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# In Vitro Reconstitution of Endosome Motility Along Microtubules

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## 1. Introduction

In higher eukaryotic cells, the secretory and endocytic membrane systems are comprised of a series of biochemically and morphologically distinct compartments. Dynamic associations with cytoskeletal networks have been shown to mediate both membrane transport between these compartments as well as maintenance of their characteriztic spatial distributions within the cell. More specifically, in endocytosis, association with microtubules and the action of microtubule-associated motor proteins are necessary for the proper positioning of late endosomes, lysosomes, and the pericentriolar recycling compartment (*1–3*). In addition, efficient transport of cargo between early and late endosomes, and transcytosis in polarized epithelial cells, also depend on microtubule-associated motors (*4,5*).

In order to study the molecular mechanisms responsible for regulating the interactions between endocytic organelles and microtubules, we, along with others (*6*), have developed assays to measure endosome motility on and association with microtubules in vitro. In this chapter, we will outline how we reconstitute and measure movement of endocytic organelles specifically labeled with fluorescent markers along fluorescently labeled microtubules in vitro, and we discuss some of the advantages and pitfalls of these techniques.

Generation of the components necessary to perform an assay to measure motility of organelles along microtubules in vitro can essentially be split into two parts. The first is the generation of cytosolic and organelle fractions. Although the exact protocols necessary for the generation of these fractions may vary, buffers and pH requirements for the maintenance of important biological functions, such as motor activities, in these fractions can serve as useful

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guides when developing motility assays with novel components. As we are interested in endosome motility along microtubules we will describe the methods we use to isolate this membrane fraction. The second is the generation of microtubules and assay chambers used in reconstituting in vitro organelle motility. These protocols should be widely applicable for visualization of movements on microtubules regardless of what membranes or molecules the investigator wishes to study. We have therefore organized the chapter into two discrete sections. The first describes the isolation of cytosol and fluorescently labeled early endosomes, and the second describes construction of the reaction chamber and synthesis of fluorescently labeled microtubules.

## 1.1. Preparation of HeLa Cytosol and Endosomal Membranes

### 1.1.1. Growth and Harvest of Cells

We use HeLa tissue culture cells that have been adapted for growth in suspension culture (s-HeLa) as the source of cytosol and early endosome fractions for our motility assays. The primary benefit of growing cells in suspension culture is the increase in cell density, which allows large numbers of cells to be grown in manageable quantities of growth medium. In addition, as the cells do not have to be released from the substrate, cell isolation is much easier. These protocols were adapted from earlier ones *(7)*, with the primary differences being slight alteration of salt concentrations of the isolation buffer and inclusion of ATP during isolation of cytosol. We generally grow 2–4 L of cells at a time ($[2–4] \times 10^9$ cells) for isolation of cytosol or endosome membranes, from which we obtain enough material for several months of in vitro motility experiments.

### 1.1.2. Internalization of Fluorescently Labeled Markers

Pioneering assays in which vesicle motility was reconstituted on microtubules relied on light microscopy to visualize vesicle movements *(8,9)*. Although these experiments elegantly demonstrated that membrane-bounded organelles did indeed move along microtubules and set the standard for further experimentation, they suffered from the fact that few enriched membrane fractions are completely pure. As a result, clear association of motility events with specific membrane-bounded compartments was difficult to confirm. We have attempted to overcome this complication by marking specific endocytic compartments using fluorescently labeled substrates, such as rhodamine-labeled transferrin, which can simply be included in the cell medium and are efficiently internalized by the cell. Because the rates of internalization and kinetics of association of substrate molecules have been extensively characterized for the endocytic pathway *(10)*, we have been able to utilize this technique to specifically label different compartments in the endocytic pathway. We have primarily used rhodamine-labeled transferrin to label early endosomes; however, fluorescent conjugates of late endosomal and lysosomal markers, such as epithelial growth factor, and low-density lipoprotein are commercially available and these compartments theoretically could also be easily labeled using similar methods. Earlier studies used internalized latex beads that fluoresce faintly in order to visualize phagocytic compartments *(6)*. Finally, we have also had some success using fusion proteins between small GTPases of the Rab family and green fluorescent protein to fluorescently label endocytic compartments (B. Sönnichsen, unpublished results).



## 1.2. The Motility Assay

### 1.2.1. Preparation of the Microscope Chamber

We observe motility utilizing an easy to make microscope perfusion chamber consisting of microscope slides and glass cover slips sealed on two sides with grease containing glass spacer beads *(11)*. We generally use between 5 and 10-μL chamber volumes. Leaving two sides of the chamber open allows for easy addition and exchange of reaction buffers. Exchange of solution can be performed by wicking up the solution from one side of the chamber with a torn piece of Whatman filter paper while simultaneously adding new buffer to the opposite side with a pipetman.

