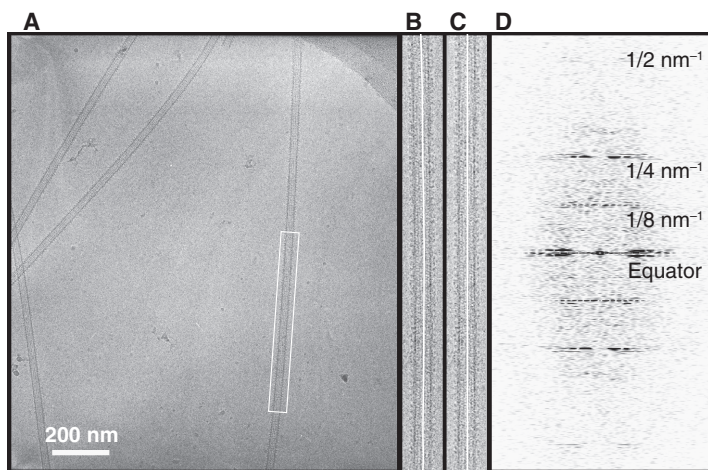


Fig. 4. Selection of MTs for 3D reconstruction. A 15-protofilament MT is selected from a micrograph (A) and is digitized (B). After the MT image is smoothly distorted so that the center line of the MT is straight (C), the Fourier transform pattern is checked (D). Sharp and strong 8-nm layer lines indicate regular and full decoration of the MT with kinesin. The 2-nm layer lines are also visible, indicating that the structure is well ordered.

2. Digitize the selected MT image (Fig. 4B). For analysis at a high resolution, it is better to use the smallest step size possible. You can downsize the image later, depending on the resolution you aim for. We usually digitize the images with 7- μm steps (equivalent to $\sim 1.4 \text{ \AA}$ on a $\times 50,000$ micrograph), and then reduce the resolution to 14 $\mu\text{m}/\text{pixel}$ or 28 $\mu\text{m}/\text{pixel}$ for analysis.
3. Smoothly distort the digital MT image to make the fitted axial line perfectly straight (from Fig. 4B to Fig. 4C).
4. Calculate Fourier transform (Fig. 4D). The Fourier pattern is assessed to see whether further processing of the data is worthwhile. Many images are discarded at this stage. For example, the layer lines in the pattern may not be sharp, indicating that the helical structure of the MT in the image is not well ordered, or the positions of the minor layer lines may not be consistent with a fully helical 15- or 16-protofilament structure. The extent of kinesin-decoration is also checked at this stage from the intensity of the 8-nm layer line.

3.5.2. Boxing MT Images and Correcting for Tilt and Shift

1. For the selected MT images, determine the axial repeat distances accurately by auto-correlation. Box MT lengths of 2 (or 1) repeat distances [approximately 300 nm (or 150 nm)] and interpolate so that the layer lines register exactly with the sampling raster (Fig. 5A; see Note 12).

