

Chapter 2

Visualizing In Vitro Trafficking

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Abstract

Here we present a detailed guide for performing in vitro trafficking assays. These are high-resolution light microscopy assays designed to look at the cytoskeletal filament-based trafficking of cellular organelles. The strategy is to partially purify organelles from lysed mammalian cells and freeze them as single-use aliquots. The organelles are then thawed and allowed to bind microtubule and actin filaments that have been coated onto handmade optical microchambers. Time lapse multichannel fluorescence microscopy is then performed to identify specific vesicles and associated proteins and to observe and quantify how the material is transported. These protocols were initially developed to study rodent liver endosomes but are adapted here for the study of cultured cells and include commentary on their use with other types of organelles.

Key words: Microtubules, Actin, In vitro trafficking, Cellular organelles, Time lapse imaging, Endosomes

1. Introduction

Vesicular trafficking involves the movement of membrane and protein under nonintuitive topological and thermodynamic constraints. In vitro trafficking assays have been designed to investigate this partially purified world that is neither a fully functioning cell nor a precise assembly of known components. The assays described here follow this general scheme:

1. Biochemically isolate intracellular vesicles.
2. Incubate vesicles with cytoskeletal filaments within disposable microscope chambers, and wash.
3. Begin live cell fluorescence microscopy and add ATP to activate the motor proteins.

Vesicle trafficking requires active motor proteins and ATP-releasable attachment to the filaments. For endocytic vesicles isolated

from rat liver, ATP induces motility in less than a second and in our hands lasts for 1–2 min. In living cells, motility is continuous, oscillatory, 3-dimensional, and very complex, all of which can hinder quantification. The trafficking assays provide a 2-dimensional, optically clean matrix, and an experimental starting point for quantitation. They are ideal for dissecting the direct chemical or protein contribution to motility since the responsible machinery is solution accessible. For instance, *in vitro* assays were crucial in identifying the cytosolic microtubule motor proteins, kinesin and dynein (1, 2). In contrast, in whole cell knockdown studies cells have time to adapt and changes in activities may stem from secondary effects. This Chapter attempts to provide an easy-to-understand step-by-step description of all that is needed for performing *in vitro* vesicle trafficking assays. Significant commentary is also included with observations that have helped our own exploration of these assays.

2. Special Materials

The following is a list of required materials that may not be present in a bioscience laboratory: large coverslips, DEAE-Dextran, polylysine, phalloidin, paclitaxel (Taxol), double-sided tape, clear nail polish, tubulin, rhodamine-labeled tubulin, GTP, ATP, Airfuge, kinesin motor domain, rhodamine-labeled actin, orosomucoid, Texas Red sulfonyl chloride, fluorescent transferrin, fluorescent EGF, protease inhibitors, tabletop ultracentrifuge, casein, primary antibodies, fluorescent secondary antibodies, multi-fluorescence channel heated microscope system, and image analysis software.

2.1. Microscope Chambers

The chamber described here is designed for an inverted microscope where the objective lens is beneath the chamber and out of the way. It is easily customized (e.g., (3)). A treated coverslip forms the optical, bottom surface, supports a 5 mm wide channel formed from two pieces of double-sided tape, and is topped with a piece of glass cut from a slide and held in place by the tape (Fig. 1). The chamber is inexpensive, easy to construct, contains an internal volume of approximately 5 μ L, and allows rapid exchange of contents by pipetting at one end and wicking from the other. Microtubules and actin filaments do not readily adhere to glass and therefore coverslips are first coated with a binding agent such as polylysine, sigmacote, or DEAE-dextran. In some cases adequate microtubule attachment may be observed in the absence of a binding agent (4). We have found that DEAE-dextran is easy to handle, does not form aggregates, and allows microtubules to bind to the surface as single filaments. The binding agents can bundle microtubules and this can inhibit or alter motility, so the coverslip should be washed thoroughly following treatment. We have not found it necessary to additionally clean the coverslips.

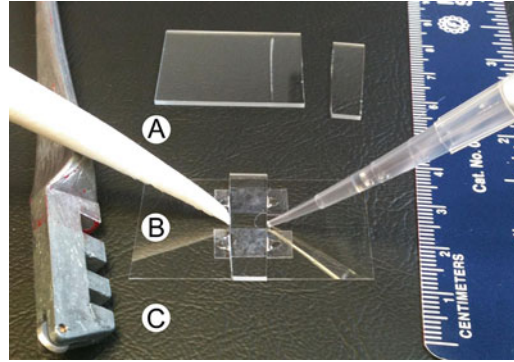


Fig. 1. Construction of disposable glass microchambers. (A) A standard glass slide is scored with a wheeled glass cutter and broken into small rectangles that are used for the tops of chambers. (B) The assembled chamber with pipette tip dispensing 5–10 μ L into the chamber where they are wicked at the other end with a folded Kimwipe. The chamber should not be allowed to dry once material has been added. (C) A wheeled glass cutter. These can be found at hardware stores or from companies specializing in stained glass work.

2.2. Construction of Chambers

1. Sequentially submerge large rectangular coverslips (22 \times 40 mm, Corning #2940 224) into deionized water containing 30 μ g/mL DEAE-dextran (e.g., Sigma-Aldrich #D9885) for 1 min and wash extensively. Allow coverslips to air-dry, or use compressed air to dry. For attachment of F-actin which requires stronger adhesive, substitute 50 μ g/mL polylysine (Sigma-Aldrich P8954) for DEAE-dextran.
2. Using a razor blade and appropriate cutting surface, cut strips of double-sided tape (3M Scotch #655, 12 mm wide, 0.076 mm thick) into rectangles of approximately 15 mm \times 6 mm and attach these to the DEAE-coated coverslips leaving a 5 mm wide channel.
3. Construct glass tops of the chambers by scoring standard slides (e.g., 25 \times 75 \times 1 mm, Fisher Brand #12-550D) with a wheeled glass cutter and breaking at the score, creating multiple 25 \times 6 mm pieces. Affix the tops to the double-sided tape channel as shown in Fig. 1.
4. Add small drop of clear nail polish at the cut glass–tape junction to improve the chamber integrity. A piece of folded Scotch or lab tape at the corner can serve as a handle and provide a label.