### 1.2.2. Synthesis of Fluorescently Labeled Microtubules

We synthesize microtubules in one of two ways depending on whether we wish to analyze the extent of motility of an organelle fraction or the direction(s) in which organelles travel along microtubules. If we want to quantify the extent of motility that an organelle fraction exhibits, it is important that, first, the number of motility events that can be observed per experiment and, second, the reproducibility of this number from one experiment to the next are optimized. In both of these cases, an evenly distributed and relatively dense carpet of microtubules upon which the organelles move is required. Obtaining this is most easily done with Taxol-stabilized microtubules polymerized in vitro from purified tubulin. If, however, we want to determine the directionality of this movement, having a source of microtubules with easily identified plus and minus ends is most important. In this case, we nucleate microtubule asters on purified centrosomes stuck to the surface of the microscope chamber. In either case, to visualize microtubules by fluorescence microscopy we utilize fluorescently conjugated tubulin and polymerize microtubules using purified components. Protocols describing the purification of tubulin *(12)* (*see also* Chapter 1), its subsequent modification with fluorescent molecules *(13)*, and purification of centrosomes *(14)* have been described in detail elsewhere and, therefore, will

not be described here. In vitro synthesis of fluorescently labeled microtubules is performed essentially as described *(15)*.

#### 1.2.3. Reconstituting Early Endosome Motility on Microtubules

Photobleaching is a major problem encountered during the extended illumination of fluorescently labeled microtubules and organelles that occurs while recording motility events. In order to minimize photobleaching, we utilize both a shuttered light source in combination with a sensitive video camera, and an oxygen scavenging system originally used for observation of actin filaments *(16)* and subsequently modified for use in microtubule-based motility assays *(6)*. This antifade buffer consists of a mixture with catalase, glucose with glucose oxidase, β-mercaptoethanol, and hemoglobin.

#### 1.2.4. Videomicroscopes and Data Acquisition

A variety of different light microscopes and video cameras can be used to view fluorescently labeled specimens. In essence, all one needs is a microscope with epifluorescent illumination and appropriate filters. We have used Zeiss Axioskop and Axiovert microscopes fitted with 100X/1.40 plan-Apochromat lenses. In order to reduce the amount of photobleaching during time-lapse imaging, we use a computer-controlled shutter with illumination by a 100-W mercury arc lamp attenuated with heat-reflection and heat-absorbance filters. At present, several commercial software packages are available that allow for computer control of attached video cameras. We use a relatively simple system consisting of a COHU 4910 series video camera with on-chip integration controlled by the NIH-Image software package. This camera and software combination is relatively inexpensive, yet sufficient for most imaging requirements. For more detailed information, we refer the reader to the NIH Image website, http://rsb.info.nih.gov/nih-image/Default.html.

## 2. Materials

### 2.1. Preparation of HeLa Cytosol And Endosomal Membranes

#### 2.1.1. Growth and Harvest of Cells

1. s-HeLa cells.
2. s-MEM containing L-glutamine, nonessential amino acids (Biochrom KG, Berlin. cat. no. K-0293), fetal calf serum (5%), penicillin (10 IU/mL), and streptomycin (10 µg/mL) (store at 4°C).
3. Trypsin–EDTA (Sigma Chemical Co., cat. no. T-3924; store at 4°C).
4. Phosphate-buffered saline (PBS) buffer.

#### 2.1.2. Internalization of Fluorescently-Labeled Transferrin

1. $CO_2$-independent modified Eagle's media (MEM) plus 0.2% bovine serum albumin (BSA) (store at 4°C).



2. Transferrin from human serum, conjugated to tetramethylrhodamine or fluorescein (Molecular Probes Inc., cat. nos. T-2872, and T-2871).
3. SIM buffer: 250 m*M* sucrose, 3 m*M* imidazole, 1 m*M* $MgCl_2$ (pH 7.4) (store at 4°C).

#### 2.1.3. Preparation of Cytosol

1. KEHM buffer: 110 m*M* KCl, 50 m*M* HEPES–KOH (pH 7.4), 2 m*M* $MgCl_2$, 2 m*M* Mg–ATP (make fresh).
2. 1000× CLAAP in dimethyl sulfoxide (DMSO): chymostatin (6 mg/mL), leupeptin (0.5 mg/mL), antipain (10 mg/mL), aprotinin (0.7 mg/mL), pepstatin A (100 mg/mL) (store at –20°C).
3. 1000× p-amidinophenyl-methanesofonyl fluoride; (APMSF) (10 mg/mL) in DMSO (store at –20°C).

#### 2.1.4. Cell Cracking

Ball-bearing Cell Homogenizer 8.020-mm size with 8.002-mm ball bearing (EMBL Workshop, Heidelberg, Germany).

#### 2.1.5. Purifying Endosome Membranes

1. 62% (v/v) sucrose solution: 80.49 g sucrose, 3 m*M* imidazole in 100 mL $H_2O$ (make the night before).
2. 35% (v/v) sucrose solution: 40.3 g sucrose, 3 m*M* imidazole in 100 mL $H_2O$ (make the night before).
3. 25% (v/v) sucrose solution: 27.59 g sucrose, 3 m*M* imidazole in 100 mL $H_2O$ (make the night before).

### 2.2. The Motility Assay

#### 2.2.1. Preparation of the Microscope Chamber

1. Microscope slides (76 mm × 26 mm × 0.8/1.0 mm thick; Select Micro Slides, washed; Chance Propper Ltd.,Warley, UK, cat. no. KTH 360).
2. Glass cover slips (18 mm × 18 mm, No.1; Clay Adams, Gold Seal, Reorder No. 3305).
3. Grease with glass bead spacers: Apiezon M (Carl Roth GmbH+Co D7500 Karlsruhe 21, Art. 1683). Glass beads (200–300 µm, acid washed; Sigma Chemical Co., cat. no. G-1227).