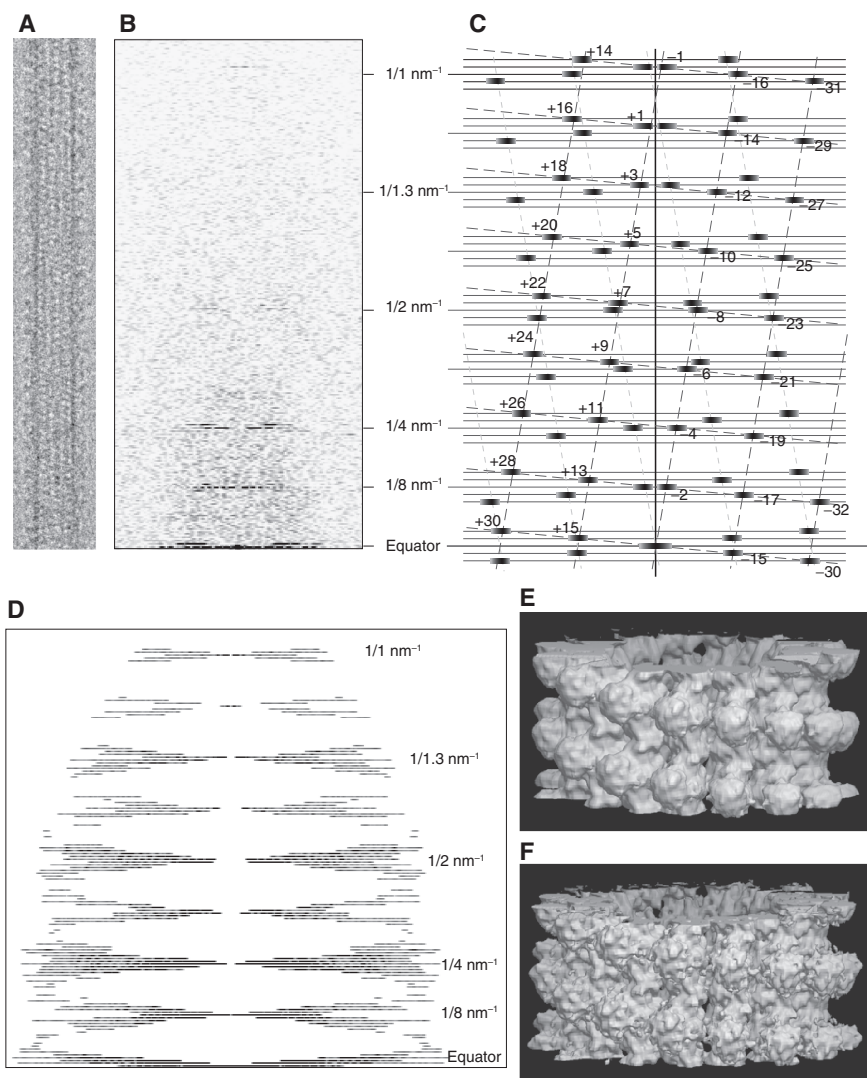


Fig. 5. 3D reconstruction of a kinesin–MT complex. (A) Part of the image in **Fig. 4C**, with a MT length of two repeat distances (approximately 300 nm). After interpolation of the digitized image so that the layer lines in its Fourier transform register exactly with the sampling raster, the final Fourier transform is calculated (B). The effect of image interpolation is evident: even though the size of the image in A is smaller than that in **Fig. 4C**, the layer lines in B look sharper than those in **Fig. 4D**, with the layer line at 1 nm^{-1} visible. For 15-protofilament MTs, layer lines are indexed (C). After the phases are corrected for the out-of-plane tilt and origin shift, data from different images are averaged. From the averaged layer line data, the area suitable for 3D reconstruction is extracted (D). (E) Surface representation of the 3D map of a MT decorated with a kinesin-family motor Kar3, calculated from the averaged layer lines in D. (F) 3D map calculated from the same averaged layer line data, but with the higher-order layer lines weighted, as described in **Subsection 3.5.4**.

2. Further refine the repeat distance and tilt, to make the layer lines as sharp as possible.
3. Calculate a Fourier transform of the refined box (**Fig. 5B**). Indexing of the layer lines from a 15-protofilament MT is shown in **Fig. 5C**.
4. Correct the out-of-plane tilt and origin-shift, by checking the phases of the peaks in the main layer lines.

3.5.3. Averaging of Data Sets and 3D Reconstruction

1. Adjust phases in the transform data from different images by allowing for an axial phase-origin shift and a rotation about the axis, so that they match, as well as possible, those of a reference image.
2. Correct each individual layer line data set for the contrast transfer function, which is determined by fitting a ring pattern to amplitudes in the Fourier transform of an area of empty carbon from the same micrograph as the MT (**27**).
3. Average the layer line data and select the range to be used for 3D reconstruction (**Fig. 5D**).
4. Calculate a 3D density map by Fourier–Bessel transformation (**Fig. 5E,F**).

3.5.4. Checking the Resolution of the Data

1. The individual data sets are sorted randomly into two groups, and two independent sets of average layer lines are calculated.
2. The layer lines of each set are interpolated to give a 2D array with values spaced at intervals that are the same as those between the layer lines (i.e., $1/c$, where c is the repeat distance in **Subsection 3.5.2**).
3. The array is used as input for the RF3 function in SPIDER, which calculates the Fourier shell correlation (FSC) coefficients as a function of R , the radius in Fourier space, as well as a critical value for each radial shell (**Fig. 6**; see **Note 13**).

3.5.5. Sharpening the Higher-Resolution Data

High resolution features may be sharpened (**Fig. 5F**) (see **Note 14**) by weighting the data as a function of R . We used a smooth linear function $(1 + 40R)$, where the reciprocal radius R is measured in $\text{\AA}^{-1}$.

3.6. Docking Crystal Structures into EM Maps

1. The program ‘O’ (**28**), or something similar, should be set up on a graphics workstation to view the structures in stereo.
2. A 3D map containing one kinesin + one tubulin dimer is calculated (**Fig. 7A–C**), and the EM map density is displayed as a mesh at a chosen contour level; then the crystal structures are docked into the mesh (**Fig. 7D–F**) (see **Note 15**).
3. Adjust the positions and orientations of the crystal structures while viewing the map in stereo and in different viewing directions, so that they fit best in all orientations. The contour level of the EM map can also be varied for checking the fit in