The optical surface of the chamber should remain clean and the entire bottom surface should be free of tape or debris to avoid affecting the focal plane. 10–20 chambers can be made for a day's experiments and mounted to a black platform for handling. The black surface helps to visualize the chambers and their contents. The DEAE-coating will last for a day and possibly longer. Once solutions are added to the chamber, care must be taken to

prevent drying or formation of air bubbles as these can destroy the contents. If perfusions are completed but the contents require further imaging, a drop of glycerol can be added to both ends to semi-permanently seal the chambers.

2.3. Polymerization of Fluorescent Microtubules

Chemical labeling of purified tubulin with a fluorophore has provided a convenient means to view microtubules. Unlabeled microtubules can also be detected by bright field or DIC microscopy (2) but it can be difficult to distinguish whether microtubules are bundled. On the other hand, fluorescent modification of tubulin may affect the binding of proteins to microtubules. This, however, can be addressed by diluting labeled tubulin with unlabeled tubulin to the limit of detection, or by comparing motor activities with unlabeled tubulin under DIC microscopy.

Our recommended protocol is the following:

1. Purchase unlabeled (cat #TL238-A, 250 μ g lyophilized aliquots) and rhodamine-labeled tubulin (cat #TL590M, 20 μ g lyophilized aliquots) from Cytoskeleton Inc. (Denver, CO). We have found that these tubulins preserve best at -80°C , dehydrated.
2. Add 25 μL of cold MT buffer (80 mM $\text{K}_2\text{-PIPES}$, 1 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM MgCl_2 , 3% glycerol, 1 mM GTP, pH 7.0) to lyophilized unlabeled tubulin to create “U-tub” stock (concentration, 10 $\mu\text{g}/\mu\text{L}$), and store on ice.
3. Add 12 μL of U-tub to a 20 μg aliquot of rhodamine tubulin to create “Rh-tub” stock (concentration, approximately 11.7 $\mu\text{g}/\mu\text{L}$) at 1:6 molar ratio of labeled to unlabeled tubulin, and store on ice.
4. Clarify (i.e., remove aggregates and debris from) both tubes by centrifugation for 5 min at 14,000 rpm ($20,000\times g$), 4°C . Collect the supernatant, carefully avoiding any potential pellet.
5. Optionally, dilute Rh-Tub with U-tub to obtain optimal brightness for your imaging conditions.
6. Polymerize batches of Rh-tub by warming 6 μL to 37°C in a water bath for 15 min. Polymerize a second batch for 25 min.
7. Stabilize and terminate the polymerization reaction by addition of 190 μL of pre-warmed MT buffer containing 20 μM Taxol (Sigma-Aldrich #T1912).
8. Pellet the microtubules in a Beckman Airfuge (Beckman Coulter, Brea, CA) at 15 psi for 5 min to remove unpolymerized tubulin. The pink microtubules should be just visible as a pellet.
9. Remove supernatant and resuspend the microtubules to approximately 0.7 $\mu\text{g}/\mu\text{L}$ with 100 μL MT buffer plus 20 μM

Taxol by repeated pipetting, being careful not to create air bubbles.

10. Store the microtubules at room temperature in the dark. They should be usable for 2 weeks. Dilute 50-fold (or to desired density) to use in motility assays.

Microtubule quality should be examined by dilution onto standard glass slides or chambers in MT buffer plus Taxol. Thick filaments indicate MT bundling and that attachment reagent should be washed more thoroughly or buffers should be remade from fresh stocks. The 15-min or 25-min polymerization point may show longer filaments or better polymerization. Asters of microtubules generally indicate Taxol-induced polymerization while puncta can indicate aggregates that should have been removed in the clarification step. Some differences may be observed between different lots of tubulin. Longer microtubules can be obtained by polymerizing at lower concentration but polymerization may then be less robust. The microtubules will typically anneal and lengthen over 1–2 days. Note that some loss occurs with pipetting and centrifugation of small volumes; minor adjustments may be required.

2.4. Polarity-Marked Microtubules

Microtubules have a fast-growing “plus” end and slow-growing “minus” end, and this polarity is critical to their cellular function. Our preferred strategy for visualizing the polarity is to polymerize dimly labeled tubulin and shear these into short “seeds” from which to grow bright fluorescent tubulin. The plus end is then identified as the long bright end of the filament.

1. Prepare and clarify Rh-tub and U-tub as for “polymerization of fluorescent microtubules,” steps 1–4 above (Section 2.3), and store on ice.
2. Make dim “Seed-tub” stock by adding 1 μL of Rh-tub to the 13 μL of the U-tub stock, giving 14 μL of approximately 10 $\mu\text{g}/\text{mL}$ stock at molar ratio of approximately 84:1 unlabeled to labeled tubulin (or adjust ratio as desired).
3. Polymerize 4 μL of Seed-tub by warming to 37°C for 5 min. Warm up 8 μL of MT buffer.
4. Shear the polymerized seeds by rapidly pipetting up and down 15 times with pipette set to 2 μL , press pipette tip against tube, and avoid air bubbles.
5. Add 2 μL of the seeds to the 8 μL of warm MT buffer and quickly add 3 μL of Rh-tub using a cut tip, and mix by up-and-down pipetting, avoiding shearing.
6. Allow to polymerize at 37°C for 6 min. This polymerizes 2.7 $\mu\text{g}/\mu\text{L}$ tubulin from 1.5 $\mu\text{g}/\mu\text{L}$ seeds, and the Rh-tub is approximately 14 \times brighter than the seeds.

7. Stabilize and terminate the polymerization by addition of 190 μL of pre-warmed MT buffer containing 20 μM Taxol, final tubulin concentration, approximately 0.4 $\mu\text{g}/\mu\text{L}$.
8. Pellet the microtubules in a Beckman Airfuge at 15 psi for 5 min to remove unpolymerized tubulin.
9. Remove supernatant and resuspend the polarity-marked microtubules to approximately 0.1 $\mu\text{g}/\mu\text{L}$ with 500 μL MT buffer plus 20 μM Taxol by gentle, repeated pipetting. This lower concentration will reduce the amount of microtubule annealing.
10. Store microtubules at room temperature and use within 3 h. The accuracy will degrade over time due to annealing and breaking.