#### 2.2.2. Synthesis of Fluorescently Labeled Microtubules

1. Purified, cycled, unlabeled tubulin (20 mg/mL; store at –80°C).
2. Purified, cycled, fluorescently labeled tubulin (20 mg/mL; store at –80°C).
3. Purified centrosomes (store at –80°C).
4. 10 m*M* GMP–CPP (store at –20°C).
5. 0.1 *M* GTP (store at –20°C).

6. 10 m*M* taxol in DMSO (store at –20°C).
7. BRB80 buffer: 80 m*M* K–piperazine-*N,N-bis*[2-ethanesulfonic acid] (PIPES) (pH 6.8), 2 m*M* $MgCl_2$, 1 m*M* EGTA (store at room temperature).
8. G-buffer: 1 m*M* GTP in BRB80 (make fresh).

#### 2.2.3. Reconstituting Early Endosome Motility on Microtubules

1. Antifade buffer: 10 μ*M* taxol (from 10 m*M* stock; store at –20°C), 10 m*M* glucose (from 1 *M* stock; store at 4°C), 4 m*M* $MgCl_2$, 50 μg/mL glucose oxidase (from 10 mg/mL stock; store at –20°C), 50 μg/mL catalase (from 10 mg/mL stock; store at –20°C), 0.1% of 2-mercaptoethanol (store at –20°C), into 1 mL BRB80, sit at 37°C for 1–2 min prior to use (make fresh).
2. Bovine hemoglobin (30 mg/mL; make fresh).

## 3. Methods

### 3.1. Preparation of HeLa Cytosol and Endosomal Membranes

#### 3.1.1. Growth and Harvest of Cells

1. Grow four confluent medium-sized flasks (175 $cm^2$) of s-HeLa in s-MEM.
2. To release the s-HeLa cells from the flask, incubate with trypsin–EDTA at 37°C until the majority has been released into the medium. Add just enough volume to cover the surface of the flask. Then, add these cells to 400 mL of s-MEM media in a 1 L spinner flask. Place this flask on a stirrer at 37°C for 24 h.
3. The next day, transfer these spinning cells into a 3 L spinner flask containing 2 L of fresh s-MEM media.
4. Grow the cells on a stirrer at 37°C until cells reach a density of 0.8–1.2 × $10^6$ cells/mL. This should take 4 d. If the cells are too dense they will not internalize, so dilute them and grow into log phase again.
5. Collect the cells by sedimentation in a Sorvall GS-3 rotor at 4500*g* for 10 min at room temperature.
6. Gently resuspend the cell pellet in PBS, swirling the flask by hand until the pellet is in solution. It is okay if there are still large chunks of aggregated cells, as long as the pellet has released from the flask.
7. Spin again in a Sorvall GS-3 rotor at 4500*g* for 10 min at room temperature.
8. Again, gently resuspend the cell pellet in 50 mL of PBS, transfer this slurry to a 50-mL Falcon tube and spin in a Sorvall tabletop centrifuge at 3000*g* for 10 min at room temperature. If preparing endosome membranes, continue with internalization protocol. If preparing cytosol, proceed directly to **Subheading 3.1.3.**

#### 3.1.2. Internalization of Fluorescently-Labeled Transferrin

1. Transfer the cell pellet to 10–15 mL prewarmed $CO_2$-independent MEM plus 0.2%BSA containing 30 μg/mL transferrin conjugated to rhodamine or fluorescein-isothiocyanate (FITC).
2. Incubate the cells in a 37°C water bath for 10 min and then dilute the cells to 50 mL with ice-cold SIM buffer.



3. Spin down the cells at 4°C in a refrigerated tabletop centrifuge at 3000*g* for 10 min.
4. Wash the cells with ice-cold SIM buffer and resediment cells in tabletop centrifuge at 4°C twice more.
5. Resuspend the final cell pellet in 2 cell volumes of ice-cold SIM buffer containing freshly added 1× CLAAP, 1× APMSF, and 1 m*M* dithiothreitol (DTT). Proceed to **Subheading 3.1.4.**

#### 3.1.3. Preparation of Cytosol

Resuspend cells in 0.7 vol of ice-cold KEHM buffer to which 1× CLAAP, 1× APMSF, and 1 m*M* DTT have recently been added. Proceed to **Subheading 3.1.4.**

#### 3.1.4. Cell Cracking

1. Assemble the cell cracker (made at EMBL) using ball-bearing size 8.002 and two 5- or 10-mL syringes, depending on the cell volume. Precool the cracker on ice, or in a cold room.
2. Passage the cells through the cell cracker six or seven times, applying quick, short bursts of pressure to the syringe plungers while frequently alternating the direction of flow through the cell cracker (*see* **Note 1**).
3. Check under a phase-contrast microscope to assess the degree of cell breakage. To do this, simply unscrew one of the syringes from the cell cracker and remove a few microliters of sample with a pipetman (*see* **Note 2**).
4. Continue with cell breakage until the majority of cells are broken. Typically, we can observe up to 90% breakage after 10–13 passages. However, more or less passages will be required depending on the strength of the researcher.
5. If endosome membranes are to be isolated, proceed to **Subheading 3.1.5.** If cytosol is to be prepared, spin the cell homogenate in a Beckmann TLA100.4 rotor at 278,000*g* for 30 min at 4°C. Remove the supernatant, saving an aliquot for protein concentration determination, and snap-freeze the rest in liquid nitrogen. Aliquots of snap-frozen cytosol should be stored at –80°C.

