CHAPTER 13

Microtubule Dynamics Reconstituted *In Vitro* and Imaged by Single-Molecule Fluorescence Microscopy

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Abstract

In vitro assays that reconstitute the dynamic behavior of microtubules provide insight into the roles of microtubule-associated proteins (MAPs) in regulating the growth, shrinkage, and catastrophe of microtubules. The use of total internal reflection fluorescence microscopy with fluorescently labeled tubulin and MAPs has allowed us to study microtubule dynamics at the resolution of single molecules. In this chapter we present a practical overview of how these assays are performed in our laboratory: fluorescent labeling methods, strategies to prolong the time to photobleaching, preparation of stabilized microtubules, flow-cells, microtubule immobilization, and finally an overview of the workflow that we follow when performing the experiments. At all stages, we focus on practical tips and highlight potential stumbling blocks.

I. Introduction

Microtubules are highly dynamic polymers that undergo spontaneous transitions from growing to shrinking phases (Mitchison and Kirschner, 1984). This behavior, termed dynamic instability, is coupled to the hydrolysis of guanosine triphosphate (GTP) and is regulated by many proteins (Howard and Hyman, 2009), including depolymerizing kinesins (Helenius *et al.*, 2006; Varga *et al.*, 2009), polymerases (Brouhard *et al.*, 2008), and plus-tip proteins (Akhmanova and Steinmetz, 2008). The discovery of dynamic instability was contingent on the ability to visualize individual microtubules. Bulk assays such as turbidity, which detects only the total amount of polymer, are insensitive to the dramatic length changes that individual

microtubules undergo when a solution containing tubulin and GTP is under steady-state conditions (Mitchison and Kirschner, 1984).

With advances in fluorescence microscopy, especially the development of total internal reflection fluorescence (TIRF) microscopy (Axelrod, 2008; Axelrod *et al.*, 1984; Funatsu *et al.*, 1995) and the discovery of genetically encoded fluorescent proteins (Zhang *et al.*, 2002), the visualization of individual molecules is becoming routine. The principle underlying single-molecule fluorescence is visualization by localization. When a fluorophore is free in solution it diffuses very quickly ($\sim$100 $\mu m^2/s$), and within the typical exposure time of a camera (0.1 s), the fluorophore will be "smeared out" over an area of several square microns, corresponding to many camera pixels, which are typically $\sim$0.01 μm^2. However, when the molecule binds, for example to a microtubule, its rate of diffusion slows dramatically. All the fluorescence is now localized to a small number of pixels during the exposure time of the camera, giving a signal that exceeds the background, even if there are many fluorophores free in solution. TIRF microscopy is important because by exciting only those molecules near the surface where binding takes place, the background is reduced. However, other techniques that reduce the out-of-focus fluorescence, such as confocal microscopy, can also be used for single-molecule studies (Gell *et al.*, 2006).

Single-molecule fluorescence assays have provided many new insights into the movement of motor proteins. For example, the sizes of the steps taken by myosin-V along actin filaments (Yildiz *et al.*, 2003) and kinesin-1 along microtubules (Yildiz *et al.*, 2004) have been measured. Single-molecule assays have also provided new insights into the regulation of microtubule dynamics: kinesin-13 targets the microtubule end through diffusion on the lattice prior to capture at the end (Helenius *et al.*, 2006); XMAP-215 is a processive depolymerase that "surfs" on the growing end of a microtubule as it adds many tubulin dimers (Brouhard *et al.*, 2008); EB1 binds only transiently to the comet tail that it forms at the growing end of a microtubule (Bieling *et al.*, 2007).

The application of single-molecule techniques to study microtubule dynamics is technically much more challenging than its application to motor proteins. The reason is that motors move quickly, often with speeds on the order of 1 μm/s, and therefore make many elementary steps in a second. By contrast, the dynamics of microtubules is slow. Growth is often only on the order of microns per minute, and the transitions from growth to shrinkage (catastrophe) and shrinkage to growth (rescue) take place at rates less than 1/min. Thus, microtubule dynamics must be observed over timescales of many minutes. This leads to several problems. The main one is that the fluorescent molecules will slowly accumulate on surfaces. Nonspecific binding to the chamber surfaces leads to an increase in the background, making detection of the fluorophores interacting specifically with the microtubules more difficult to observe. Furthermore, nonspecific binding leads to a reduction in the concentration of molecules in solution, causing an apparent decrease in activity. This chapter focuses on experimental procedures to reduce nonspecific binding to surfaces, as well as to increase the fluorescence lifetime of the fluorophores.

II. Single-Molecule TIRF Microscopy

Within the last decade, TIRF microscopy has emerged as a key tool in biophysics, particularly in single-molecule fluorescence studies (Gell *et al.*, 2006; Selvin and Ha, 2007). Incorporated into conventional fluorescence microscopes, TIRF allows imaging limited to a thin layer (~100 nm) above a glass substrate (for reviews see Axelrod *et al.*, 1984; Gell *et al.*, 2009; Thompson and Steele, 2007). This removes the distraction of out-of-focus fluorescent material, thereby providing sufficient signal-to-background to detect single molecules. Two methods to implement TIRF are commonly found in the literature: one uses a prism and the other a microscope objective as the condenser (Conibear and Bagshaw, 2000). In this section, we present an overview of the advantages of TIRF in the context of microtubule assays, provide a brief introduction to TIRF theory, and discuss its implementation in a multicolor objective-based system. Finally, we detail useful points to consider when using TIRF for dynamic microtubule studies.

A. TIRF Microscopy for Microtubule Assays

To study microtubule growth or shrinkage, and the mechanism by which microtubule-associated proteins (MAPs) enhance or inhibit growth or shrinkage (Brouhard *et al.*, 2008; Helenius *et al.*, 2006; Varga *et al.*, 2006, 2009), fluorescent microtubules (see Sections IV-VI) are immobilized (see Section VIII) onto glass surfaces (see Section VII and Fig. 1). Typically, high concentrations (100 nM-1 μM) of MAPs and/or tubulin heterodimers are added to the solution, some proportion of which are fluorescent (see Section IX and Fig. 1). Because only the interactions of the MAP/tubulin with the microtubule are of interest, the microscope is focused on the stationary, surface-immobilized, microtubules. Fluorescence images are recorded, with typical exposure times of ~100 ms, over a period of time. Visualization of single molecules is mediated through their localization on the microtubules (as explained above), typically restricting them to a few pixels over the exposure time of each frame. In contrast, molecules not interacting with the microtubule have their signal spread over many pixels in a single frame (and also move out of focus) due to their comparatively rapid, three-dimensional diffusion in solution. In standard epifluorescence illumination, the large number of rapidly diffusing molecules in solution above the microtubules are illuminated and fluoresce, causing a high background signal. TIRF illumination allows a very thin layer (~100 nm) of solute above the glass surface to be exclusively illuminated. Thereby, fluorescent molecules that interact with the microtubules are excited efficiently. Molecules in the bulk solution reside only fleetingly within the illuminated layer and therefore contribute little to the detected fluorescence signal. This combination of restricted observation volume and localization of molecules at the microtubules allows us to visualize single fluorescent molecules.

B. TIRF Theory

In TIRF, fluorescent molecules are excited using an evanescent field generated by the total internal reflection (TIR) of a laser beam at the interface between a glass surface and an aqueous solution (Axelrod *et al.*, 1984), see Fig. 2.