The accuracy of the polarity marks can be assessed with gliding assays that utilize a single species of motor protein. In these, motor protein is added directly to untreated chambers followed by washing, and addition of microtubules and then ATP (5). Commercially available kinesin motor domain (KR01, Cytoskeleton Inc.) can be used for this purpose, although motility may be quite slow and light sensitive. This construct has recently changed but is indicated to function in motility assays. Some microtubules will contain ambiguous marks and must be eliminated from scoring. Since marked microtubules can only be used for a single day, and because the microtubules must be carefully scored by hand, these assays require considerable effort, and there is certainly room for improvement. Axonemes from tetrahymena or sea urchin have been used to nucleate microtubules and indicate the microtubule minus ends (6, 7). The GTP analogue, GMPCPP, will stabilize microtubule seeds and improve reproducibility of microtubule marks (8), but this can be difficult to obtain commercially. Treatment of tubulin with N-ethylmaleimide inhibits polymerization of tubulin more strongly at the minus end, and tubulin can be diluted with NEM-treated tubulin to preferentially inhibit minus end polymerization and increase polarity accuracy (6). Soppina and colleagues have used magnetic avidin beads to bind biotin-labeled, nonfluorescent tubulin seeds to mark microtubule minus ends (9). Other groups have shown that MTs will align with the direction of force from either an electric field (10) or fluid flow (11) during kinesin-based gliding assays. The microtubules could then be chemically fixed in place and potentially used for vesicle trafficking assays.

2.5. Polymerization of Fluorescent Actin

Actin filaments can also be imaged directly by fluorescence microscopy in vitro (12, 13), and we have developed actin trafficking assays in the style of the microtubule assays. Because of their different diameters and effective brightness, it is possible to distinguish actin and microtubule filaments in the same fluorescent

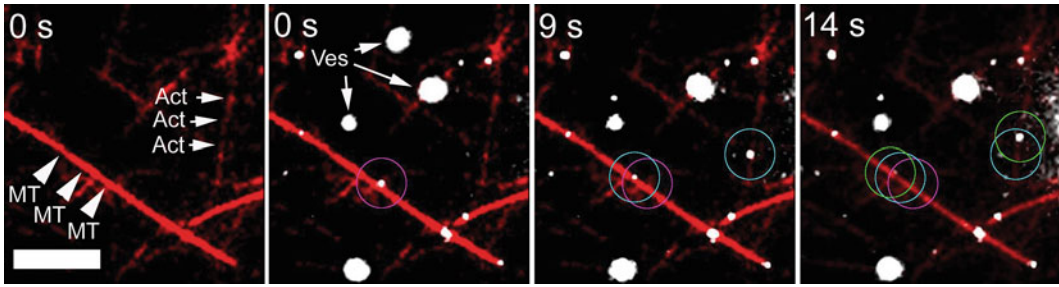


Fig. 2. Motility of rat liver vesicles along microtubule and actin filaments. Following the procedures described in Sect. 2.9, rat liver vesicles were bound to actin and microtubules within microchambers and 1 mM ATP was added to initiate vesicle trafficking. In this example 2 μ M bodipy ceramide was used to stain the vesicles. Imaging data was captured in the bright field (not shown), FITC (vesicles), and rhodamine (microtubules and actin) fluorescence channels. Microtubules (MTs) are seen as thick bright filaments while actin (Act) filaments are dimmer. A vesicle (*circled* at time 0 s) moves to the left on the MT, while another vesicle at 9 s (*circled*) binds to an Act filament and moves upwards. This demonstrates that both MT and Act motors are active in the vesicle preparation. Bar = 5 μ m.

channel (Fig. 2), and many options for imaging actin, microtubules, and vesicles are possible. Our recommended protocol is the following:

1. Obtain rhodamine-labeled actin (Cat #AR05, lyophilized aliquots, Cytoskeleton Inc.), and store at -80°C .
2. To a single 20 μg aliquot add 50 μL of cold “G Buffer” (5 mM Tris, 0.2 mM CaCl_2 , 0.2 mM ATP, 0.5 mM DTT pH 8.3), incubate on ice for 1 h to allow depolymerization of actin oligomers, and clarify by centrifugation for 5 min at 14,000 rpm ($20,000\times g$), 4°C . Collect the supernatant, containing 0.4 $\mu\text{g}/\mu\text{L}$ (9.3 μM) globular “G-actin.”
3. Polymerize overnight at approximately 6.2 μM (0.27 mg/mL) by adding 10 μL G-actin, 3 μL of 5 \times polymerization buffer (the 5 \times polymerization buffer contains 250 mM KCl, 10 mM MgCl_2 , 5 mM ATP, made fresh from single species stocks of KCl and MgCl_2 and frozen stocks of ATP), and 2 μL deionized H_2O .
4. Warm to room temperature and stabilize actin filaments (F-actin) by addition of 0.07 μM phalloidin (1.7 μL from a 0.7 μM phalloidin stock).
5. Store both G- and F-actin on ice in the dark. Both are usable for approximately 1 week.

Actin, unlike tubulin, does not depolymerize at low temperature and both F- and G-actin are stored cold to prevent degradation. Polymerization is induced by raising the KCl and magnesium concentration.

2.6. Isolation of Vesicles

We have developed protocols for isolating endocytic vesicles from rodent liver that contain active, endogenous microtubule and actin motors. In these, hepatocytes within the liver are allowed to endocytose fluorescent ligand, and vesicles are isolated by floatation through a sucrose gradient.