#### 3.1.5. Purifying Endosome Membranes

1. Transfer the cell homogenate from **step 4** of **Subheading 3.1.4** to a 15- or 50-mL Falcon tube and sediment nuclei and unbroken cells in a Sorvall tabletop centrifuge at 4000*g* for 20 min at 4°C.
2. Collect the postnuclear supernatant (PNS) (*see* **Note 3**).
3. Using a refractometer, adjust the sucrose concentration of the PNS fraction to 40.6% (v/v) with an ice-cold 62% (v/v) sucrose stock solution. Begin the adjustment by adding 600 μL of 62% sucrose per 500 μL PNS.
4. Split the density-adjusted PNS obtained from 2.4 L of spinner culture into six SW40 tubes using a syringe to apply equal amounts to the bottom of each tube. Carefully overlay these fractions with 4 mL of ice-cold 35% (v/v) sucrose solu-

tion, followed by 4 mL of ice-cold 25% (v/v) sucrose solution. Balance the tubes by adding to or removing from the 25% sucrose layer.
5. Spin these tubes in a SW40 rotor at 220,000*g* for 6 h at 4°C and collect the early endosome band at the 25–35% sucrose interface (*see* **Note 4**).
6. Aliquot the early endosomes in 200-µL fractions, saving a portion for protein concentration determination, and snap-freeze the rest in liquid nitrogen.

## 3.2. The Motility Assay

### 3.2.1. Preparation of the Microscope Chamber

1. Warm approx 20 g Apiezon M grease on a heating plate until the viscosity is low enough that the grease is easy to stir. Add 2 g of 200- to 300-µm glass beads and stir until they are well mixed. Pour the mixture of grease and beads into a syringe tipped with blunted 18-gage needle. Push in the plunger and remove the majority of trapped bubbles. Let the grease cool to room temperature. This grease dispenser can be stored at room temperature and will last for several months worth of experiments.
2. Extrude two lines of grease onto a glass cover slip about 1.5 cm apart and place a cover slip on top. Gently tap the cover slip with a pipet tip to generate an even seal with the grease lines (*see* **Note 5**).
3. At this point, 5 µL of previously synthesized microtubules (*see* **Subheading 3.2.2.1.**) can be added to the perfusion chamber. Alternatively, 5 µL purified centrosomes can be added for the nucleation of microtubule asters (*see* **Subheading 3.2.2.2.**). Gently tap the cover slip down with a pipet tip to reduce the chamber volume until the 5-µL reaction fills the entire chamber.
4. If prepolymerized microtubules are being used, let the chamber sit at room temperature for 5 min to allow nonspecific sticking of the microtubules to the glass surfaces of the chamber, then proceed to **Subheading 3.2.3.** If microtubule asters are being nucleated, proceed to **Subheading 3.2.2.**2.

### 3.2.2. Synthesis of Fluorescently Labeled Microtubules

#### 3.2.2.1. Synthesis of Polarity-Marked Microtubules In Vitro

1. To synthesize brightly labeled microtubule seeds, dilute 1 µL of freshly thawed, fluorescently labeled tubulin (20 mg/mL) into 5 µL BRB80 and add 0.5 µL of 10 m*M* GMP–CPP (*see* **Note 6**). Incubate this mix for 10–20 min in 37°C water bath.
2. Meanwhile, add 10 µL freshly thawed 0.1 *M* GTP into 1 mL of BRB80, producing 1 mL G-Buffer. Take 30 µL of G-Buffer and mix with 10 µL freshly thawed, cycled tubulin (20 mg/mL) and 1 mL freshly thawed, fluorescently labeled tubulin. Add this mixture to the brightly labeled microtubule seeds and incubate at 37°C for an additional 20–40 min.
3. While the microtubules are polymerizing, dilute 1 µL of 10 m*M* taxol dissolved in DMSO into 1 mL BRB80. Vortex this solution well to ensure proper suspension of the taxol (*see* **Note 7**). Prewarm a Beckmann airfuge rotor to room temperature (*see* **Note 8**).



4. Remove the polymerized microtubules from the 37°C water bath and dilute into 200 µL of BRB80 +10 µ*M* taxol solution. If polymerization was successful, one should observe a marked viscosity of the microtubule-containing solution. Transfer the diluted microtubules to an airfuge tube, make a balance tube, and sediment the microtubules at 30 psi for 5 min at room temperature.
5. Aspirate the supernatant and gently resuspend the pellet in 50 µL of BRB80 + 10 µ*M* taxol. These microtubules are now stable for up to 1 wk at room temperature. To minimize photobleaching, wrap the storage tube with aluminum foil.