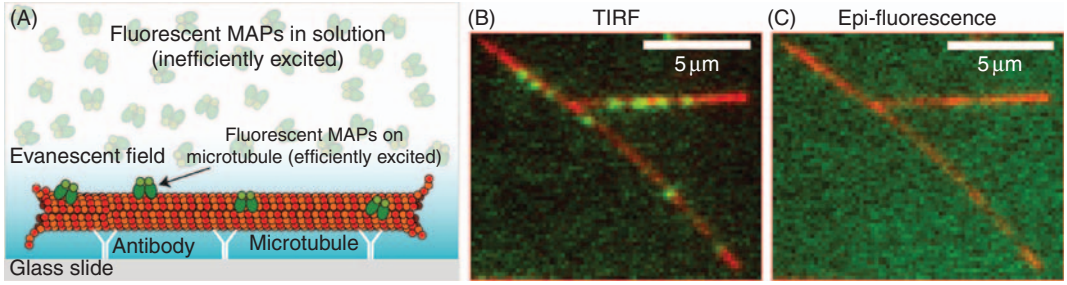
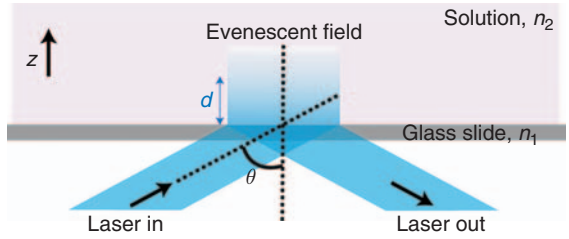


Fig. 1 (A) Schematic of the total internal reflection fluorescence (TIRF) experimental setup. Fluorescent-labeled microtubules (red) are immobilized onto glass surfaces using antibodies, fluorescent-microtubule-associated proteins (MAPs) (green), and/or fluorescent-tubulin (not shown) are added to the solution. The evanescent field for total internal reflection illumination is shown (blue). (B) and (C) Dual-color overlays of fluorescence micrographs of the same sample area. Different illuminations, TIRF (B) and epifluorescence (C), were used to excite enhanced green fluorescent protein-tagged (see Section IV) kinesin-1 (Leduc *et al.*, 2007). With TIRF illumination, the individual microtubule-interacting molecules are clearly visible. (See Plate no. 13 in the Color Plate Section.)



$$I(z) = I_0 \exp\left(-\frac{z}{d}\right)$$

$$d = \frac{\lambda}{4\pi\sqrt{\left(n_1^2 \sin^2\theta - n_2^2\right)}}$$

Fig. 2 Simplified optical geometry for total internal reflection evanescent wave generation, highlighting the key parameters (see text). The two equations describing the decay profile and penetration depth of the evanescent field are also shown.

The intensity of the evanescent field (I) decays exponentially in a direction (z) perpendicular to the glass surface and into the lower refractive index aqueous solution, with characteristic decay length (d, the distance at which the intensity has fallen e-fold). The decay length of the evanescent field, often called the penetration depth, is a function of the wavelength of light (λ), the refractive index of the glass (n_1), the average refractive index of the imaging solution (n_2), and the angle of incidence of the light (θ) (Gell *et al.*, 2009).

C. Two-Color TIRF Implementation

We achieve two-color TIRF in a through-the-objective-based configuration (Axelrod *et al.*, 1984; Conibear and Bagshaw, 2000; Gell *et al.*, 2006) installed into a standard inverted fluorescence microscope. A laser is focused onto the periphery of the back focal plane (BFP) of a high numerical aperture microscope objective (α Plan-Apochromat 100×, 1.46 NA, Carl Zeiss, Jena, Germany), as shown in Fig. 3. A narrow (<1 mm) collimated beam emerges from the objective, impinging on the coverglass/solution surface at an angle determined by the distance of the point of focus from the optical axis of the objective. The angle of incidence of the light at the interface can then be adjusted by translating the focused beam across

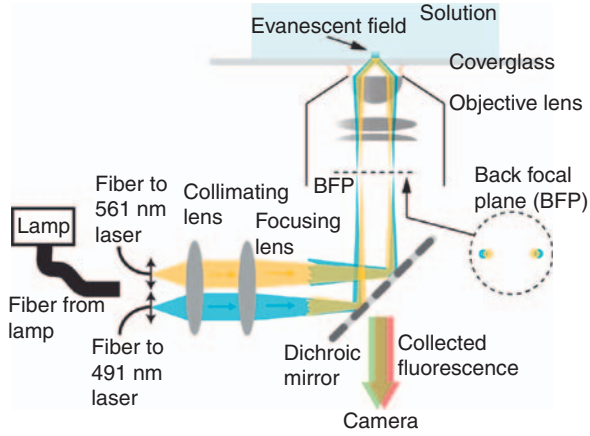


Fig. 3 Schematic of the layout of the two-color TIRF microscope used in our laboratory, see text for a description. (See Plate no. 14 in the Color Plate Section.)

the BFP. In our microscope, excitation light is provided using two lasers. One operates at a wavelength of 491 nm and the other at 561 nm (Cobolt Calypso and Jive, Cobolt, Sweden). The output from these lasers is coupled into two single-mode optical fibers (Oz Optics, Ottawa, Canada). The output ends of these fibers are mounted onto independent translation stages at the back port of an inverted microscope (Zeiss, Axiovert 200 M). Beams are collimated (lens, $f = 80$ mm, 25-mm diameter) to a beam diameter of $\sim$10 mm and focused (lens $f = 120$ mm, 25-mm diameter) using a system based on a commercial condenser assembly (Visitron Systems, Puchheim, Germany). A dual-band polychroic mirror (zt488-491/561rdc, Chroma Tech. Corp., Bellows Falls VT, USA) then directs the light toward the BFP of the objective. This dual-fiber geometry allows the two lasers to be focused onto opposite sides of the back aperture of the objective. Importantly the incident angle, and therefore the penetration depth, as well as the focus, of each laser can be varied independently; this is in contrast to systems delivering multiple illumination wavelengths through a single fiber. Emitted fluorescence is collected by the same microscope objective and directed toward an electron multiplying charge-coupled device camera (Andor iXon+, Andor Tech., Belfast, UK). For simultaneous dual-color imaging, we use a beam splitter (W-View, Hamamatsu, Japan) in front of the camera that incorporates filters from Chroma Tech. Corp.

D. Using a TIRF Microscope—Practical Tips and Pointers

- For single-molecule imaging we typically use a laser beam power of $\approx$5 mW, measured as it exits the microscope objective.
- Temperature control: for dynamic microtubule assays we use an objective heater (#0280.004, with controller 37-2, Pecon GmbH, Erbach, Germany). We find this effectively regulates the temperature of the region of the sample around the objective focus.
- The epifluorescence arc-lamps on many microscopes generate considerable heat. They can significantly affect the temperature of the microscope, causing unwanted sample drift, but also heating the sample itself. Therefore, we mount our arc-lamp

off-scope and couple the light to the microscope through a large multimode optical fiber (see Fig. 3).

- Uniform illumination across the entire field of view (typically $\approx$80 μm^2) is desirable. Two parameters affect this. Firstly, the divergence of the focused light in the objective BFP determines the illumination area at the coverglass surface; it should be adjusted for the field of view required. Secondly, interference with reflections of the laser light from the optical components and diffraction from dust on the surfaces of optical components (even when not in a conjugate plane) can cause fringes and nonuniform illumination. Therefore, keep your microscope clean, covered when not in use, and use good antireflection-coated filters and lenses.
- Accurate focusing on the BFP can be achieved by observing the transmitted beam emerging from the objective some meters away on the ceiling. Adjustment of the position of the focusing lens can then be performed.