Further purification is not necessary since the endosomes are defined by their fluorescent ligand. We have traditionally utilized the ligand, asialoorosomucoid (ASOR), which has been extensively characterized in historic papers of endocytic classification (14, 15), and which is well behaved in solution and released upon lysis of endosomes. Fluorescent ASOR is prepared by removing terminal sialic acid residues from orosomucoid (alpha-1 acid glycoprotein, Sigma Cat. #G-9885) and labeling with an amine-reactive fluorescent dye (e.g., Texas Red sulfonyl chloride, cat #T1905, Life Technologies (5)). Numerous alternate ligands (e.g., Transferrin, EGF, or LDL, GFP—your favorite protein) can be utilized; although if endocytic uptake is not performed, the identity of the vesicles should be confirmed by other methods. ASOR binds specifically to hepatocytes via the highly expressed asialoglycoprotein receptor (ASGPR) (16, 17), and a single rat liver contains approximately a billion hepatocytes (18), providing an abundance of concentrated endosomes that are required for the microscopy assays. Although in some cases freezing can reduce motor protein activity (19), it has been essential to freeze vesicles as single-use aliquots so that a reliable stock of vesicles is available for the quantitative assays. For mice, we use between 2 and 5 livers and maintain these on ice prior to homogenization. For cultured cells, attaining enough concentrated vesicles can be difficult and therefore a gel filtration step is omitted and screening the cells and ligands for high binding capacity is recommended (see below). Many related strategies have been used to isolate populations of vesicles that are active for in vitro assays and these can be combined with live cell imaging studies to bolster results (4, 20, 21).

2.7. Protocol for Isolating Motile Fluorescent Early Endocytic Vesicles from Cultured Cells

1. Culture Huh7 or other cell line to confluence in sixteen 15-cm dishes.
2. Wash cells in Hepes-buffered saline (HBS, 135 mM NaCl, 1.2 mM MgCl₂, 0.8 mM MgSO₄, 28 mM dextrose, 2.5 mM CaCl₂, 25 mM Hepes, pH 7.4) and incubate in HBS for 30 min to remove cell surface-bound proteins that may occupy the receptor.
3. Cool cells to 4°C by addition of cold HBS, place cells on ice, and add approximately (see below) 5 µg/mL fluorescent ligand (i.e., ~0.1 µM ASOR) for 30 min. Fluorescent ASOR, transferrin (Life Technologies Cat #T23364), EGF (Life Technologies Cat #E-35350), dextran, and other ligands have been used in our studies. At 4°C, cell surface binding will occur without

internalization (note that ASOR binding requires calcium). After 30 min, wash cells in cold HBS.

4. Initiate endocytosis by addition of 37°C HBS, and allow endocytosis to proceed for 5 min.
5. Wash cells in cold MEPS buffer (5 mM MgCl₂, 5 mM EGTA, 35 mM K₂-PIPES, 0.25 M sucrose, pH 7.4). This low-calcium buffer will remove surface-bound ASOR. Subsequent steps are performed at 4°C.
6. Scrape cells from dishes with rubber policeman and pellet whole cells at 240×*g* (1,500 rpm) for 5 min.
7. Resuspend the cells in 600 μL MEPS buffer plus 4 mM DTT, 2 mM phenylmethanesulfonyl fluoride (PMSF) and a 1:50 dilution of protease inhibitor cocktail (Sigma Cat. #P-8340).
8. Lyse the cells by 20–30 passages through a 25-gauge syringe needle.
9. Remove nuclei and cell debris by centrifugation at 950×*g* (3,000 rpm) for 10 min; collect supernatant (PNS), which should be approximately 600 μL; and re-add 4 mM DTT, 2 mM PMSF, and 1:50 dilution of protease inhibitor cocktail. An opaque supernatant will indicate effective lysis; if clear, re-lyse the pellet.
10. Bring PNS to 1.4 M sucrose by the addition of 2.5 M sucrose MEPS buffer (i.e., MEPS buffer that contains 2.5 M sucrose instead of 0.25 M). A handy calculation is that the volume of added 2.5 M sucrose should equal 1.045× the volume of the PNS (total volume equals 2.045× the PNS).
11. Add the 1.4 M sucrose PNS to the bottom of tube fitted for a tabletop ultracentrifuge swinging bucket rotor (e.g., Beckman Coulter TL-100 centrifuge, TLS-55 rotor, and 347357 tube). On top of this load approximately 1 mL of 1.2 M MEPS buffer, followed by 0.1 mL of 0.25 MEPS buffer, creating a 1.4 M/1.2 M/0.25 M sucrose step gradient.
12. Centrifuge for 2 h at 101,000×*g* (39,000 rpm) during which time the low-density membranes, including the endocytic vesicles, will float up through the 1.2 M sucrose.
13. Collect approximately 200 μL of the opaque, membrane-containing material from the 1.2 M/0.25 M interface and store frozen in 12 μL aliquots at –80°C. It is critical that the vesicle density be very high so that 20–60 vesicles can be seen in a single microscope field during the trafficking assays.

The binding of ligand to the surface of the cells should be examined prior to performing vesicle preparations to ensure adequate uptake and to determine optimal ligand concentration. This can be done by culturing cells on coverslip-bottomed chambers

(MatTek, Ashland, MA) and treating cells with ligand for 5 min at 37°C followed by washing and imaging. The receptor for ASOR is expressed in hepatocyte cell lines but often at low levels. Similarly, transferrin receptor expression can be low, but this may be increased by addition of deferoxamine to the culture medium 1–2 days prior to the experiment (22). Cell density, passage number, or culture conditions may affect cell surface receptor expression. Investigators may wish to purify other types of organelles, and for this a survey of the vesicle trafficking literature is recommended (e.g., (4, 7, 21, 23)). Liver, brain, and different kinds of cultured cells may require different strategies and our recommendation is to plan for many isolations with various permutations (buffers, lysis conditions, etc.), and to perform fast, crude isolations in the initial stages, and test these for trafficking activity as isolation conditions become more complex.

2.8. Vesicle Trafficking Assay

Here we describe how to combine the vesicles with cytoskeletal filaments to produce reliable trafficking assays. Trial experiments should be performed to assess proper dilutions of microtubules, vesicles, antibodies, and imaging exposure times and lamp intensity. Once these are established, a series of DEAE-dextran-treated chambers are assembled, coated with microtubules, washed, incubated with vesicles, washed, and optionally incubated with antibodies, washed, and placed on ice in humid container to prevent dehydration. The chambers are brought to the microscope and one by one placed on the microscope stage and imaged during addition of ATP. Vesicles bound to microtubules and washed free of soluble material in this manner should retain their motile capacity for many hours, whereas solutions of thawed vesicles can rapidly lose activity.