#### 3.2.2.2. In Situ Nucleation of Microtubule Asters on Purified Centrosomes

1. Perfuse 5 µL of purified centrosomes diluted to preferred concentration in BRB80 into a microscope chamber (described below). Incubate the chamber in an inverted position for 1–2 min to allow centrosomes to stick to the glass cover slip.
2. Mix 1 µL of fluorescently labeled, cycled tubulin (20 mg/mL) with 10 µL of unlabeled cycled tubulin (20 mg/mL) and dilute into 50 µL of G-Buffer. Perfuse 5 µL of this mixture into the microscope chamber using a ripped piece of Whatman filter paper; wick out the preexisting chamber fluid on the opposite side.
3. Incubate the chamber in a humid box at 37°C for 20 min to polymerize the microtubules.
4. Perfuse the chamber with 10 µL of BRB80 + 10 µ*M* taxol to remove unpolymerized tubulin and stabilize the microtubules. The microscope chamber can now be stored at room temperature for several hours.

### 3.2.3. Reconstituting Early Endosome Motility on Microtubules

1. Prepare fresh antifade buffer, hemoglobin, and BRB80 + 10 m*M* taxol solutions.
2. Mix 4 µL antifade buffer, 1 µL hemoglobin (30 mg/mL), 2 µL HeLa cytosol (10–15 mg/mL), and 3 µL of fluorescently labeled early endosomes (5–7 mg/mL), and perfuse all 10 µL into a microscope chamber preloaded with microtubules or microtubule asters.
3. Place a drop of immersion oil on the glass cover slip and visualize fluorescent endosomes and microtubules with appropriate immunofluorescence filters on a time-lapse video microscope. Examples of endosome motility on in vitro synthesized microtubules or microtubule asters are shown in **Figs. 1** and **2**, respectively.

## 4. Notes

1. Avoid the inclusion of bubbles in the syringes, as they can lead to a vapor lock of the cell cracker. Also, as high pressures are generated within the cell cracker apparatus during this procedure, it is important to wear protective goggles or glasses to prevent injury if a syringe breaks.
2. It is good to save a small aliquot of unbroken cells to compare against those processed in the cracker. Intact cells can be distinguished from broken cells by presence of cytosol around a highly refringent nucleus. Alternatively, a drop of

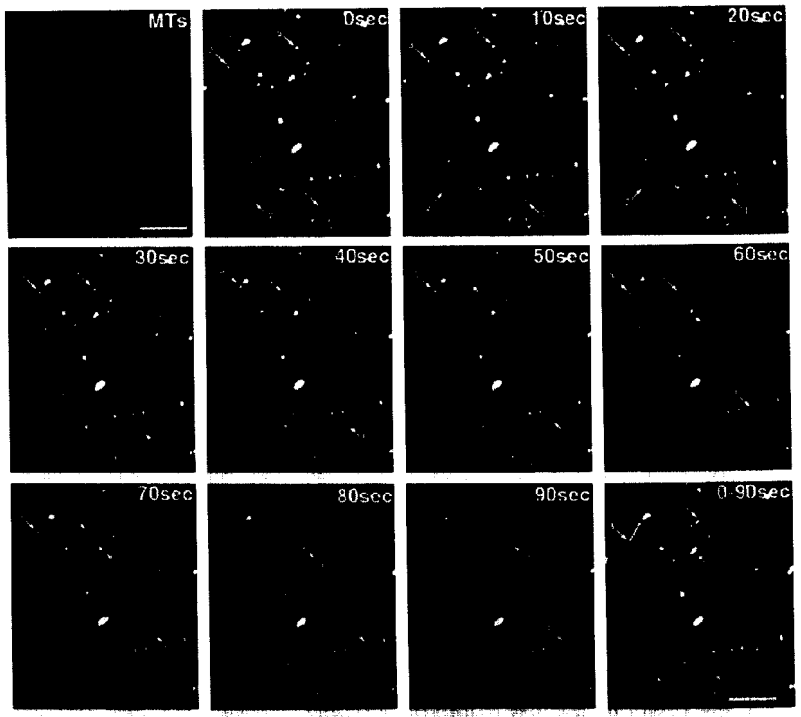


Fig. 1. Fluorescently labeled endosomes moving along in vitro synthesized microtubules. Oregon-green labeled microtubules (MTs) were adsorbed to a glass cover slip. Images of rhodamine–transferrin-labeled endosomes are shown at 10-s intervals. Moving endosomes are indicated by numbered arrows. In the final panel, a merged stack of consecutive images of fluorescently labeled endosomes (2-s intervals) is superimposed on an image of the microtubules. When represented in this manner, moving endosomes produce a series of closely spaced dots that line up with fluorescent microtubules. Scale bar: 5 μm.

1% trypan blue solution dissolved in water can be applied to the cells. Nuclei from broken cells will stain blue, whereas those of unbroken cells remain clear.

3. If it is difficult to distinguish between the supernatant and the pellet fractions, hold the tube up to a light source to help observe this interface.
4. To more easily see the endosomal membranes, place the tubes in a rack in front of a piece of black paper. We typically use a straight metal tube connected to tubing threaded through a peristaltic pump to collect the interface fractions.