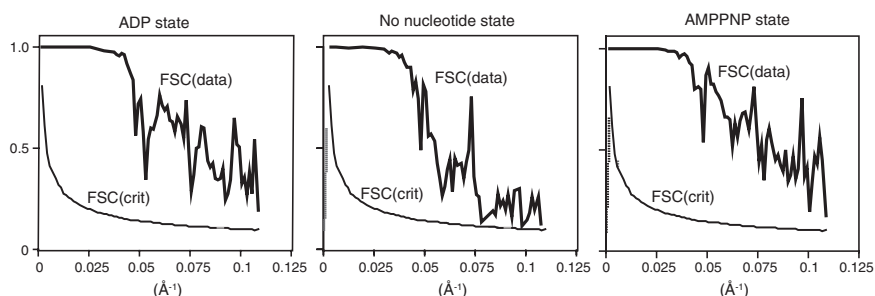


Fig. 6. Fourier shell correlation (FSC) coefficients plotted as a function of radius in reciprocal space ($\text{\AA}^{-1}$). Images were divided into two groups and their layer line data were averaged to give pairs of independent data sets. The average coefficients correlating equivalent points in the pairs of data sets were calculated for shells with a width of $0.005 \text{ \AA}^{-1}$. FSC(crit) is a theoretical critical minimum that depends on the number of data points in each shell (29,30). Points corresponding to the centers of the layer lines project well above the background, showing that the layer lines all contain significant signal.

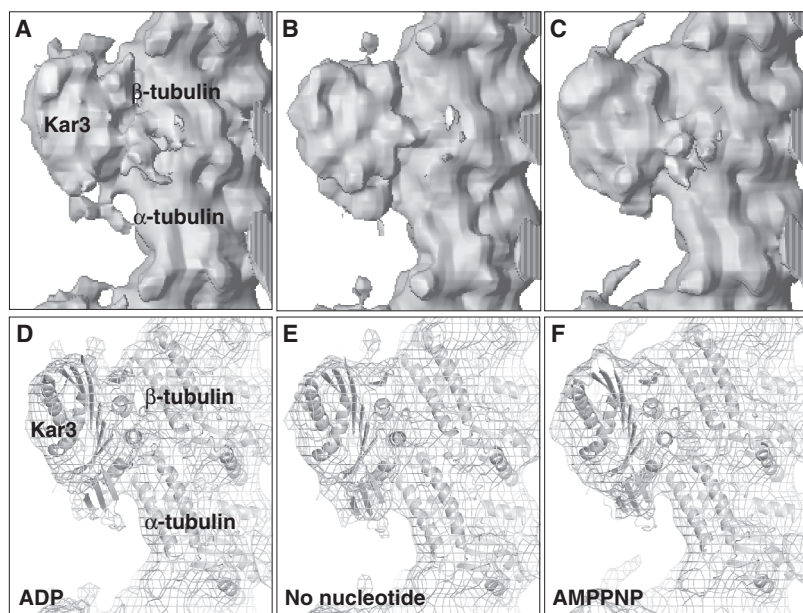


Fig. 7. Surface representation of the 3D map of Kar3 bound to a MT in the presence of ADP (A), absence of nucleotide (B), and in the presence of an ATP analogue, AMPPNP (C). The same density maps are displayed as a mesh (D–F), with the crystal structures of Kar3, α -tubulin, and β -tubulin docked into the mesh.

different regions of the map, as the protein density is weaker for more mobile parts of the molecules.

4. If the map has a high enough resolution, try dividing the crystal structure into several domains and docking them separately.

3.7. Calculation of Difference Maps

1. To identify significant differences, the maps being compared need to be on the same scale, as accurately as possible. We have followed Trachtenberg and DeRosier (31) in scaling each map to have an average density of 0.0 and a power (sum of the squares of the density values in the 3D array representing the map) of 1.0.
2. If the motor domains are very disordered in one or more states and thus appear to have a lower average protein density than the MT to which they are bound, it may be best to determine the scale factors only from regions containing tubulin.
3. One way to help to decide whether differences are significant (*see Note 16*) is to compare difference maps between different states with those between same-state maps, calculated from the independent halves of the data sets for the FSC computation.