1. Prepare 2.5 mL of “10×” PMEE (350 mM K₂-PIPES, 50 mM MgCl₂, 10 mM EGTA, 5 mM EDTA, 20 mg/mL bovine serum albumin (BSA) pH 7.4, made daily from single chemical liquid stocks and powdered BSA), 25 µL of 10 mM ATP, pH 7.0 stored as single-use frozen stock, 0.5 mL of 200 mM DTT made fresh, 50 µL of 1 mM Taxol stored as frozen stock in dimethylsulfoxide, 1 mL of 100 mg/mL Na-ascorbic acid made fresh (optional antioxidant), 0.5 mL of “1×” PMEE plus 20 µM Taxol, 2 mL of Assay Buffer (1× PMEE plus 20 µM Taxol, 4 mM DTT, 2 mg/mL Na-ascorbic acid), and 0.5 mL of Blocking Buffer (Assay Buffer plus 5 mg/mL casein, Sigma Cat #C7078, undissolved casein removed by filtration).
2. Prepare 12 motility chambers coated with DEAE-dextran.
3. Dilute fluorescent microtubules, prepared as above, 1:50 (to 14 µg/mL, concentration will vary) in 1× PMEE plus Taxol, and mix gently (optional: use cut tips to prevent shearing of microtubules).

4. Add 5 μL of microtubules to the chambers, incubate for 3 min at room temperature, and wash 3 \times with Blocking Buffer and 2 \times with Assay Buffer. The Blocking Buffer (casein) is used to decrease the binding of vesicles directly to glass.
5. Thaw fluorescent vesicle aliquots and immediately add 5 μL of the vesicles to each chamber and incubate for 10 min at room temperature in a humid environment. Longer incubation times will decrease motility; shorter incubation times may not allow sufficient binding to microtubules. A drop of assay buffer at the edges can prevent dehydration and insure a nonoxidizing environment.
6. Wash off unbound vesicles with Assay Buffer and store chambers on ice in humid environment.
7. At the microscope, heated to 37°C, remove a single chamber, wash in Assay Buffer, and focus on a field of microtubule-bound vesicles, avoiding excess excitation light.
8. Initiate image acquisition and add 20 μL of 50 μM ATP in Assay Buffer. Capture images at 1 frame per 3 s for 120 s.
9. Repeat trafficking assay for all chambers.

Motility is sensitive to oxidative damage from the microscope excitation light. Fluorescence excitation should be minimized, for instance by lowering the intensity, using transmitted light or long-wavelength light when focusing, and by capturing at low frame rates. Many laboratories use oxygen-scavenging systems to reduce photodamage (e.g., 0.1 mg/mL glucose-oxidase, 0.018 mg/mL catalase, 2.3 mg/mL glucose (24, 25)). In our hands these components can bundle microtubules and do not increase motility during the short acquisition times of these assays. We do include the antioxidants, DTT and ascorbic acid. An ATP-regenerating system (e.g., 0.16 mg/mL creatine phosphokinase, 8 mM phosphocreatine) may be employed but did not increase the motility of our purified vesicles (5, 26). The above assay uses a low concentration of ATP (50 μM), which may limit extraneous ATPase activity. Many factors can inhibit motility and initial negative results should not be a cause for despair. Motor proteins are sensitive to salt, oxidation, ATPases, free phosphate, ADP, and other factors, and buffer stocks can go bad. It has been very helpful to have a source of active motor proteins or vesicles as a positive control. For instance, microtubule gliding assays (5) can be performed in the presence of isolated vesicles to determine if motility inhibitors are present. The endocytic vesicle preparation typically contains enough motors to cause some bending or sliding of microtubules even when attached by DEAE-dextran. Microtubule-bound vesicles also frequently exhibit short, back-and-forth diffusive movement prior to ATP addition. If vesicles show no motility, no microtubule bending,

and no diffusive movement, they may be cross-linked to the microtubules. Higher salt or removal of the cross-linking molecule(s) may be required.

2.9. Trafficking Assay Adapted for Actin and Microtubules

Assays for vesicle trafficking along actin filaments can be performed in the same manner as for trafficking along microtubules with a few *key differences*. Because these filaments can be distinguished by their diameter and brightness (Fig. 2), both actin and microtubules can be imaged in the same field.

1. Prepare buffers as described in Vesicle Trafficking Assay (Sect. 2.8). Instead of “1×” PMEE plus Taxol, prepare 0.5 mL of *AM Buffer* (5 mM Tris, 50 mM KCl, 2 mM MgCl₂, 0.07 μM phalloidin, 20 μM Taxol, pH 8.3).
2. Prepare 12 motility chambers coated with 50 μg/mL *polylysine*. The polylysine can bundle microtubules. To prevent this, both actin and microtubules are diluted into the high pH AM buffer prior to their introduction into the microscope chambers.
3. Dilute fluorescent F-actin 1:150 (to 1.8 μg/mL or 41 nM) and fluorescent microtubules 1:200 (to 3.5 μg/mL, 64 nM tubulin) into AM Buffer. Actin and microtubules are not stable in this buffer and should be used immediately.
4. Add 5 μL of the microtubules and actin to the polylysine chambers and *immediately* wash 2× with AM Buffer followed by 2× with Blocking Buffer and 2× with Assay Buffer.
5. Incubate vesicles with the actin and microtubules for 5 min, wash, and store chambers on ice.
6. Initiate motility experiments with 1 mM ATP at 30°C. Higher ATP was found to be more effective for actin motility, and 37°C was found to be inhibitory.

With this assay we have found that vesicles isolated from rat liver move short distances (<2 μm) or release from actin filaments upon addition of ATP whereas microtubule-based movement lasts for 40–80 s, covering 10 to 100s of microns (5, 27). Therefore it may be preferable to add both ATP and vesicles during microscopy acquisition, rather than pre-binding the vesicles. In Fig. 2 microtubule and actin-based movement is observed in the same field of view. Microtubules appear as long, thick, filaments while actin is dim. A microtubule-bound vesicle moves left during the 14-s sequence while a second vesicle binds an actin filament at 9 s, moves up, and releases after 14 s. This indicates that endogenously expressed actin and microtubule motors are present on the vesicles.