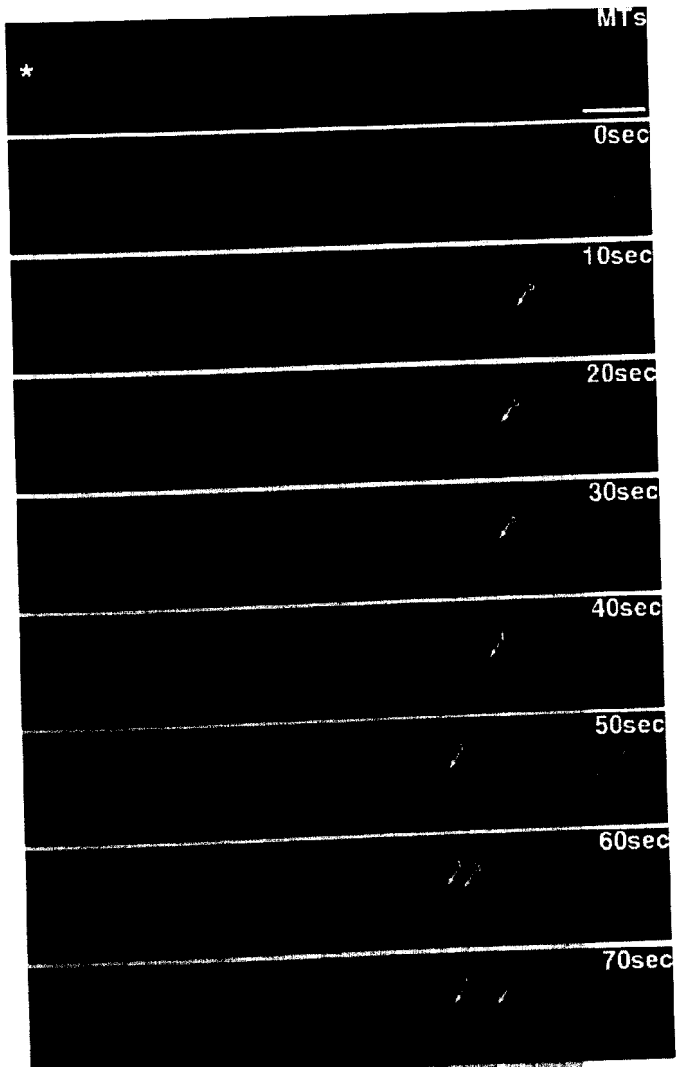


Fig. 2. Fluorescently labeled endosomes moving along microtubule asters toward both plus and minus ends. Oregon-green labeled microtubule asters (MTs) were nucleated on centrosomes (asterisk) adsorbed to a glass cover slip. Images of rhodamine–transferrin-labeled endosomes are shown at 10-s intervals. Moving endosomes are indicated by numbered arrows. *(continued)*

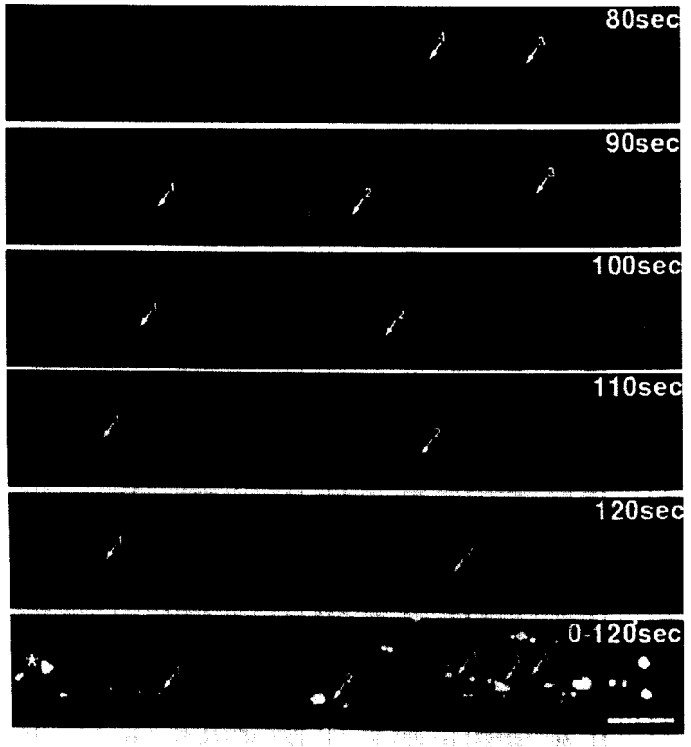


Fig. 2. *(continued)* In the final panel, a merged stack of consecutive images of fluorescently labeled endosomes (2-s intervals) is superimposed on an image of the microtubule aster. Scale bar: 5 µm.

5. When applying the grease, it is better to apply too much than too little as the chamber can be tapped down to create the proper reaction volume. The flow of buffer through the chamber and, by extension, the extent of washing in any given area of the chamber will be affected by its cross-sectional area; therefore, it pays to make the chambers as similar to one another as possible. To accurately lay the two lines of grease at set distances apart, we have found it useful to generate the chamber on a piece of graph paper using the underlying grid to apply the grease lines 1.5 cm apart.
6. GMP–CPP is unfortunately not available commercially and must be synthesized *(17)*. Alternatively, short microtubule seeds can be synthesized in the presence of glycerol *(15)*.