E. Considerations for Dynamic Microtubule Assays

- Using a dual laser beam setup, with beam splitter, allows fluorescence to be collected simultaneously from two differently labeled proteins with single-molecule resolution. For dynamic growth assays, two lasers may not be necessary. Generally the microtubule extensions are very bright, due to the large number of incorporated fluorescent labels. Thus, a laser with a wavelength far from the excitation maximum of the tubulin–dye is usually sufficient to produce a detectable signal.
- The growing ends of dynamic microtubules are not fixed to the surface and can move outside the TIRF evanescent field. When possible, choose longer wavelengths and shallower angles of incidence of the TIRF laser to increase penetration depth. Some labs have employed methyl cellulose to reduce growing microtubule fluctuations (Bieling *et al.*, 2007).
- Chromatic aberrations both change the focus and distort recorded images. Therefore, it is essential to use an objective corrected at two wavelengths for chromatic aberration (an apochromat) when performing simultaneous dual-color TIRF imaging at two different wavelengths. This is necessary even when using a beam splitter such as the Hamamatsu W-View that incorporates some built-in correction.

III. List of Reagents

In this section, we present an alphabetical list of the reagents used throughout this chapter. We give details of product codes and manufacturers where possible, comment on storage and handling as well as details of preparation.

- Alexa Fluor 488 (Invitrogen, Molecular Probes, Karlsruhe, Germany, A30005), used for tubulin labeling (see Hyman *et al.*, 1991), we use the tetrafluorophenol conjugate.
- Antibeta-tubulin SAP4G5 (Sigma, Munich, Germany, T7816), typically a 1:50–1:200 dilution from the stock is used, in BRB80, stored at 4°C for several weeks.
- Antitetramethylrhodamine (Invitrogen, Molecular Probes, A6397), typically 10–20 μg/ml in BRB80, stored at 4°C, for several weeks.

- β-Mercaptoethanol (BME, Sigma, M3148), stored in ~100 µl aliquots in small tubes at 4°C.
- BRB80 buffer (80 mM PIPES/KOH, pH 6.9, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM $MgCl_2$). This buffer is commonly used to allow efficient polymerization of tubulin (Brinkley, 1997; Olmsted and Borisy, 1975; Weisenberg, 1972). We use KOH rather than NaOH because potassium is the main intracellular cation, and sodium ions enhance the hydrolysis of guanylyl-(alpha, beta)-methylene-diphosphonate (GMPCPP) in the microtubule lattice (Caplow *et al.*, 1994). Buffer is filtered (0.22 µm vacuum filter), degassed, and the stock stored in 50 ml aliquots at −20°C.
- Bovine serum albumin (BSA, Sigma, A3059), stock solution is made at 10 mg/ml in BRB80, filtered, and the pH checked; solution is stored at 4°C for daily use for up to several months.
- Catalase (Sigma, C9322), stock solution is made at 1 mg/ml (4.2 uM) in BRB80, 10 µl aliquots, snap-frozen in liquid nitrogen, stored at −20°C.
- Dichlorodimethylsilane (DDS, Aldrich, Munich, Germany, 440272), stored upright, in sealed container at 4°C—avoid agitating when handling.
- Dithiothreitol (DTT, Sigma, D0632). Aliquots stored at −20°C.
- EGTA (Sigma, E4378).
- Ethanol (Merck, Haar, Germany, 1.00983.2511).
- F127, Pluronic F127 (Sigma, P2443), stock solution of 1% F127 is dissolved in BRB80 overnight, filtered (0.22-µm syringe filter), stored at 4°C.
- D-Glucose (Sigma, G7528) stock solution is made at 2 M in water, 10 µl aliquots, stored at −20°C.
- Glucose oxidase (Sigma, G7016) stock solution is made at 2 mg/ml (12 uM) in BRB80, 10 µl aliquots, snap-frozen in liquid nitrogen, stored at −20°C.
- guanylyl-(alpha, beta)-methylene-diphosphonate (GMPCPP) (Jena Biosciences Jena, Germany, #NU-405), 10 mM stock stored in 10 µl aliquots at −80°C.
- Hydrogen peroxide (H_2O_2, 30%) (Sigma, 21676-3), stored at 4°C.
- Sulfuric acid (H_2SO_4, 97%) (Roth, Karlsruhe, Germany, X944.1).
- Magnesium chloride ($MgCl_2$, Merck, 1.05833.0250).
- Methanol (Merck, 1.06009.2511).
- Potassium hydroxide (KOH, 0.1 M, Sigma, P-6310). Add KOH slowly to BRB80 buffer to set the pH 6.9, the solution may warm.
- Neutravidin, 5–50 µg/ml (Pierce, Rockford, IL, USA, 31000). Dissolve neutravidin at the required concentration in phosphate-buffered saline. It can be stored at 4°C for several weeks.
- PIPES (Sigma, P6757).
- Trichloroethylene (TCE, Sigma, 251402).
- Trolox (Sigma, 238813), see notes in text, Section V.
- Tubulin, unlabeled (purified from porcine brain, see Chapter 7 by Miller *et al.*, this volume).
- Tubulin, biotin labeled (Cytoskeleton Inc., Denver, CO, USA, T333-B)—for a labeling procedure (see Hyman *et al.*, 1991).
- Tubulin, fluorescent labeled (we label tubulin purified from porcine brain; see Chapter 7 by Miller *et al.*, this volume; Hyman *et al.*, 1991).
- TAMRA (Invitrogen, Molecular Probes, C1171), the succinimidyl ester conjugate is used for tubulin labeling (see Hyman *et al.*, 1991).
- Water, ultrapure (>18 MΩ/m).

IV. Choice of Fluorophore/Protein Labeling

The right choice of fluorescent label is important for the success of a dynamic microtubule experiment. It depends on the experimental approach, number of components in the assay, and available instrumentation. In this section, we highlight some of the considerations that affect the choice of labeling method and dye. We refer the reader to Bane *et al.* (this volume) and also the following papers (Gell *et al.*, 2006; Hunter *et al.*, 2003; Hyman *et al.*, 1991; Selvin and Ha, 2007) for more detailed information regarding labeling protocols.

A. Common Labeling Strategies

- *Chemical posttranslational dye labeling*

  Typically amine or thiol-reactive dyes are used to covalently bind fluorophores to primary amines, the N-terminus, or cysteine groups of expressed proteins.

  Advantages: Protein is expressed a single time and can be used for multiple labeling reactions with different dyes.

  Disadvantages: Labeling conditions can be harsh; labeling site is often unspecific and unknown; labeling can occur at multiple sites, or not, in an uncontrolled manner; the protocol generally needs optimization for each biomolecule; thiol-labeling first requires the production of a cysteine-free mutant.

  Successfully used: This method has been used successfully to label tubulin (e.g., Hyman *et al.*, 1991) and kinesin-1 (e.g., Mori *et al.*, 2007).
- *Fusions with intrinsically fluorescent proteins*

  Make a fusion protein of green fluorescent protein (GFP) or a GFP-variant gene and the gene of interest.

  Advantages: Excellent specificity; control over number of labels; defined stoichiometry; GFP derivatives available across the visible spectrum.

  Disadvantages: Genetic modification can be time consuming; needs to be repeated if different fluorescent proteins (colors) are required; overexpression of some fusion proteins can fail; the photo-physical properties of GFP and its derivatives are not ideal (Dickson *et al.*, 1997); may alter protein activity.

  Successfully used: We have used this method to label a variety of MAPs (Brouhard *et al.*, 2008; Helenius *et al.*, 2006; Varga *et al.*, 2006).
- *Fusions with SNAP-tag (or similar systems, e.g., HALO, ACP)*

  The SNAP-tag (New England Biolabs, Frankfurt am Main, Germany) is a small enzymatic protein that can be expressed as a fusion with the protein to be labeled, whose substrate can carry a dye that becomes covalently attached to the SNAP-tag (Gautier et al., 2008).