4. Notes

1. The structure of the myosin–actin complex can be studied with the same helical methods because actin filaments have a helical symmetry. In the case of dynein, however, calculation of the 3D structures is more difficult, because a dynein head is too large to decorate each tubulin dimer, so that another method such as the single-particle analysis should be used.
2. For obtaining good 3D maps, it is critical to use MTs fully and regularly decorated with kinesin. At the same time, nonspecific binding of kinesin, aggregation of proteins, and bundling of MTs should be avoided so far as possible. Regularity of decoration can depend on minor differences in the kinesin construct, and the solution used for kinesin decoration. It is worth trying different constructs of kinesin, different concentrations of the proteins, and different buffers to find the best conditions for forming the most regular complexes.
3. For helical reconstruction, it saves time if the MT preparation contains a high proportion of 15 (or 16)-protofilament (pf) MTs. The proportion of 15- or 16-pf MTs increases when DMSO is added to the polymerization buffer (5). However, a high concentration of DMSO also increases formation of flat, tubulin sheets. As a compromise, we use 10% (or 15%) DMSO. The proportions of MTs with different protofilament numbers and that of tubulin sheets vary greatly between different tubulin preparations, even when the same concentration of DMSO is used. With 10% DMSO, the percentages of the 15-protofilament MTs we obtained were 25–45% in five different preparations. If your preparation does not contain enough 15-protofilament MTs, it is worth trying again. In our experience, use of freshly purified tubulin results in a good MT preparation without many sheets.

4. Addition of pure DMSO directly to tubulin tends to cause immediate polymerization of tubulin into sheets, so it is better to dilute DMSO first (e.g., to 50%) with PEM buffer.
5. GTP contained in the polymerization buffer can influence the binding state of kinesin to MTs and is better removed (*see Steps 5–7 in Subsection 3.1.*).
6. It is very difficult to dilute MTs uniformly without breaking them. We usually dilute them in two or three steps (*see Steps 2 and 3 in Subsection 3.2.*). Also, MTs diluted to a low concentration should be used within ~1 h, because they tend to depolymerize even when taxol is present.
7. Optimum dilution for cryo-EM can vary, depending on the MT preparation. For cryo-EM, it is better to check by applying the diluted MTs on a holey grid, blotting with a filter paper (without staining, just as you do for preparing a grid for cryo-EM), drying, and then observing under an EM using a high degree of defocus.
8. Be careful in handling liquid ethane. Wear goggles to protect your eyes.
9. When you handle frozen grids in liquid nitrogen, be careful to avoid water condensation in the liquid nitrogen as much as possible. Use of a dehumidifier helps, especially if you are in a humid country.
10. The time for blotting excess solution (*see Step 7 in Subsection 3.3.*) determines how thin the layer of vitreous ice will be and consequently influences the quality of the images. With experience, rather than timing the blotting, the expanding circle of absorbed liquid on the paper may be used as a guide, before the grid is rapidly plunged vertically into the ethane slush. We use the color of the circle as a guide. First, it looks transparent, because there is liquid between the grid and filter paper. Then, it becomes white, because a layer of air forms between the grid and the filter paper.
11. Sometimes the grid is covered with solidified ethane, which goes away in several days during storage in liquid nitrogen.
12. This process (*see Step 1 in Subsection 3.5.2.*) is important when you aim to calculate 3D maps at a high resolution; otherwise, the relative amplitudes of the layer lines are not correct (compare **Fig. 4D** and **Fig. 5B**).
13. The FSC plots show graphically how the amplitudes in the Fourier data drop dramatically after about 25 Å resolution. Noise affects the phases so that when different data sets are averaged as complex numbers the amplitudes of the average are lower than those of individual data sets and high-resolution features may not stand out very well. After sharpening, we found that ~10 Å features, already detectable in the unsharpened maps, were easier to see (compare **Fig. 5E** and **Fig. 5F**). Obviously, it is important to avoid oversharpening, which would mean the introduction of artifactual fine features.
14. We know that the data in individual data sets go out to a certain resolution because layer lines at that resolution are detectable above the background. However, data from different specimens will only agree if the contrast transfer functions have been properly corrected for, and we need to check that the averaged data are good to high resolution. The Fourier shell correlation (FSC) (**29,30**) was invented for

single (i.e., globular) particle reconstruction. When used for helical particles, data at radii corresponding to the central regions of the layer lines are seen to correlate better than points further out along the layer lines, producing a series of peaks above the falling background (see Fig. 6). This correlation reflects the nonuniformity of the resolution for long helical structures, which tend to produce higher resolution along the axis than in directions at right-angles to the axis. Most of the data are well above the critical level. In most cases, there are peaks out to 10 Å resolution that are above the (somewhat arbitrary) level of 0.5 which is often used to determine the resolution of single-particle reconstructions.

15. Docking of near-atomic models of tubulin and kinesin obtained by X-ray crystallography or nuclear magnetic resonance (NMR) into EM reconstructions of MTs has mainly been carried out manually. The alternative possibilities are semi-automatic fitting using a reduced-vector representation of both the model and the data (32) or, more directly, of density correlation (33,34).
16. Difference maps are most useful when some component is present in one map but not another or when a substantial feature moves its position as a rigid body. Fluid-like movements produce small but complex differences that may be less informative than simply comparing the maps directly.