7. Taxol does not remain active through several freeze–thaw cycles; therefore, we store at the 10 m*M* taxol stock solution in 1-µL aliquots at –20°C. Additionally, as at cold temperatures, taxol precipitates out of aqueous solutions, always store at these tubes at room temperature.
8. Alternatively, microtubules can be sedimented by spinning at 100,000g in a Beckmann TLA 100 rotor for 5 min at 37°C.

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# Approaches to Study Interactions Between Kinesin Motors and Membranes

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## 1. Introduction

Determining the subcellular location of molecular motors is an essential step in understanding their function and regulation. Although much attention has been focused on the ability of kinesin-like motors to bind reversibly to microtubules, the interaction of this family with cargo has not been extensively studied. Thus, the interactions of kinesin with membrane-bound organelles (MBOs) remains poorly understood, although kinesins are widely thought to serve as motors for movement of MBOs (*see also* Chapter 12). Methods presented in this chapter were developed to study the regulation of the membrane association of kinesins.

Immunofluorescence, nerve ligation, and pulse label axonal transport studies suggest that conventional kinesin is bound to MBOs ***(1–3)***. In standard subcellular fractionation studies, however, the majority of kinesin is recovered in soluble cell fractions ***(4)***. A possible explanation for this discrepancy between biochemical and whole-cell approaches may be that the regulation and proper compartmentalization of activities that control kinesin association with membranes in intact cells becomes disrupted during tissue homogenization. As a result, during biochemical fractionation, kinesin may become accessible to improperly regulated/localized activities that artifactually increase the amount of kinesin in the soluble fraction. Although this is useful for the purification of motor proteins, the release of kinesin from normal cargoes hampers the study of cellular functions for kinesin. By carefully controlling extraction conditions and modulating enzymatic activities that can affect protein–protein interactions during cell fractionation, the release of MBOs from kinesin can be mini-

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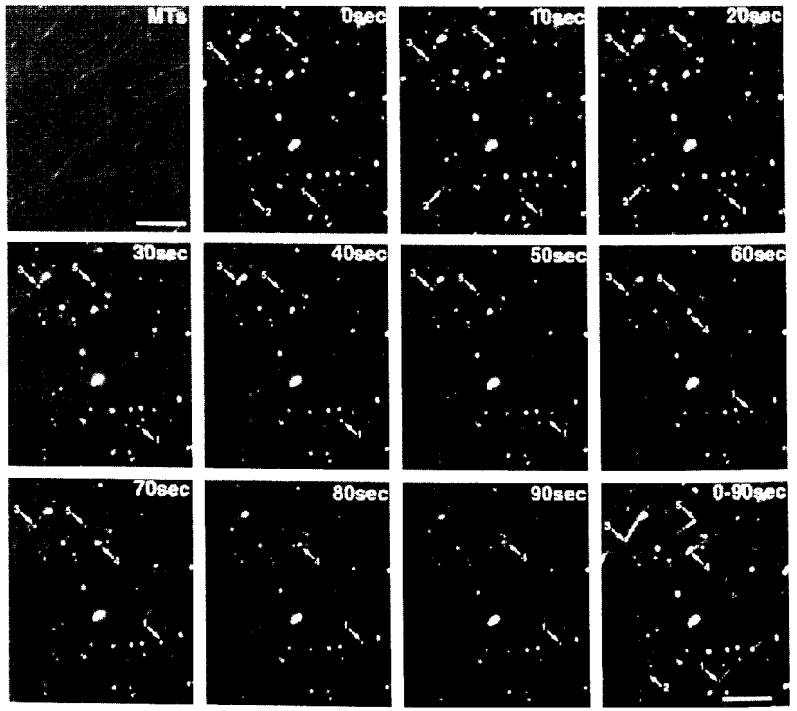


Fig. 1. Fluorescently labeled endosomes moving along in vitro synthesized microtubules. Oregon-green labeled microtubules (MTs) were adsorbed to a glass cover slip. Images of rhodamine–transferrin-labeled endosomes are shown at 10-s intervals. Moving endosomes are indicated by numbered arrows. In the final panel, a merged stack of consecutive images of fluorescently labeled endosomes (2-s intervals) is superimposed on an image of the microtubules. When represented in this manner, moving endosomes produce a series of closely spaced dots that line up with fluorescent microtubules. Scale bar: 5 μm.

1% trypan blue solution dissolved in water can be applied to the cells. Nuclei from broken cells will stain blue, whereas those of unbroken cells remain clear.

3. If it is difficult to distinguish between the supernatant and the pellet fractions, hold the tube up to a light source to help observe this interface.
4. To more easily see the endosomal membranes, place the tubes in a rack in front of a piece of black paper. We typically use a straight metal tube connected to tubing threaded through a peristaltic pump to collect the interface fractions.