2.10. Labeling Vesicle Proteins with Fluorescent Antibody

In addition to imaging fluorescent-labeled ligand, we typically quantify the localization and motility of various proteins that are present. This can be achieved by immunofluorescence labeling of epitopes that are found on the exterior (cytosolic) face of the vesicles.

Fixatives are usually avoided so that motile activity is retained and because they are unnecessary. Since the experimental volume and diffusion barriers are low, high antibody concentrations can be used with short incubation times, and typically vesicles can be imaged in less than an hour.

After step 5 of the Vesicle Trafficking Assay (i.e., after 10-min incubation of vesicles with the microtubules, Section 2.8):

1. Wash vesicles $3 \times 15 \mu\text{L}$, using Blocking Buffer for this and subsequent washes and dilutions. This serves to remove unbound vesicles and to block potential nonspecific antibody-binding sites. Alternate blocking reagents may be used.
2. Perfuse $3 \times 15 \mu\text{L}$ of primary antibody, incubate for 6 min, and wash $5 \times$. Suggested starting antibody concentration is $5 \mu\text{g/mL}$; monoclonal antibodies frequently require higher concentrations. Incubations are typically performed on ice, although some may work better at room temperature.
3. Perfuse $3 \times 15 \mu\text{L}$ of fluorescent-labeled secondary antibody (e.g., Anaspec, Inc Cat #61056-1-H555), incubate for 6 min, and wash $5 \times$; suggested antibody concentration is $10 \mu\text{g/mL}$.
4. Continue with step 7 of the Vesicle Trafficking Assay (i.e., bring to microscope and initiate motility experiments).

Antibodies must be carefully evaluated. Western blots showing significant nonspecific reactivity should be excluded. Although western blots and unfixed vesicles presumably evaluate denatured and native protein, respectively, in our experience most antibodies with good signal by western blot also exhibit good signal for vesicle immunofluorescence, in contrast to whole cell or histological immunofluorescence where epitopes may be blocked or destroyed by the fixative. The primary and secondary antibodies should be titrated to include zero concentration and a specific increase in fluorescence should be observed. If possible, additional antibodies to the same protein should be used. It is also helpful to compare the immunofluorescence to that of “total membrane” in the field to determine if the signal is concentrated to particular vesicles. The total membrane can be estimated using high-contrast bright field or phase-contrast microscopy (28). We have also found that $2 \mu\text{M}$ bodipyFL C_5 ceramide (Life Technologies Cat #B22650) will label all the vesicles in a rat liver preparation with uniform intensity.

2.11. Microscope Systems

Many microscope systems will function for vesicle trafficking assays. The main components to consider are the following:

1. Low excitation intensity light, high signal detection: Laser scanning confocal systems have strong excitation light and confocal imaging is not necessary since the contents are perched on a single focal plane. Similarly, spinning disk confocal systems, although less photodamaging, may not have significant

advantage over non-confocal, wide-field imaging. If using confocal we recommend opening the pinhole and reducing laser intensity. Also because longer wavelength light has lower intensity, motility may be more robust in the red and far red wavelengths. Total internal reflection imaging can provide high signal-to-noise ratio and can allow high-resolution automated tracking (29), although switching between multiple wavelengths may be difficult and photodamage is a concern. The objective lens should have high numerical aperture (≥ 1.4) and high magnification (e.g., 60 \times). EMCCD cameras give the highest quantum efficiency (signal detected per photon). However their many settings can complicate image quantitation. Additionally, EMCCDs have large pixel size and lower spatial sampling. sCMOS cameras appear to offer many advantages as they are less expensive than EMCCD and have high quantum efficiency, and high frame rates. We have had success with wide field fluorescence imaging and cooled CCD camera that has approximately 60% quantum efficiency (CoolSnap HQ, Photometrics, Inc.).

2. Moderately fast, multichannel fluorescence image acquisition: The assays described have a recommended acquisition speed of 1 set of images per 3 s or faster with up to four fluorescence channels. At 200 ms exposure, this leaves 550 ms to switch channels and to store images. In practice, delays creep into the system and a goal of 100 ms channel switching capability is recommended. This can be accomplished with a white light source and filter wheels (excitation, dichroic, emission), with lasers, or notably with new solid-state excitation sources such as the SOLA Light Engine (Lumencor Inc.). Our system uses a DG-4 xenon 175 W excitation lamp (Sutter Instrument Company) with four excitation filters, a Sedat Quad dichroic mirror (Chroma Technologies), and a Lambda 10–20 emission filter wheel (Sutter Instrument Company, Novato, CA) with proper emission filters for DAPI, FITC, Cy3, and Cy5 or equivalent wavelengths. All imaging is directly to the camera (the eye piece has been removed) and displayed on the computer screen. The devices are run with Metamorph software (Molecular Devices, Sunnyvale, CA). It should be noted that very rapid vesicle movement may be missed at these acquisition speeds and that higher frame rates may reveal novel features of vesicle trafficking.
3. Heating the stage: Most experiments are performed at 37°C and any thermal gradients will alter the focal plane. An enclosure placed around the microscope stage and a hot air heater (Airtherm, World Precision Instruments) can suffice to equilibrate the stage; the temperature at the lens should be carefully monitored.

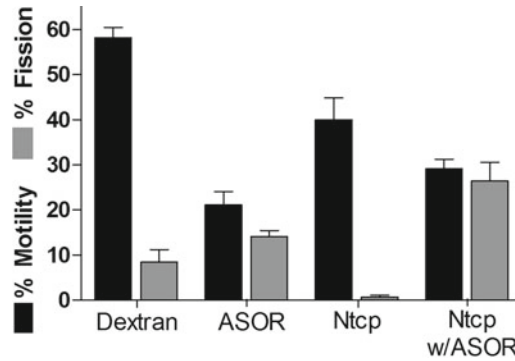


Fig. 3. Quantifying the motility and fission of different populations of vesicles. Vesicle trafficking assays were performed as described in Section 2.8. Different populations of vesicles (as indicated) exhibit different levels of motility and fission, and the level of fission is independently regulated from motility. Texas Red-labeled amino dextran and Texas Red ASOR endocytic vesicles were purified in separate isolations from rat liver as described (Sections 2.6 and 2.7). Rat liver vesicles were labeled by immunofluorescence for the bile acid transporter, Ntcp, according to methods of Section 2.10, and vesicles were colocalized for the presence of both Ntcp and ASOR. Interestingly these vesicles had moderate motility (29%) but very high fission (26%), suggesting that vesicles containing proteins directed toward different cellular compartments, i.e., the plasma membrane (ntcp) and lysosome (ASOR), may have higher rates of fission. Error, standard error of the mean.