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Chemical-Genetic Inhibition of Sensitized Mutant Unconventional Myosins

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Summary

The construction of a sensitized mutant unconventional myosin is an excellent method for determining the function of the individual myosin against a background of related myosins with partially overlapping functions. In this chapter, we outline the steps involved in sensitizing myosin by mutation and screening them against panels of nucleotide analogs, including transfection, microinjection, and actin co-sedimentation *in vitro*. We also describe conditions and considerations involved in designing functional experiments after a mutant and cognate analog have been identified. The powerful strategy of chemical genetics, when correctly applied to unconventional myosins, enables both the specific and selectable inhibition of the target motor with outstanding internal controls.

Key Words: ATP hydrolysis; chemical genetics; myosin-Vb; myosin-Ic; unconventional myosins.

1. Introduction

Myosins are the only family of actin-based molecular motors. Analyses suggest that there are at least 15 distinct classes within the myosin family (1,2), but the most detailed analysis to date revealed 37 different domain combinations (3). Unconventional myosins (all except class II, which includes the muscle myosins) have been implicated in vesicle transport, actin organization, cell motility, and apoptosis (4), but understanding of their functions has lagged behind their identification. The myosin-V family (5) and the myosin-I family (6,7) are the best studied classes of unconventional myosins and represent two of the three likely ancestral types (3). The “tail” or cargo-binding domains differ dramatically between families. The 80-kDa catalytic myosin “head” domain is responsible for coupling ATP hydrolysis to mechanical force through confor-

mational change, and it is well-conserved within the myosin superfamily; this is of immense value in applying our strategy to the dozens of myosin family members. This high homology allows us to align unconventional myosins and easily identify specific residues in the ATP-binding pocket to mutate (**Fig. 1**). To date, two unconventional myosins have been selectively and specifically sensitized to ADP analogs and introduced as transgenes or knock-ins in mice: myosin-1c (formerly myosin-I β) (**8**) and myosin-Vb (formerly myr 6) (**9**).

The chemical-genetic strategy allows very acute inhibition of a single myosin over a short time scale, with the use of stringent negative controls for possible side effects. Chemical genetics allows both the temporal and spatial application of inhibitors to dissect myosin function in multiple biological processes. It is particularly valuable as a complement to other techniques, such as dominant-negative overexpression.

Our strategy represents a reverse chemical-genetic approach because the sensitizing mutation is introduced genetically first into the protein before synthetic inhibitors are utilized. This method is opposed to forward chemical genetics, in which chemicals are first used to generate a phenotype in a given biochemical pathway (**10**). This strategy was first applied to protein kinases such as v-Src (**11**) and JNK (**12**) and has been an invaluable tool in the identification of their substrates. The similarity of kinase ATP binding to that of

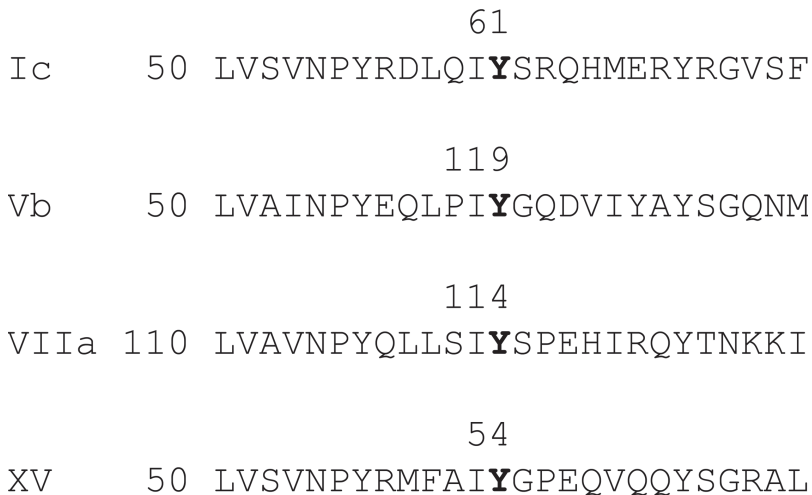


Fig. 1. Alignment of unconventional myosin sequences. The tyrosine from the ATP-binding pocket that has been (Ic and Vb) or will be (VIIa and XV) mutated in each sequence is in bold and labeled with a residue number above it.

myosins led to use of the strategy on these molecules as well. Here, the primary goal is the inhibition of the motor and its associated processes through tight actin binding, not substrate identification as with the kinases. The initial method (13) involved baculovirus expression of myosin-1c and in vitro screening of analogs before expression in transgenic mice (14). In this chapter, we present a more complete description of the more streamlined method we used for myosin-Vb (15), in which identification of an analog is accomplished by transfecting cultured cells and expression for additional in vitro characterization is accomplished by expression in MDCK cells.