  Advantages: After production of SNAP fusions, ready-to-use labeling substrates with a large range of chemical dyes are available; labeling is specific; protein is singly labeled or unlabeled; labeling is with dyes with optimized photo-physical properties.

  Disadvantages: Initial genetic modification procedure can be a time-consuming process; requires a reliable overexpression system; may alter protein activity.

  Successfully used: We have used this method to label kinesin-1 (unpublished).

Tip: *Whatever the choice of labeling protocol, it is essential to check that the activity of the labeled molecule is comparable to the wild type.*

Tip: *We have experienced particular problems with some dyes (e.g., Atto 425) when labeling microtubules. Some dyes seem to stabilize the microtubules significantly, others prevent polymerization with labeled tubulin. Controls to check function should always be performed.*

Tip: *Fluorescent dye- or biotin-labeled tubulin is also commercially available (Cytoskeleton Inc.).*

B. Dye-Selection Considerations for Multicolor, Dynamic Microtubule Assays

- *General considerations for single-molecule detection*

  For single-molecule detection, look for dyes with high quantum yields and extinction coefficients at the laser wavelengths to be used. Note that literature values for these should be used only as a guide; the photo-physical properties of a dye will change depending on the molecule it is conjugated to and the solution used. Common dyes that have been used with success in the literature include TAMRA (Helenius *et al.*, 2006; Varga *et al.*, 2006), Cy dyes (Yildiz *et al.*, 2004), Alexa Fluor dyes (Bieling *et al.*, 2007), and various GFP fusions (Helenius *et al.*, 2006; Varga *et al.*, 2009).
- *Monitoring microtubule growth/shrinkage and a single MAP at single-molecule resolution*

  Choose dyes with sufficiently separated fluorescence emission so that available emission filter sets can effectively separate the signals without overlap of one dye's fluorescence emission with the transmission range of the other dye's filter.

  Choose a shorter wavelength dye for the MAP than the labeled microtubule: The emission curves of dyes typically have long tails toward longer wavelengths, but not to shorter wavelengths. As the signal from the microtubule will be bright, this could limit contrast in the single-molecule channel.

  Choosing a shorter wavelength dye for the MAP, which is observed at the level of a single molecule using TIRF, generally means that the same laser can provide sufficient excitation of the growing/shrinking microtubule. This means that simultaneous dual-color experiments can be performed with a single laser and beam splitter.
- *Monitoring the behavior of two MAPs at single-molecule resolution*

  Choose labels optimized for the two laser wavelengths, the filter sets, and the camera available.

  Spectral overlap is less of an issue.

  Microtubules should be either unlabeled or labeled with a third color (again choose a dye that does not overlap with the fluorescence spectrum of either MAP).

V. (Anti-)blinking/Photo-Toxicity/Photo-Bleaching Cocktails

Many fluorescent molecules used for single-molecule TIRF display unwanted blinking on the millisecond to second timescale, often, but not exclusively, due to the dye being excited into a triplet state (Aitken *et al.*, 2008; Rasnik *et al.*, 2006). Population of dye triplet states is also thought to be an important precursor to irreversible loss of fluorescence (photo-bleaching) (Aitken *et al.*, 2008; Rasnik

et al., 2006). Dye triplet states are effectively quenched by molecular oxygen, but the by-product of quenching is a singlet oxygen that is highly reactive and can induce oxidative damage to both dyes and proteins (Aitken *et al.*, 2008). Oxidation of dyes leads to photo-bleaching; oxidation of tubulin can, for example, cause the spontaneous breaking apart of microtubules (Guo *et al.*, 2006). The effects can be reduced, and concomitantly, the time-to-bleaching of fluorescent molecules can be prolonged, using enzymatic oxygen scavenger systems with additional triplet-state quenching chemicals. In this section, we detail the steps we typically take to reduce these effects in TIRF (and standard epifluorescence)-based microtubule assays in our laboratory.

A. Glucose Oxidase/Catalase with BME as an Oxygen Scavenging/Antifade System

D-Glucose is oxidized to gluconic acid by glucose oxidase, depleting oxygen in solution. This reaction generates hydrogen peroxide as by-product, which is degraded by the catalase in a second reaction step. BME in solution increases time-to-bleaching through triplet-state quenching as well as helping to maintain a protein-friendly reducing environment.

Include in the final reaction solution the following concentrations of components, typically in BRB80 buffer (see the reagent list in Section III, and the tips later, for further details of the reagents, their preparation, and use):

250 nM glucose oxidase in BRB80
64 nM catalase in BRB80
40 mM D-glucose in water
1% BME

Tip: *Store all components, separately, at −20°C in aliquots of 10 µl at a 100× concentration. Catalase and glucose oxidase are dissolved in BRB80 and snap frozen in liquid nitrogen. When thawed and stored on ice, aliquots will maintain activity for several hours. Once mixed together use the solution within 1 h, thereafter we have observed a loss of effectiveness. For consistency, do not refreeze thawed aliquots.*

Tip: *Add the glucose oxidase last and just before actually using the imaging buffer. This will initiate the first part of the reaction, which depletes the oxygen from solution.*

Tip: *DTT can be substituted for BME. Use a final DTT concentration of 10 mM. Note, however, BME and DTT have both been demonstrated to have negative effects on some dyes (Aitken et al., 2008). For example, DTT appears to dramatically reduce the signal from the dye Alexa Fluor 488, but BME does not (Aitken et al., 2008*).

B. Troubleshooting (Anti-)blinking/Photo-Toxicity/Photo-Bleaching Cocktails

If you observe significant bleaching or photo-induced damage of microtubules, check whether the glucose oxidase/catalase (GODCAT)/BME system works in a reference assay. We use a robust standard casein-gliding assay with kinesin-1 for this purpose (see Chapter 14 by Nitzsche *et al.*, this volume). Other points to consider are

1. GODCAT enzymes might adsorb to an incompletely blocked surface (see Section VIII and Chapter 28 by Bieling *et al.*, this volume), reducing their concentration.

2. BME (and DTT) activity decreases with exposure to air and can be a major reason for ineffective cocktails. Make small amounts (1–5 ml), aliquot to smaller sizes once, and store at 4°C in a desiccator. BME (and DTT) activity can be determined by assaying the amount of oxidized reagent using a spectrometer (Iyer and Klee, 1973; Mickey and Howard, 1995).
3. If the buffer that you use has a low buffering capacity and/or your imaging solution exchanges a lot of oxygen with the environment, pH can drop quite drastically over time due to production of gluconic acid from the oxidation of D-glucose by glucose oxidase (Selvin and Ha, 2007). In these cases, try to raise buffer capacity and/or reduce oxygen exchange with the environment.
4. The effectiveness of different oxygen scavenging/triplet quenching systems/dye systems is variable; it may be necessary to experiment to find the best cocktail for a given dye (see Aitken *et al.*, 2008; Rasnik *et al.*, 2006). We have found the protocols detailed here effective with TAMRA, GFP, RFP, Alexa 488, and Alexa 568.

C. Trolox as an Alternative Triplet-State Quencher

While BME (or DTT) has been a traditional component in many groups' oxygen scavenging/antiphoto-bleaching cocktails, it is known to cause slow blinking with some fluorescent dyes. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) has recently been characterized as an alternative triplet-state quencher (Aitken *et al.*, 2008; Rasnik *et al.*, 2006). In our assays, we keep BME present to maintain a reducing environment.