2.12. Data Analysis

Initially, an investigator may wish to know how much motility is observed for different populations of vesicles and under various treatments. This can be answered by identifying vesicles and manually scoring whether movement occurs, relative to the cytoskeleton, upon addition of ATP. It is helpful to establish rigorous distance and time intervals for scoring since back-and-forth diffusive movement is also observed. Gliding or bending or other factors may preclude scoring particular vesicles. The vesicles should be identified prior to scoring since their movement will increase their visibility and lead to overestimation of the motility. Some vesicles undergo fission, or splitting of their contents (30). With endocytic vesicles this can be manually scored as the separation of fluorescent puncta after addition of ATP. Fission has been shown to be a specific, independently regulated activity of endocytic vesicles and not a result of the movement overlapping vesicles (31). Figure 3 demonstrates the motility and fission of different populations of vesicles using this technique. Rat liver vesicles that contain the endocytic marker, fluorescent dextran, exhibit high motility (58%) with moderate fission (8%) while vesicles containing the ASOR ligand show moderate motility (22%) with relatively high fission (14%). Interestingly, vesicles that contain both lysosomally directed ASOR and a plasma membrane-directed bile acid transporter, ntcp, show highest fission (26%), suggesting that high rates of fission may correlate with

vesicles that contain proteins that are destined for different cellular compartments.

Many detailed parameters of vesicle trafficking can also be quantified, and the public domain program, ImageJ, developed by Wayne Rasband at the US National Institutes of Health (<http://rsbweb.nih.gov/nih-image/>) is an excellent resource for quantification methods. For instance, the position of vesicles can be tracked using ImageJ Manual Tracking software “plugin” to reveal run length, instantaneous velocity, and movement periodicity. Additional technical advances using high-contrast, rapid image acquisition have allowed automated particle tracking with nanometer precision (4, 32) and combined with super-resolution techniques (33) may provide spatial-temporal “signatures” for specific vesicle trafficking events, although this has so far remained elusive. The direction of motility relative to microtubule polarity mark has been scored manually in trafficking assays and has revealed the bidirectional nature of early and late endocytic vesicles from rodent liver, HeLa, and other cell types (20, 34). Chemical inhibitors, when used appropriately, can help distinguish the activity of different motors. For instance, inclusion of 5 μ M vanadate or 1 mM AMP-PNP along with ATP in the trafficking assays has been shown to inhibit dynein- and kinesin-based motility, respectively (1, 5, 35). We have also found that phosphoinositide 3-kinase inhibitors will reduce vesicle motility, and that a specific protein kinase C ζ inhibitor will block motility of vesicles that contain the bile acid transporter, ntcp, but not late endosomes (35). Another means of determining motor specificity is the use of inhibitory antibodies. Specific antibodies to kinesin and dynein have been effective at inhibiting motility in vitro and when microinjected into cells (36). Typically these are incubated with the microtubule-bound vesicles for 5 min prior to washing and initiation of motility.

2.13. Protein Colocalization

A major advantage of vesicle trafficking assays is that they can be used to quantify protein colocalization. Scoring colocalization can be automated using macro programs within the ImageJ software. The strategy is to allow biochemically isolated organelles to attach either directly to the glass surface or else to cytoskeletal filaments that are bound to microscope chambers. Soluble material is washed away and immunofluorescence is performed. This can be repeated for multiple primary antibodies provided that they do not cross-react. Because the isolated vesicles are attached to a 2-dimensional substrate, image complexity and background intensity are minimized and it is straightforward to “segment” the image into fluorescent spots and background. To report colocalization, users can measure the total area of the spots that overlap, or they can count the percentage of spots that show fluorescence overlap (28).

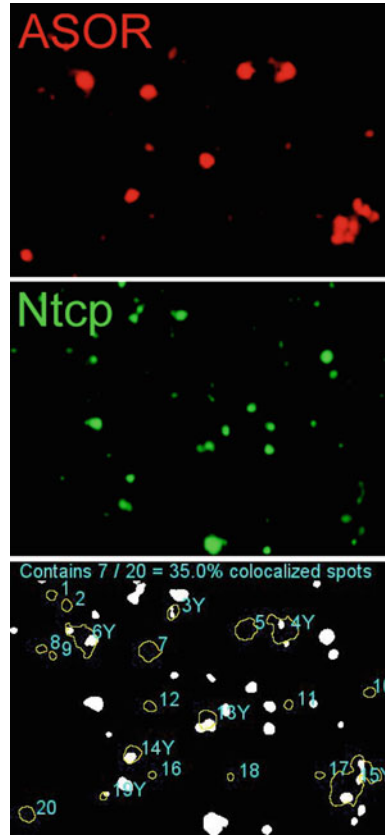


Fig. 4. Demonstration of the quantification of protein colocalization using endocytic vesicles and the “simple colocalization” macro presented in Sect. 3.1. Rat liver vesicles containing fluorescent-labeled ASOR (*top panel*) were incubated within an untreated microchamber. The vesicles bound to the chamber were co-stained for the bile acid transporter, Ntcp (*middle panel*), and imaged. An individual cropped, $9 \times 13 \mu\text{m}$ field is presented for the two fluorescent channels as well as a final image (*bottom panel*) that results from running the macro within ImageJ, resulting in a score of 35% colocalization. Typically 1,000s of vesicles and numerous experiments are quantified, and image processing, thresholding, and error checking are performed to suit the image data sets.