2. Materials

2.1. Generation of Sensitizing Mutation

1. Alignment software.
2. Quik-Change site-directed mutagenesis kit (Stratagene).

2.2. Transient Mammalian Transfection for Single-Cell Analysis

1. Mammalian cell line, such as HeLa, grown at 5% CO₂ and 37°C in DMEM supplemented with 10% fetal calf serum and 1X Gluta MAX-1 (Gibco).
2. Plasmid preparation of mammalian expression vector (at least 1–2 µg) from a commercial kit (such as Qiagen's Plasmid Mini Kit).
3. Lipofectamine 2000 (Invitrogen).
4. OPTIMEM 1 (Gibco).
5. Sterile 0.5-mL microcentrifuge tubes.

2.3. Designing Chemical-Genetic Assays for Myosin Function: Inhibitor Microinjection

1. Transfected mammalian cells.
2. Coverslips or Delta TPG dishes (Fisher).
3. 10X microinjection buffer: 1 M KCl, 80 mM KPO₄, pH 7.4.
4. Nucleotide stocks for final cell concentrations of 1 mM ATP and 500 µM N⁶-substituted ADP analogs.
5. 10 mg/mL fluorescent dextran.
6. Eppendorf Microloader pipet tips.
7. Inverted microscope (Nikon TE-2000E).
8. Narishige micromanipulator.
9. Injector (Medical Systems Corp. PLI-100).
10. P-87 needle puller (Sutter Instrument).
11. BF100-78-10 borosilicate microcapillary tubing (World Precision Instruments).
12. Charge-coupled device (CCD) camera (Photometrics Quantix).
13. Imaging software/hardware (MetaMorph version 6.4 software on a standard laboratory PC).

2.4. Transient Mammalian Transfection and Expression for Protein Purification

1. 100-mm culture dish per expression construct, 70%–90% confluent with MDCK cells. Incubator conditions should be 10% CO₂ and 37°C, with the medium DMEM supplemented with 10% fetal calf serum and 1X GlutaMAX-1 (Gibco).
2. 10 µg plasmid preparation for mutant and wild-type mammalian expression constructs.
3. Lipofectamine 2000 (Invitrogen).
4. OPTIMEM 1 (Gibco).
5. Sterile 1.5-mL microcentrifuge tubes.

2.5. Protein Purification from Mammalian Cells

1. CLP protease inhibitor stock: 10 µg/mL each chymostatin, leupeptin, and pepstatin in DMSO.
2. Base lysis buffer: 150 mM KCL, 50 mM HEPES, pH 7.4, 1 mM ethyleneglycol-tetraacetic acid (EGTA), 1 mM MgCl₂, 1 mM Eagle's basic medium (BME), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/mL chymostatin, leupeptin and pepstatin (CLP).
3. Calmodulin (bovine).
4. Triton X-100.
5. ATP precomplexed with Mg²⁺.
6. Ultracentrifuge (Beckman L8–60M).
7. Cell scraper (Costar Cell Lifter).
8. Cell homogenizer (1/4 inch with a 15-µm bearing clearance).
9. Nickel affinity resin (HIS-Select HF nickel; Sigma).
10. Wizard minicolumns (Promega).
11. Wash buffers: 1.5 mL base lysis plus 1 mM ATP, 1.5 mL base lysis plus 10 mM imidazole, and 2 mL base lysis with 150 mM NaCl, respectively.
12. Elution buffer: 200 mM imidazole, 300 mM KCl, 20 mM HEPES, and 2 µM bovine calmodulin (commercially available or phenyl sepharose preparation; *see Note 1*).

2.6. Screening In Vitro: Actin Co-Sedimentation

2.6.1. Actin Co-Sedimentation Assay

1. Phalloidin-stabilized actin (from rabbit skeletal muscle).
2. Ultracentrifuge (L8–60M), cellulose propionate tubes (7 × 20 mm), and 42.2 Ti rotor (all Beckman).
3. 10X ATPase buffer: 10 mM MgCl₂, 1 mM EGTA, and 150 mM HEPES at pH 7.5.
4. ATP, ADP, and ADP analog stocks appropriate for final concentrations of 100 µM, 300 µM, and 300 µM, respectively.

2.6.2. Actin Co-Sedimentation Analysis

1. SDS-PAGE: 4%–20% 10 × 10 cm PAGEr Gold precast gels (Cambrex).
2. Hoefer Mighty Small II electrophoresis gel box and Mighty Slim SX250 power supply.
3. Western transfer apparatus (Idea Scientific Company Genie Blotter).
4. Nitrocellulose membrane.
5. Blocking solution: Tris-buffered saline (pH 8.0) plus Tween-20 (TBST) with and without 5% nonfat dried milk.
6. Ponceau S.
7. Appropriate primary (protein-specific or epitope tag-specific) and horseradish peroxidase (HRP)-conjugated secondary antibodies.
8. Pierce SuperSignal West Dura Extended Duration Substrate.
9. BioRad VersaDoc imaging system or X-ray film and cassette.

