CHAPTER EIGHT

Reconstituting Dynamic Microtubule Polymerization Regulation by TOG Domain Proteins

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Abstract

Microtubules (MTs) polymerize from soluble αβ-tubulin and undergo rapid dynamic transitions to depolymerization at their ends. Microtubule-associated regulator proteins modulate polymerization dynamics *in vivo* by altering microtubule plus end conformations or influencing αβ-tubulin incorporation rates. Biochemical reconstitution of dynamic MT polymerization can be visualized with total internal reflection fluorescence (TIRF) microscopy using purified MT regulators. This approach has provided extensive details on the regulation of microtubule dynamics. Here, I describe a general approach to reconstitute MT dynamic polymerization with TOG domain microtubule regulators from the XMAP215/Dis1 and CLASP families using TIRF microscopy. TIRF imaging strategies require nucleation of microtubule polymerization from surface-attached, stabilized MTs. The approaches described here can be used to study the mechanism of a wide variety of microtubule regulatory proteins.

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1. INTRODUCTION

Microtubules (MTs) are polarized dynamic intracellular polymers that are required for producing and organizing forces during cell division and development. MTs serve as tracks for motor proteins and generate pulling and pushing forces by dynamic polymerization and depolymerization activities at their plus ends. MTs polymerize from αβ-tubulin dimers at MT plus ends, and GTP hydrolysis is activated in newly polymerized tubulin dimers near plus ends. Tubulin GTP hydrolysis at plus ends leads to stochastic switch-like transitions, where MT ends become unstable and depolymerize rapidly. These switch-like transitions, termed "dynamic instability," allow dynamic MTs to reorganize cells and produce physical pulling forces while coupled to kinetochores, chromosomes, or other MTs (Akhmanova & Steinmetz, 2008; Desai & Mitchison, 1997). Over the past three decades, diverse and conserved classes of MT-associated proteins (MAPs) emerged with unique functions in regulating MT organization, polymerization, and dynamic transitions (Akhmanova & Steinmetz, 2008; Al-Bassam & Chang, 2011). MT polymerization regulators that bind soluble αβ-tubulin dimer while bound at MT plus ends can influence MT polymerization and dynamic transitions (Al-Bassam & Chang, 2011). The End Binding (EB) protein family, such as EB1, bind polymerizing MT plus ends by recognizing the GTP-like state of newly polymerized tubulin dimers (Maurer, Bieling, Cope, Hoenger, & Surrey, 2011). In contrast, two unique classes of tumor overexpressed gene (TOG) domain proteins regulate MTs by recruiting soluble tubulin dimer to MT plus ends. XMAP215/Dis1 proteins, such as Alp14 and XMAP215, increase MT dynamic polymerization rates by recruiting soluble αβ-tubulin via their conserved TOG domains to polymerizing MT plus ends (Al-Bassam et al., 2012; Brouhard et al., 2008). The related CLASP family proteins decrease MT transitions to depolymerization (termed MT catastrophe) and activate polymerization reinitiating transitions of MT polymerization (termed MT rescue) by recruiting soluble tubulin via their TOGL to depolymerizing MT plus ends (Al-Bassam & Chang, 2011; Al-Bassam et al., 2010). The effects of these regulators on MT dynamics in cells have been extensively studied (Al-Bassam & Chang, 2011). However, it is also critical to observe and understand their mechanism using *in vitro* reconstitution of dynamic MT polymerization using purified recombinant MAPs in combination with soluble tubulin. Here, I describe the approach

and methods for purifying, reconstituting, and visualizing mechanisms of recombinant TOG domain-MT regulators during dynamic MT polymerization using total internal reflection fluorescence (TIRF) light microscopy.

1.1. The use of light microscopy methods for the *in vitro* reconstitution of dynamic MTs

Over the past two decades, the interactions of recombinant or purified MAPs with MTs polymerized from purified tubulin have been studied using light microscopy approaches. Although differential interference contrast (termed DIC) microscopy methods were initially used to directly visualize MTs and their dynamic polymerization (Walker et al., 1988), fluorescence-based light microscopy methods rapidly replaced DIC methods to study MTs *in vitro*, due to increased signal to noise and the ability to visualize the localization of fluorescent MAPs along MTs (Bieling, Telley, Hentrich, Piehler, & Surrey, 2010; Gell et al., 2010; Telley, Bieling, & Surrey, 2011). In the past decade, TIRF microscopy methods emerged as an extremely robust method to study MT-based motors and regulator MT dynamic polymerization mechanisms (Bieling et al., 2007; Brouhard et al., 2008). TIRF microscopy visualizes MT polymerization through their proximity to glass surfaces, and fluorescent MAPs, which bind along these MTs or at their ends. TIRF microscopy is particularly effective in visualizing dynamic MTs polymerization from purified tubulin to explore mechanisms of MT regulators in influencing dynamic MT transitions or organization (Bieling et al., 2010; Gell et al., 2010). Here, I describe a general approach to study dynamic MT polymerization regulators. I will use recent studies as examples for how to study TOG-domain-MT regulators from the XMAP215/Dis1 and CLASP families (Al-Bassam et al., 2010, 2012). The approach described here can be adapted to a variety of MT polymerization regulators.