Figure 4 shows an example of a field of ASOR vesicles that has been co-stained for the bile acid transporter, ntcp, and quantified for the percentage of colocalized (overlapping) vesicles using the “Simple Colocalization” macro presented below. This macro numbers the ASOR vesicles and displays a “Y” (yes) for any vesicle showing ntcp colocalization. The total is summarized directly on the image. The code for this Simple Colocalization macro is provided below to let the reader explore the ImageJ macro language in this context. The ImageJ Web site provides excellent help and many macro examples. Some familiarity with computer programming may be necessary.

3. Note

3.1. Simple Colocalization Macro Program for ImageJ to Demonstrate Colocalization of Fluorescent Spots

Download the ImageJ program at <http://rsbweb.nih.gov/ij/download.html>.

Start ImageJ and create a new text window (File → New → Text Window). Then copy and paste the text below into this window. Open two images of the same size. They should be 8-bit or 16-bit gray scale (Image → Type → 8-bit). For instance one can copy and paste the images of Fig. 2 into ImageJ. Run the macro (in the text window: Macros → Run Macro). Note that “//” denotes a comment, is not read by the program, and instead provides a running description of the operations that are being performed.

```
macro 'Simple Colocalization [z]' // Press 'z' as shortcut to run the macro
{
    // curly brackets enclose the macro operations
    requires("1.44e"); // ImageJ version 1.44e required for 'Overlay' text functions
    MinSpotSize = 30; // Spot must be greater than this number of pixels
    MaxSpotSize = 2000; // Spot cannot be greater than this number of pixels
    MinimumForColocalization = 2; // Min % overlap to be scored as positive for colocalization
    SpotsWithColocalization = 0; // Initialize variable
    setLineWidth(1); // Line width of drawings
    setForegroundColor(255, 255, 0); // Outline color, Yellow
    setColor("Cyan"); // Text color, Cyan
    setFont("SanSerif", 20, "antialiased");
    run("Set Measurements...", "centroid redirect=None decimal=2");
    selectImage(2); // Select 2nd, "Green" image that was opened
    GreenImage = getTitle();
    setAutoThreshold("Triangle dark"); // Automatic thresholding using the 'Triangle' method
    // Below: find the spots, make 'Mask' used for colocalization
    run("Analyze Particles...", "minimum=" + MinSpotSize + " maximum=" + MaxSpotSize + " bins=20");
    show=Masks display clear "";
    MaskGreen = getTitle();
    run("Invert"); // Invert the image, black to white
    run("RGB Color"); // Convert to color image
    selectImage(2);
    close(); // Close "Green" image
    selectImage(1); // Select 1st, "Red" image opened, base image
    run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel"); // Remove any distance scaling
    RedImage = getTitle();
    setAutoThreshold("Triangle dark");
    // Below: find the spots on base, Red image, make 'Mask' used for colocalization
    run("Analyze Particles...", "minimum=" + MinSpotSize + " maximum=" + MaxSpotSize + " bins=20");
    show=Masks display clear "";
    MaskRed = getTitle();
    run("Invert");
    selectImage(1);
    close(); // Close original "Red" image
    NumberOfSpots = nResults; // Stores the number of spots
    for (i = 0; i < NumberOfSpots; i++) // For loop: Go thru each spot and check for colocalization
    {
        selectWindow (MaskRed);
        // Below: Use wand tool at positions from 'Analyze Particles' function
        doWand(getResult("X", i), getResult("Y", i));
        selectWindow (MaskGreen);
        run("Restore Selection");
        // Get number of black pixels, counts [0], white pixels, counts [255], in spots
        getHistogram(values, counts, 256);
        FluorescencePixelsInSpot = counts[255];
        TotalPixelsInSpot = counts[255] + counts[0];
        PercentColocalizationOfSpot = FluorescencePixelsInSpot / TotalPixelsInSpot * 100;
        if ( PercentColocalizationOfSpot > MinimumForColocalization )
        {
            IsSpotColocalized = "Y"; // Spot colocalizes? "Yes"
            SpotsWithColocalization++;
        } else
    }
```

```

IsSpotColocalized = ""; // Empty "" when not colocalized
run("Draw"); // Draw selection
//Below: Number the spots, "Y" if colocalized
Overlay.drawString( (i+1)+ IsSpotColocalized, getResult("X", i) + 10, getResult("Y",i) );
Overlay.show();
} // for loop
//Below: print % colocalization on the image
TotalColocalizedSpots = SpotsWithColocalization / NumberOfSpots * 100;
setColor("Black"); // Draw black box beneath text for visibility
setLineWidth(10);
Overlay.drawRect(10, 5, 372, 10)
setColor("Cyan");
Overlay.drawString("Contains " + SpotsWithColocalization + " / " + NumberOfSpots +
" = " + d2s (TotalColocalizedSpots ,1) + "%" + " colocalized spots", 10 , 16 );
Overlay.show();
} // End Simple Colocalization macro

```

The macro may give error messages or problems with some images. Please contact the author with any questions. For actual analysis, alternate thresholding methods are employed along with image processing and error detection. Determining the threshold method is critical for quantifying data and this should be investigated by the user. Most commands within ImageJ can be automated and incorporated into macros using the “record” function (Plugins → Macros → Record). The ability to quantify protein colocalization in this manner has, for instance, allowed studies of protein sorting during endocytic fission in vitro and, when combined with proteomic analysis, has helped demonstrate an unexpected role for Rab1a in allowing early endosome trafficking progress toward protein degradation ([31](#), [37](#)).

4. Summary

Instructions for performing cytoskeletal-based in vitro vesicle trafficking assays have been provided along with an overview and discussion of how these can be quantified and interpreted. The goal has been to be as explicit as possible so that investigators with varying experience will find them helpful. The assays themselves are especially suitable for identifying the immediate and primary effects of protein or chemical treatments on vesicle trafficking, since reagents can be added directly to organelles and their effects studied instantaneously in an environment free of cytosol. The assays are also easy to quantify since they avoid the complicated 3-dimensional environment of the living cell. The primary difficulties in producing reliable data are the vesicle isolation procedure and the imaging system, which should be minimally damaging to proteins. These approaches should continue to be useful in answering important questions of cell biology such as how motor proteins are regulated and how populations of vesicles are differentiated.

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