3. Methods

3.1. Generation of Sensitized Mutation

1. Align unconventional myosin protein sequence either manually or with the help of alignment software to the tyrosine residue corresponding to Y61 in myosin-1c or Y119 in myosin-Vb. This site is extensively, but not entirely, conserved throughout the superfamily (*see Note 2*).
2. Utilize a commercial kit such as the Quik-Change Kit (Stratagene) to introduce an amino acid substitution at the conserved tyrosine residue to a glycine by vendor standard protocol (*see Note 3*).

3.2. Transient Mammalian Transfection for Single-Cell Analysis

1. Grow cells to 50%–70% confluency under normal culture conditions.
2. Follow vendor protocol (Lipofectamine 2000; Invitrogen) for transfection.
3. Select in a G418 concentration that provides expression at less than 30% endogenous levels; for myosin-Vb, the concentration was 400 µg/mL for selection and 200 µg/mL for maintenance.

3.3. Designing Chemical-Genetic Assays for Myosin Function Inhibitor Microinjection

1. Grow mammalian primary or cultured cells either on coverslips or Delta TPG dishes.
2. (*See Note 4* for setup.) Make inhibitor solutions in 1X microinjection buffer and supplement to 10 mM ATP, 2.5 mM *N*⁶-substituted ADP analog, and 1 mg/mL fluorescent dextran.
3. Backload needles with Eppendorf Microloader pipet tips, typically with 1–3 µL clarified (10 min, 14 K rpm in minifuge) solution.
4. Microinject cells with balance pressure to approximately 10% cell volume of solution, the injections totaling five minutes in time (approximately 20–50 cells) (*see Note 9*).

5. Allow cells to recover for 10–20 min at 37°C before fixing [4% paraformaldehyde in 1X phosphate-buffered saline (PBS) for 5 min] and follow with standard immunocytochemistry techniques.
6. Continuously record single cells before and after the injection of inhibitor for live-cell imaging experiments. Controls include injecting wild-type cells with inhibitor and also injecting cells (wild-type versus knock-in or transgenic) with solutions minus analog.
7. Quantitate the assay. In our work with myosin-Vb, fluorescently labeled transferin was imaged (15). (See Note 5.)

3.4. Transient Mammalian Transfection for Protein Purification

1. Grow MDCK cells to 70%–90% confluency in at least a 60-mm plate (ideally, a 100-mm plate) per construct.
2. Aliquot 10 µg (for 100 mM culture plates) of plasmid in one tube with 1.5 mL OPTIMEM 1 and in another tube 1.5 mL OPTIMEM 1 with 50 µL Lipofectamine 2000.
3. Invert each tube several times, incubate 5 min, combine, mix by inverting again, and incubate an additional 20 min at room temperature.
4. Take the 70%–90% confluent plate(s) and trypsinize during this incubation period. Pellet the cells (144g for 3 min in a clinical centrifuge) and resuspend at 5 mL/plate in DMEM.
5. Add this 5 mL of cells with the 3 mL combined DNA/lipofectamine 2000/OPTIMEM 1 onto a 100 mM plate; put in incubator at 37°C.
6. Replace medium 4–6 h posttransfection if desired (see Note 6).

3.5. Protein Purification from Mammalian Cells (See Note 7)

1. Harvest 18–24 h transiently transfected cells in 1.5 mL base lysis buffer with a cell scraper.
2. Lyse in prechilled cell homogenizer (20 full passes) using 3-mL plastic syringes.
3. Add calmodulin to 2 µM, Triton X-100 to 0.5%, ATP to 1 mM, and an additional 150 mM KCl. After a short (5 min) ice incubation, clarify 30 min at 105,000g in a Beckman Ti70 or equivalent.
4. Remove supernatant and batch for 1 h at 4°C while shaking with 100 µL metal affinity resin slurry (HIS-Select HF) utilizing a Wizard minicolumn affixed to a 3-mL syringe.
5. After step 4, expel lysate gently through the column and wash resin in syringe with 1.5 mL base lysis plus 1 mM ATP, 1.5 mL base lysis plus 10 mM imidazole, and 2 mL base lysis with 150 mM NaCl, respectively.
6. Remove column from syringe and place in 1.5-mL tube to drain excess wash by gravity. Elute protein with 2 × 50 µL 200 mM imidazole, 300 mM KCl, 20 mM HEPES, and 2 µM calmodulin by gravity flow.
7. Pool fractions for in vitro screening.