Include in the final reaction solution the following concentrations of components (see Section III, and the tips later, for further details of the reagents, their preparation and use):

250 nM glucose oxidase
64 nM catalase
40 mM D-glucose
1% BME
1 mM Trolox

Tip: *10 mM Trolox is dissolved for 1 h in the buffer that is used for imaging. This solution is then filtered (0.22-μm syringe filter) and the concentration checked using absorption at 290 nm and an extinction coefficient of 2350/M/cm. The pH should also be checked and adjusted to pH 6.9. The Trolox solution can then be stored at 4° C and used within 2 weeks.*

D. Effect of (Anti-)blinking/Photo-Toxicity/Photo-Bleaching Cocktails

The effect of using different anti-bleaching and anti-photo-toxicity components is illustrated in Fig. 4. Single molecule fluorescence intensity trajectories were recorded from surface-immobilised Alexa Fluor 488 dye-labeled tubulin, using TIRF. An increase in the time-to-bleaching of the molecules is observed upon addition of the GODCAT/BME cocktail. However, blinking is induced by using BME alone as a triplet state quencher. The blinking is reduced when also using Trolox in the cocktail.

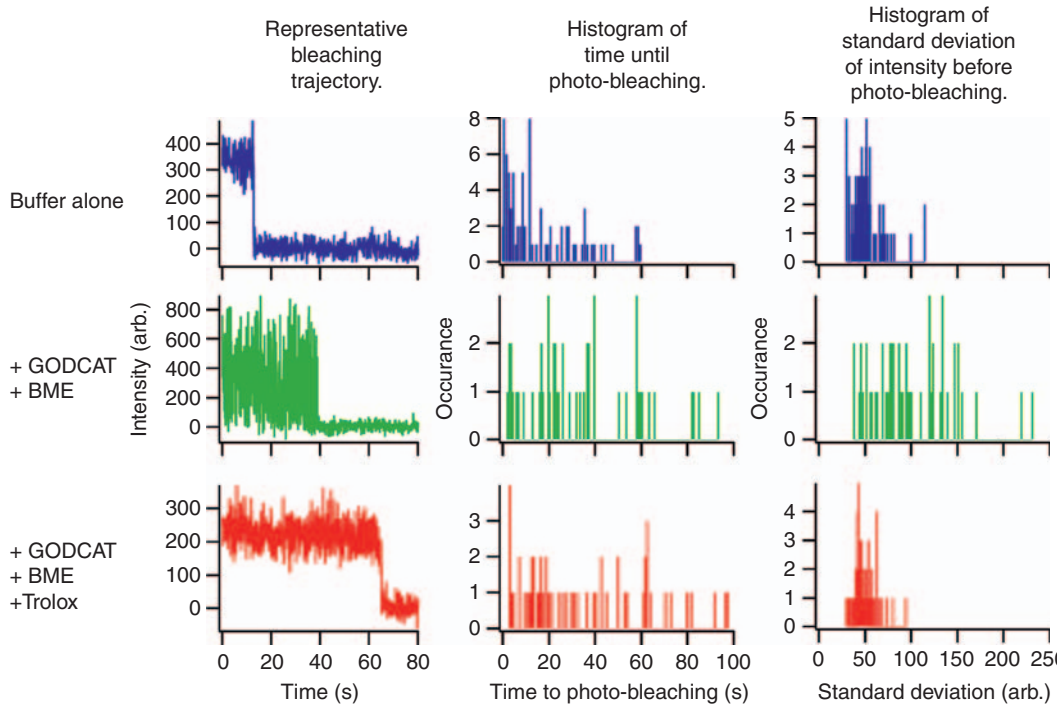


Fig. 4 The effectiveness of different cocktails is quantified by measuring the fluorescence versus time trajectories of single molecules. Here are shown example trajectories for single Alexa Fluor 488-labeled tubulin heterodimer immobilized on a glass slide and imaged using TIRF, histograms of the time until photo-bleaching from many such trajectories, and finally histograms of the standard deviation of the intensity from many single molecules before photo-bleaching occurs, each in buffer alone, with GODCAT + BME and with GODCAT + BME + Trolox. (See Plate no. 15 in the Color Plate Section.)

VI. Preparation of GMPCPP-Stabilized Microtubules

Stabilized microtubules for use as substrates for microtubule depolymerization assays, or as seeds upon which to nucleate the growth of microtubule extensions, are prepared by polymerization of tubulin in the presence of the slowly hydrolyzed GTP analogue, GMPCPP (Hyman *et al.*, 1992). The distribution of microtubule lengths obtained, and the propensity of the microtubules to spontaneously depolymerize, can be tailored by altering the concentration of tubulin used in the polymerization reaction and by additional cycles of polymerization, respectively.

A. Single-Cycled Microtubules

Step 1: For microtubules with an average length of ~3.5 µm (suitable as seeds from which to grow extensions), the following mix is first incubated on ice for 5 min, then at 37°C for 30 min.

20 µM tubulin in BRB80
1 mM GMPCPP

Or, for microtubules with an average length of ~6.5 μm (suitable as substrates for depolymerization assays), the following mix is first incubated on ice for 5 min, then at 37°C for 2 h.

2 μM tubulin in BRB80
1 mM GMPCPP

Step 2: Spin microtubules in an ultracentrifuge (e.g., Beckman Airfuge, A95 rotor at 80,000 rpm/126,000 × *g*) to remove unpolymerized tubulin, discard supernatant, and resuspend microtubule pellet in the required buffer (see Sections III and VIII).

Tip: *Single-cycled microtubules are made for immediate use. These microtubules are stable for several hours postpolymerization, depending on initial length. The stability of GMPCPP microtubules can be increased by a second cycle of polymerization—see below.*

Tip: *At 20°C the average rate of spontaneous depolymerization of single-cycled GMPCPP-stabilized microtubules immobilized on coverglass surfaces is in the range of 0.02–0.03 μm/min.*

B. Double-Cycled Microtubules

To enhance the stability of microtubules, two cycles of polymerization in GMPCPP can be carried out (Caplow and Shanks, 1996). This results in an increased proportion of GMPCPP–tubulin relative to single-cycled microtubules by reducing the amount of nonexchanged guanosine diphosphate–tubulin present in the microtubule lattice; this is useful as short microtubule seeds will last longer in assays. This can also have important consequences when microtubules are used as substrates for depolymerization studies (Helenius *et al.*, 2006).

- **Cycle 1**

  Step 1: For microtubules with an average length of ~3.5 μm (suitable as seeds from which to grow extensions), the following mix is first incubated on ice for 5 min, then at 37°C for 30 min.

  20 μM tubulin in BRB80
  1 mM GMPCPP

  Step 2: To remove unpolymerized tubulin, spin incubation mix for 5 min in an ultracentrifuge (e.g., Beckman Airfuge, A95 rotor at 80,000 rpm/126,000 × *g*) and discard the supernatant.

  Step 3: Resuspend the microtubule pellet in BRB80. Choose a volume of buffer that will result in a tubulin concentration of 20 μM after addition of GMPCPP in step 5 below (~80% of the initial tubulin is routinely recovered after polymerization and resuspension).

  Step 4: Incubate resuspended tubulin on ice for 20 min to depolymerize the microtubules.

- **Cycle 2**

  Step 5: Add GMPCPP to the depolymerized tubulin to a concentration of 1 mM, resulting in a tubulin concentration of 20 μM. Incubate on ice for 5 min, then at 37°C for 30 min.

  Step 6: Spin microtubules for 5 min in an ultracentrifuge (e.g., Beckman Airfuge, A95 rotor at 80,000 rpm/126,000 × *g*) and discard supernatant.

Step 7: The microtubule pellet should then be resuspended in the required buffer (see Sections III and IX). The volume of buffer used to resuspend the pellet can be chosen to give the required concentration of polymerized tubulin (~80% of the tubulin is routinely recovered from each cycle of polymerization and resuspension).