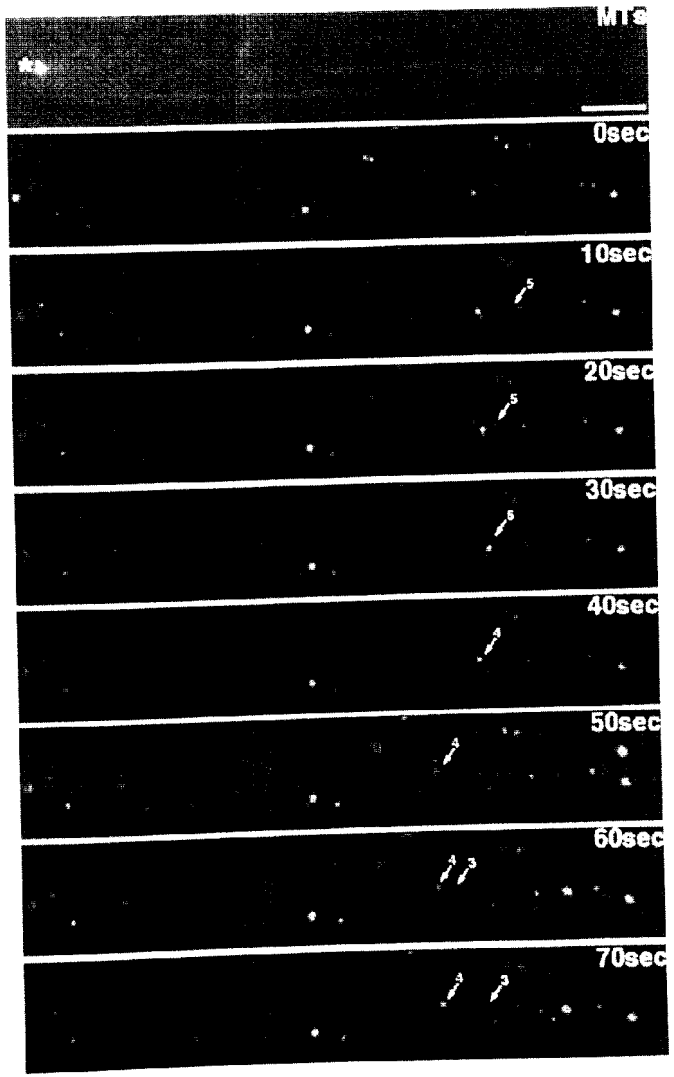


Fig. 2. Fluorescently labeled endosomes moving along microtubule asters toward both plus and minus ends. Oregon-green labeled microtubule asters (MTs) were nucleated on centrosomes (asterisk) adsorbed to a glass cover slip. Images of rhodamine–transferrin-labeled endosomes are shown at 10-s intervals. Moving endosomes are indicated by numbered arrows. *(continued)*

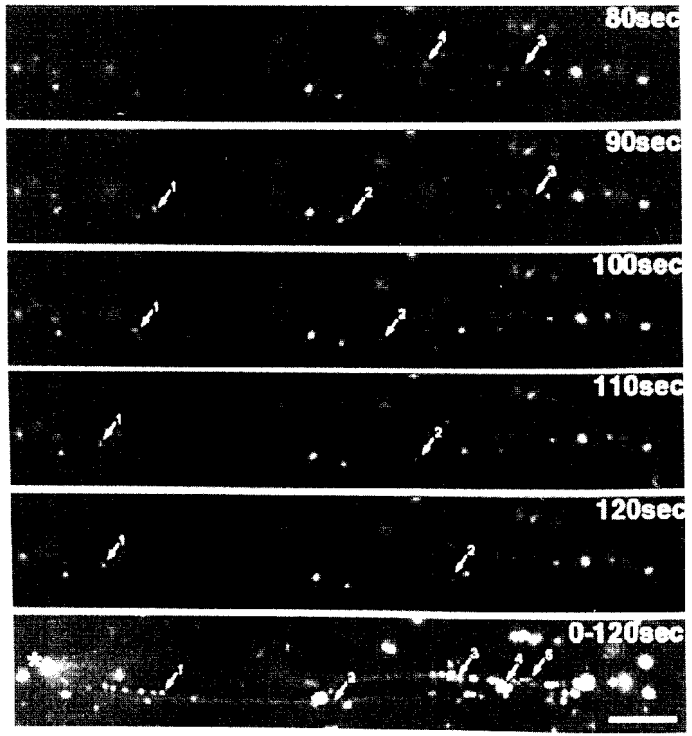


Fig. 2. *(continued)* In the final panel, a merged stack of consecutive images of fluorescently labeled endosomes (2-s intervals) is superimposed on an image of the microtubule aster. Scale bar: 5 µm.

5. When applying the grease, it is better to apply too much than too little as the chamber can be tapped down to create the proper reaction volume. The flow of buffer through the chamber and, by extension, the extent of washing in any given area of the chamber will be affected by its cross-sectional area; therefore, it pays to make the chambers as similar to one another as possible. To accurately lay the two lines of grease at set distances apart, we have found it useful to generate the chamber on a piece of graph paper using the underlying grid to apply the grease lines 1.5 cm apart.
6. GMP-CPP is unfortunately not available commercially and must be synthesized *(17)*. Alternatively, short microtubule seeds can be synthesized in the presence of glycerol *(15)*.



7. Taxol does not remain active through several freeze–thaw cycles; therefore, we store at the 10 m*M* taxol stock solution in 1-µL aliquots at - 20°C. Additionally, as at cold temperatures, taxol precipitates out of aqueous solutions, always store at these tubes at room temperature.
8. Alternatively, microtubules can be sedimented by spinning at 100,000g in a Beckmann TLA 100 rotor for 5 min at 37°C.

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