3.6. Screening *In Vitro*: Actin Co-Sedimentation (See Note 8)

3.6.1. Actin Co-Sedimentation Assay

1. Perform reactions (30 μ L) in prechilled 7 $\times$ 20 mm tubes.
2. Aliquot 15 μ L purified motor eluate (from **Subsection 3.1.2.**) into the bottom of the centrifuge tube on ice (*see Note 9*).
3. Bring to 1–5 μ M phalloidin-stabilized actin (final concentration) with the appropriate volume of KCl-free 10X ATPase buffer to a total of 8 μ L and dot on side of a 7 $\times$ 20 mm tube (*see Note 10*).
4. Spin samples in a Beckman L8–60M with a NVT 65.2 rotor 20 min at 160,000g.
5. Remove supernatant and add to 5X sample buffer, then dissolve the pellet into the appropriate amount of 1X sample buffer.

3.6.2. Actin Co-Sedimentation Analysis

1. SDS-PAGE co-sedimentation samples, supernatants and pellets, on a 4%–20% gradient gel at 100 V until the dye front has run 3/4 distance of gel length.
2. Transfer protein from the acrylamide gel by submerged Western blot (Idea Scientific Company Genie Blotter) in transfer buffer (typical Tris-base, glycine, 20% MeOH, without SDS), onto a nitrocellulose membrane, for 90 min at 18 V. Lanes can be marked by transient Ponceau S staining for 3 min, followed by a 1-min distilled water wash.
3. Block membrane for 1 h at room temperature or overnight at 4°C in TBST with 5% nonfat dried milk.
4. Probe with primary antibody for 1 h at room temperature, wash 3 $\times$ 10 min with TBST, apply HRP-conjugated secondary antibody for 45 min at room temperature, and again wash for 3 $\times$ 10 min in TBST.
5. Expose blot to film in a cassette for 1–5 min or longer, depending upon signal intensity.
6. Quantitate supernatant and pellet bands on a BioRad VersaDoc imaging system, using the rectangular tool to delineate protein bands.
7. Normalize quantitation to protein load through actin level.

4. Notes

1. Bovine calmodulin is readily available commercially. However, we purified our calmodulin by phenyl Sepharose chromatography from bovine testis lysate (**16**). This preparation does not contain the impurities associated with most commercial calmodulin isolated from bovine brain.
2. To this point, glycine substitutions of tyrosine have worked for myosin-Vb and -1c. However, this may not prove to be the case for all unconventional myosins. If the glycine substitution fails to sensitize and/or yield a functional myosin, our standing strategy is to mutate tyrosine to the next larger amino acids, for example, alanine, valine, or serine, to create a suitably-sized ATP-binding pocket that will sensitize the myosin.

3. Site-directed mutagenesis (considerations for large plasmids). Try to keep the completed plasmid under 12 kbp. We also introduce or destroy a restriction site near the substitution for PCR screening and add differential restriction sites at the 5'- and 3'-ends for polarity.
4. Our equipment includes an inverted Nikon TE-2000E microscope fitted with a Narishige micro-manipulator (Nikon), in turn fed by a Medical Systems Corp. PLI-100 injector. Injection needles were pulled on a model P-87 from Sutter Instrument Company, utilizing a 4.5-mm trough filament to generate a "bee stinger" morphology with tips less than 1 μm from 1-mm borosilicate microcapillary tubing (BF100-78-10; WPI).
5. Our imaging facility uses a Photometrics Quantix CCD camera (12-16 bit) with MetaMorph version 6.4 software to acquire and process still or time-lapse imaging.
6. Many cell lines will tolerate lipofectamine (and other) transfection reagents overnight. If the cells detach or look unhealthy the next day, it may be advantageous to replace the medium 4-6 h posttransfection. We have not observed any decrease in efficiency by changing medium.
7. An alternative to mammalian protein purification is the recombinant baculovirus system, which allows high efficiency of transfection. However, light chains (calmodulin) must be provided by additional constructs. For specifics on this purification from recombinant baculovirus-infected Sf9 cells, *see* **ref. 13** for reagents and methods.
8. Another possibility for in vitro screening is motility or actin gliding. In vitro motility (IVM), although notoriously fickle, allows a clear readout of motor function and is a very informative approach. For protocols, see the biotinylated myosin IVM assay adapted by Ostap's group (**17**) from Kron and Spudich (**18**).
9. All other solutions (nucleotides and buffer) are dotted onto the side of the tube and are combined only during centrifugation. This step keeps incubation time to a minimum and lowers the chances of the myosin hydrolyzing ATP and increasing ADP concentration, which shifts the binding equilibrium into the actin pellet.
10. 100 μM ATP (precomplexed with Mg^{2+}) is added alone, with 300 μM ADP, or with 300 μM ADP analog to a total of 7 μL , and dotted. The ATP reaction alone should keep the myosin in the supernatant, ATP/ADP should shift the myosin to the pellet, and if the ADP analog is a selective inhibitor, it will shift only the mutant myosin to the pellet.

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