Tip: *At 20°C the rate of spontaneous depolymerization of double-cycled GMPCPP-stabilized microtubules immobilized on coverglass surfaces is <0.01 μm/min.*

Tip: *Double-cycled microtubules can be stored for many months in liquid nitrogen by snap freezing immediately after resuspension. When required for use, they should be thawed by incubation at 37°C. It is best to store the microtubule samples in aliquots of ~100 μl or less so that they can be thawed rapidly (1–2 min). The ability to store microtubule seeds in this way can greatly reduce the day-to-day workload in performing dynamic microtubule assays.*

Tip: *The freeze–thaw process causes breakage of microtubules resulting in an average length of ~2 μm.*

C. Preparation of Labeled Microtubules

A suitable proportion of dye-labeled tubulin can be used in the polymerization reaction to provide fluorescent microtubules. Biotinylated tubulin can be incorporated to bind microtubules to a neutravidin-coated surface (Section VIII). Labeled tubulin can be either prepared in-house (Hyman *et al.*, 1991) or purchased commercially (e.g., from Cytoskeleton Inc.).

- **Biotin-labeled microtubules:** To facilitate attachment to a neutravidin-coated surface, 10% biotin-labeled tubulin is combined with unlabeled tubulin in any of the above polymerization protocols.
- **Fluorescent microtubules:** To obtain fluorescent microtubules up to 25% TAMRA-labeled tubulin is combined with unlabeled tubulin in any of the above polymerization protocols.

Tip: *To adjust the brightness of fluorescently labeled microtubules the ratio of labeled to unlabeled tubulin can be adjusted as required. We find that 5–25% fluorescent-labeled tubulin generally results in a good compromise between brightness and inhibition of the interaction of MAPs due to the presence of label.*

D. General Information on Handling Microtubules/Tubulin

Tip: *Unpolymerized tubulin is generally stored long-term at −80°C in small aliquots and kept on ice prior to use.*

Tip: *Microtubules should* ***NOT*** be placed on ice as low temperatures cause rapid depolymerization.

Tip: *If long microtubules (>10 μm) are required, a cut pipette tip (such that the opening of the tip has a larger diameter) should be used when resuspending the microtubule pellet to minimize shearing.*

Tip: *The concentration of polymerized tubulin in the resuspended microtubules can be determined by depolymerizing a small volume of the microtubule solution with 5× BRB80, 50 mM KCl, 5 mM $CaCl_2$, and incubation on ice for 20 min. The*

tubulin concentration is then determined by measuring the absorbance at 280 nm. The extinction coefficient for dimeric tubulin, calculated from the sequence of tubulin, including the contribution of two bound guanine nucleotides is 115,000/M/cm, assuming an approximate molecular weight of 100,000 g. Note that different labs tend to use different estimates for the extinction coefficient of tubulin.

VII. Glass Treatment and Sample Chamber Preparation

In this section, we describe the preparation and construction of sample chambers for studying microtubules in a single-molecule TIRF microscope. The aim is to produce clean, low-fluorescence glass surfaces, formed into simple flow cells, that allow convenient immobilization of microtubules or microtubule seeds and that can be effectively passivated to prevent unwanted nonspecific absorption (see Section VIII).

A. Safety

Many of the chemicals used here are potentially harmful, corrosive, or explosive. Particular care must be taken to familiarize yourself with the dangers, how to mitigate them, and how to deal with problems. All of these steps should be performed in a suitable fume hood.

B. Key Equipment

- 18 × 18 mm and 22 × 22 mm (0.17–0.19 mm thick) coverglass (e.g., Corning, No. 1½, 2870-18, and 2870-22), porcelain or polytetrafluoroethylene racks for coverglasses (e.g., Coverglass maxi-rack, Invitrogen, Molecular Probes, C24784)
- Hotplate, set to 60°C
- Fume hood, acid-resistant gloves, lab coat, eye protection, acid/chemical spill kit
- Acid-resistant tweezers, 1-ml "luer-lock" syringe, with long needle
- Several thick-walled, acid-/temperature-resistant glass containers (∼250 ml volume, suitable to immerse coverglass racks)
- Measuring cylinder
- Ultrapure water (>18 MΩ/m), H_2SO_4 (97%), H_2O_2 (30%), DDS, TCE, methanol, ethanol, KOH
- Ultrasonic bath
- Clean nitrogen gas line for drying
- Coverglass holders for microscope
- Double-sided scotch tape (we use Scotch, "Double Face," 136D MDEU)

C. Glass Cleaning and Surface Preparation

These steps result in clean, low-fluorescence glass surfaces with the correct reactivity to enable subsequent silanization, following the general approach of deCastro *et al.* (1999).

Precleaning.

1. Place coverglasses into racks, ensuring adjacent coverglasses cannot contact.
2. Sonicate for 15 min in a soap bath (2% Mucasol, 98% water); rinse in water for 1 min.

3. Bathe, sequentially, in acetone for 10 min, ethanol for 10 min, ultrapure water for 1 min.

"Piranha solution" cleaning to remove all organic matter. EXTREME CAUTION—VOLATILE AND CORROSIVE—RESEARCH THE PROPERTIES OF THIS SOLUTION CAREFULLY BEFORE USE.

1. Mix a sufficient volume of 30% H_2O_2 solution and H_2SO_4 at a ratio of 1:2 in a suitable container to fully immerse the precleaned coverglasses in their racks. Always add the peroxide to the acid, adding the peroxide slowly; note that if the peroxide concentration is greater than the acid an explosion could occur. The solution initially gets hot upon mixing, but the temperature should be maintained at 60°C throughout the cleaning process by additional heating. Prepare the solution just prior to use since it is only active for ~1 h.
2. Transfer precleaned coverglasses directly from the water to the Piranha solution. Bathe for 1 h at 60°C.
3. Transfer racks directly from the Piranha solution sequentially to 3 ultrapure water baths, bathing for 1 min each.

Bathe coverglasses in KOH solution to activate the OH groups on the glass surface for silanization.

1. Transfer racks from the third ultrapure water bath to a 0.1-M KOH bath for 15 min.
2. Transfer racks sequentially through two ultrapure water baths, bathing in each for 1 min.
3. Remove from water baths, dry coverglasses *completely* with clean nitrogen gas. Store in clean sealed glass containers.

Tip: *It is essential to prepare the coverglasses at all stages in as clean an environment as is possible; use an area dedicated to their preparation; dry slides away from any sources of dust; store the slide in double-sealed holders and bags; open the containers only briefly to remove slides.*

Tip: *For silanization (see below), use only freshly cleaned coverglass and silanize immediately after the slides have dried completely.*

Tip: *Clean glass surfaces are wetted by water. A test for cleanliness is to check whether a droplet of water spreads immediately over the complete coverglass. If a drop with a finite contact angle forms, the coverglasses are dirty! Clean glass surfaces have a high surface energy. Any dirt adsorption, in particular organic molecules (always present in ambient conditions, e.g., due to oil vapors of pumps and air conditioning), reduces the surface energy of glass and is thus energetically favorable, i.e., clean glass surfaces are an attractant for dirt. It is best to use clean surfaces right away or render them hydrophobic for storage (see below).*

Tip: *Dispose of piranha properly. Used solutions must not be stored in closed containers since gas formation continues and a closed container bares the risk of explosions.*

Tip: *Use a filter on the nitrogen gas line. Clean the nitrogen gun in acetone to remove oil.*

D. Glass Silanization

These steps functionalize cleaned glass surfaces with a hydrophobic silane that allows efficient passivation (see Section VIII). Silanization is performed with a 0.05% solution of DDS in TCE.

1. Add sufficient TCE to a clean glass container to immerse the cleaned coverglasses in their racks.
2. Add DDS (to a final concentration of 0.05%), stir while adding DDS to TCE.
3. Gently place the coverglasses in the TCE/DDS solution, bathe for 1 h.
4. Transfer the silanized glass, sequentially, through three methanol baths, placed in an ultrasonic bath for times of 5, 15, and 30 min.
5. Remove from the final methanol bath, dry coverglasses *completely* with clean nitrogen gas. Store in clean sealed glass containers.

Tip: *We have found that DDS forms insoluble crystals during storage; avoid agitating the DDS in its bottle. Use a long needle and syringe to pierce the Teflon seal on the DDS bottle and draw off the required amount from the top. Add DDS via syringe under the TCE to avoid air contact. DDS vigorously reacts with water, resulting in crosslinking and crystal formation. Crystals, if present on coverglass surfaces, greatly reduce the surface's ability to be passivated. Store DDS under nitrogen or in a desiccator.*

Tip: *We store the silanized coverglasses at room temperature and find that they last for at least 1 month; hydrophobic, low-energy surfaces attract less dirt than clean glass surfaces and are thus more suitable for storage. Alternatively, storage under 0.22-μm filtered ethanol is possible.*

Tip: *The quality of the silanization can be checked by putting a water droplet (smaller than 2 mm—the capillary length of water) onto the glass surface and measuring its contact angle. The water contact angle should ideally be larger than 100°.*

E. Sample Chamber Assembly and Use

These steps describe how we use a simple metal holder, one 18×18 mm and one 22×22 mm coverglass to make convenient, quick-to-construct sample chambers. We use small aluminum or brass holders designed to fit the microscope stage. The holder has a clear central aperture to allow approach of the microscope objective from underneath. The hydrophobic-rendered 22×22 mm slide is clamped in the holder, then typically four strips of double-sided scotch tape are laid down to define three channels. The smaller 18×18 mm slide is then pressed down onto the tape from the top. The depth of the channels is $\sim$100 μm (Fig. 5).

Tip: *Machine the holders as thin as possible; chamfer around the clear-aperture to allow access of the microscope objective to the outermost channels; do not place channels too close to the edges, else it may not be possible to image them with the microscope objective.*

Tip: *In place of double-sided tape, layers of Nescofilm (#2569.1, Bando Chemicals, Kobe, Japan) can be used to form the channels; further details can be found in Chapter 14 by Nitzsche et al., this volume.*

Tip: *Using multiple layers of tape can increase the channel volume; this decreases the surface area-to-volume ratio of the channel, mitigating the effects of any undesirable surface binding.*

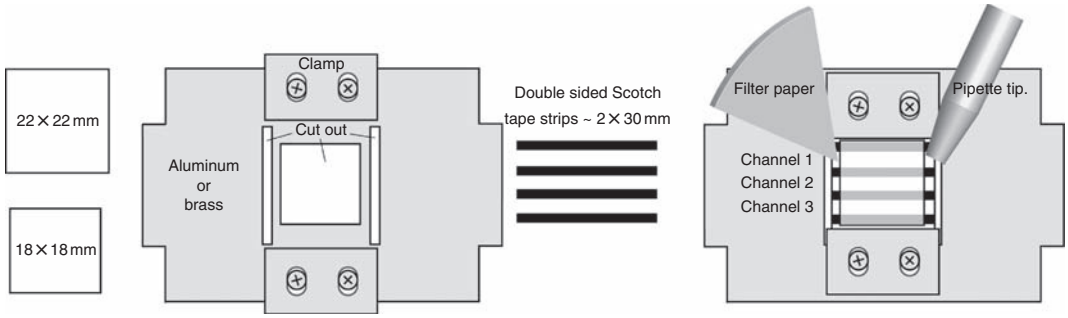


Fig. 5 Schematic of the sample holder design we used for microtubule assays. Channels defined by double-sided adhesive tape between hydrophobic-rendered coverglasses provide a convenient chamber in which to immobilize microtubules and to perfuse different solutions. Filter paper can be used to perfuse solutions, added at the opposite end of the channel using a pipette. In the case of hydrophobic channels, it may be necessary to introduce the first solution with the assistance of a vacuum line; filter paper is then used for subsequent perfusion steps.

Tip: *The flow profile in the channel is parabolic, thus there is slower movement of the solution near the channel walls. In order to exchange solution in the channels thoroughly, it is necessary to flow several channel volumes of solution.*

VIII. Binding of Microtubules and Passivation of Surfaces

Stabilized microtubules are bound to the surface *via* a spacer protein that attaches nonspecifically to the silanized coverglass surface, but specifically to the microtubule (see Fig. 1). The spacer protein holds the microtubule away from the surface, reducing unwanted surface interactions. Immobilized microtubules can be used as seeds from which to examine polymerization, substrates for depolymerization studies, and substrates for studying the mechanism of MAPs. The use of a spacer, in contrast to direct adsorption of the microtubule to the surface, ensures that the bottom surface of the microtubule is accessible and that the surface does not deform the microtubule. The spacer protein used depends on what microtubule/MAP property will be examined. One concern is microtubule-specific binding, i.e., that the surface should not bind the other components of the reaction mix used, including the additional tubulin used to grow extensions. In this section, we summarize different approaches to microtubule immobilization, highlighting their advantages and disadvantages for different types of assay. We then present a protocol for seed binding and subsequent passivation of the remaining surface.

A. Commonly Used Methods of Attachment

- Antitubulin antibody is used to bind to tubulin

  The tubulin antibody binds nonspecifically to the surface, but specifically to the microtubule.

  Advantages: No labeled tubulin required.

  Disadvantages: Free tubulin can compete for binding and cause dissociation of the microtubule from the surface.

- Antifluorophore binding to labeled tubulin (Reuther *et al.*, 2006)

  A useful method when a fluorophore such as TAMRA or Alexa488 is used to label the microtubule.

  Advantages: Free tubulin (unlabeled or differently labeled) does not compete for binding; no direct contact with microtubule lattice.

  Disadvantages: Binding dependent on labeling density of microtubule.
- Neutravidin binding to biotinylated tubulin

  Advantages: Strong binding; free (unbiotinylated) tubulin does not compete for binding.

  Disadvantages: Requires 5–10% biotinylated tubulin to be included in the microtubules, in addition to any other fluorescent labels that are used.

Tip: *The seeds should be bound tightly, but using the lowest possible surface density of attachment spacer, reducing any possible inhibition of normal binding of MAPs to the seeds.*

B. Typical Seed Binding/Surface Passivation Protocol

Step 1: The antibody/neutravidin is perfused into the hydrophobic-rendered glass flow-cell with the assistance of a vacuum (see Section VII) at the following range of concentrations:
Use one of

antibeta-tubulin SAP4G5 (typically a 1:50–1:200 dilution in BRB80)
antitetramethylrhodamine: 10–20 μg/ml (in BRB80)
neutravidin: 5–50 μg/ml (in BRB80)

Tip: *These stocks can be stored at 4°C for several weeks.*

Step 2: After 5-min incubation excess antibody/neutravidin is flushed out with ~5 channel volumes of BRB80.

Step 3: The remaining exposed surface is passivated in order to block any nonspecific surface binding using Pluronic F127. F127 is a tri-block copolymer consisting of two outer poly(ethylene oxide) (PEO)—also known as poly(ethylene glycol)—and an inner poly(propylene oxide) (PPO) blocks with 100 and 65 monomers, respectively. The PPO block is hydrophobic and strongly adsorbs onto the hydrophobic-rendered glass surface. The outer PEO parts form a polymer brush with approximately 10-nm thickness that is very effective in blocking protein adsorption in single-molecule experiments. Typically 1% Pluronic F127 in BRB80 is used.

Step 4: After 5 min incubation excess F127 is removed with ~10 channel volumes of BRB80.

Step 5: The channel is now ready for perfusion of solution containing prepared microtubules or microtubule seeds (see Section VI). The incubation time of microtubule seeds depends on the choice of immobilization method, microtubule seed, and antibody/neutravidin concentration, as well as the desired microtubule density in the channel. Typical times range from 5 to 15 min.

Tip: *The silanized coverglasses are hydrophobic. It is necessary to use a vacuum line to help draw in the first solution. After that, blotting/filter paper can be used. With care this can be done* in situ *on the microscope. Caution is needed not to introduce air bubbles into the channel.*

Tip: *The channels can dry out if left out on the bench for more than a few minutes. This can make it difficult to pull liquid through the channel ends and exposure to air will cause degradation of the microtubules. To avoid this, keep the sample chamber covered and either add additional BRB80 to the channel openings or keep a moist towel under the sample chamber. Chambers can be sealed to prevent evaporation using immersion oil.*

IX. Dynamic Microtubule Assays

In this section, we outline an assay that can be used to study the behavior of MAPs on dynamic microtubules. Firstly, we present the basic workflow for these types of assays and discuss the important components of the reaction mixture that will be perfused into the channel containing the microtubule seeds. Secondly, we discuss several important variables that must be considered and controlled for accurate experiments. Finally, we discuss the basic type of analysis carried out on these types of data (see elsewhere in this volume and Bieling *et al.*, 2007; Brouhard *et al.*, 2008, for additional information).

A. Protocol for Dynamic Microtubule Assay

Step 1: Prepare channels with fluorescently labeled GMPCPP-stabilized microtubule seeds bound to the surface (see Section VIII). Wash the channel with 5 channel volumes of BRB80.

Step 2: Place the holder on the microscope stage and bring the coverglass into contact with immersion oil on the objective. The objective is heated to the desired temperature (normally 35°C) by an objective heater (see Section II).

Step 3: Inject prewarmed reaction solution (see below) containing tubulin and/or a MAP of interest into the channel.

Step 4: Record a movie using TIRF illumination to visualize microtubule extensions growing from the seeds and/or the behavior of fluorescently labeled MAPs (see tips below).

B. Typical Reaction Solution

Prepare the reaction mixture including (1) antiblinking/photo-toxicity/photobleaching cocktail (see Section V); (2) the MAP of interest (if included), including any required nucleotide; (3) the following polymerization mix:

- tubulin (see notes for concentration and labeling ratio)
- 1 mM GTP
- 0.1 mg/ml BSA—providing additional blocking of nonspecific surface binding (competes with tubulin and MAPs for nonspecific binding sites).

C. Considerations Regarding the Reaction Solution

- Polymerization buffer

  Typically the buffer for polymerization is BRB80 (Section III). Care should be taken if some of the components of the reaction solution are not predissolved in BRB80 buffer; in this case it is possible to use a suitable amount of more

concentrated buffer when mixing the reaction solution to keep the final concentration of PIPES at 80 mM.

- Ionic strength

  MAPs differ in their requirements for the ionic strength of the reaction solution. The ionic strength can be increased by addition of KCl or decreased by using a lower concentration of PIPES. Note that changes in ionic strength also affect microtubule dynamics (Olmsted and Borisy, 1975); control experiments should always be carried out under identical buffer conditions.
- Tubulin concentration

  In the absence of MAPs that promote microtubule growth, no growth of extensions will occur below $\sim$7 μM tubulin. At high ($\geq$ 15 μM) tubulin concentrations spontaneous nucleation of microtubules can occur in the channel (Fygenson *et al.*, 1994).
- Ratio of fluorescent-labeled to unlabeled tubulin

  The soluble tubulin introduced into the reaction chamber should not be 100% labeled. Despite the thin evanescent field from TIRF, the background fluorescence will become intolerable at micromolar concentrations of fluorescent tubulin. Therefore, a mixture of labeled and unlabeled tubulin must be used. Our experience suggests that a labeling ratio of 3–5% (3–5 fluorescent labels per 100 tubulin dimers) gives good signal to noise for the dynamic microtubule extensions.
- Ratio of fluorescent-labeled-to-unlabeled MAP

  If single-molecule imaging of a MAP is desired, while investigating its effect on the microtubule dynamics, so-called "spiking" experiments can be performed (Varga *et al.*, 2009), where only a very small ratio of the MAP is labeled (see Sections II and IV). Typically the fluorescent-labeled MAP concentration should be kept below 1 nM, although the optimum concentration depends significantly on the particular characteristics of the MAP–microtubule interactions present.

Tip: *The key to obtaining reproducible growth rates in these experiments is the consistent handling of your tubulin. Each aliquot of tubulin must experience an identical procedure of thawing, incubation time on ice, lag time until use in the experiment. In addition, the concentration of tubulin should be precisely known and controlled for reproducibility.*

Tip: *When measuring microtubule growth rates at a constant concentration of tubulin, for example, generating growth versus concentration curves for MAPs such as XMAP215 (Brouhard et al., 2008), it is recommended to "equilibrate" the channel with the appropriate concentration of soluble tubulin before each sample. This step ensures that the appropriate tubulin concentration is present in the channel upon addition of the protein of interest.*

Tip: *In the absence of MAPs we typically get a growth rate of 1.5 μm/min with 10 μM tubulin at a temperature of 35°C. We find that time points taken at 5s intervals are ideal. Addition of MAPs can result in much higher growth rates (>10 μm/min). In this case, we use frame rates up to 10 Hz to ensure no events are missed during the time course.*

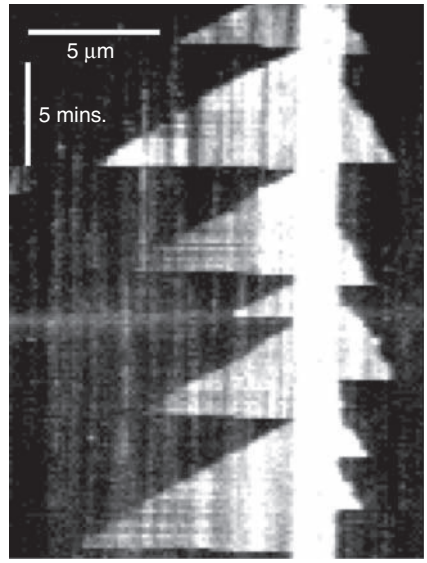


Fig. 6 Kymograph produced from a series of images, showing dynamic microtubule extensions growing from a GMPCPP stabilized seed. In the experiment 9.6 μM unlabeled and 0.4 μM Cy3-labeled tubulin was present in the channel, the objective was heated to 35°C, and TIRF images were recorded at 10-s intervals.

D. Analysis of Dynamic Microtubule Assays

The rate of extension of a growing microtubule tip can be determined through measurement of the microtubule length at different time points in a series of images recorded with TIRF illumination. However, care must be taken as thermal fluctuations will cause the ends of long microtubule extensions to move in and out of the TIRF evanescent field. This may affect the apparent measured extension size in an image. The intensity profile of the microtubule can be examined to determine if the tip has drifted out of the evanescent field. If a line scan along the microtubule shows an intensity profile that decreases exponentially, rather than decreasing abruptly, it is likely that the microtubule has bent upward and out of the field of view, making its true length impossible to measure. The dynamic properties can be determined using kymographs (see Fig. 6).

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