1.2. Advances in reconstituting dynamic MT polymerization using TIRF microscopy

Several technical advances have facilitated reconstitution studies of MT dynamics by TIRF microscopy, including (A) improvements in manufactured light microscopes, objectives lenses, and single-wavelength solid-state lasers that have made multiwavelength TIRF microscopy accessible to many users; these developments are beyond the scope of this chapter and are described previously (Gell et al., 2010). (B) Chemical strategies to clean and neutralize

hydrophobic glass surfaces to prevent aggregation of soluble tubulin and MAPs during reconstitution experiments. I will describe a single method to neutralize glass surfaces, but there are other strategies to accomplish this, which can be found in the literature (Bieling et al., 2010; Gell et al., 2010) as well as other chapters in this book. (C) Chemical cross-linking methods that generate fluorescent, polymerization-competent tubulin dimers (Hyman et al., 1991). (D) Extensive advances in recombinant protein expression methods to produce homogenous, well-behaved full-length MT regulators with fluorescent tags (Hitchman, Locanto, Possee, & King, 2011; Machleidt, Robers, & Hanson, 2007). (E) Strategies for attaching short, stable MTs (termed MT seeds) on glass surfaces, which nucleate dynamic MT polymerization at MT plus ends from soluble tubulin (Bieling et al., 2010; Gell et al., 2010). Unlike previous methods that directly attached MTs to glass surfaces, new chemical or protein scaffolds can be used to attach MTs at a defined distance above glass surfaces. This is critical to ensure that MTs are free to undergo dynamic polymerization at their ends. The rigidity of newly formed, dynamic MTs maintains them in focus in the evanescent field near the glass surface to allow imaging by TIRF microscopy.

2. METHODS

2.1. Preparing fluorescently tagged recombinant MT regulator proteins

2.1.1 Rationale

Recombinant macromolecular overexpression strategies advanced dramatically over the past two decades (Hitchman et al., 2011). Full-length MT regulators or complexes, which are relatively large molecular weight proteins, can be produced using overexpression in yeast or insect cells and purified to homogeneity using biochemical purification strategies. MT regulators can be engineered to add fluorescent tags or mutated to inactivate functional domains to study function (Al-Bassam et al., 2010, 2012). A general overexpression and purification strategy is described below, including dual affinity steps as well as ion-exchange and size-exclusion chromatography techniques. Reconstitution studies with MT regulators using TIRF microscopy require well-behaved proteins purified by size-exclusion chromatography. I will describe a strategy for the purification of fission yeast Cls1 or Alp14 (Al-Bassam et al., 2010, 2012) using insect cells baculovirus expression, using his-Maltose-binding protein (MBP) fusion dual affinity strategy (Sun, Tropea, & Waugh, 2011).

2.1.2 Method

1. Baculoviruses are generated from engineered transfer vectors, which can be prepared using a variety of well-described methods. 6×his-MBP sequences are fused at the N-termini of full-length MT regulators such as Alp14 and Cls1. 9×10^5 Sf9 insect cells are transfected in 6-well plates using lipids or calcium phosphate strategies. Three rounds of virus amplification (P1-P3), each taking 72 h by infecting 9×10^5 Sf9 insect cells with the previous round. The final P3 virus is then utilized for protein expression in the following steps.
2. 1–2 L of 1×10^6 Sf9 or Hi5 insect cells are infected with P3 virus and diluted 50–100-fold dilution to initiate protein expression.
3. Cell pellets are collected 60–72 h post infection. Cells are lysed using lysis buffer (50 m*M* Hepes 300 m*M* KCl, pH 7.0 0.2% Triton X 100, 5 m*M* β-mercaptoethanol, 15 m*M* imidazole supplemented with protease inhibitors to prevent protein degradation) using a dounce homogenizer with 1–2 strokes every 20 s for 5–10 min.
4. Lysates are clarified using centrifugation at 60,000 × *g* for 30 min.
5. His-MBP-Alp14 is bound to Ni-NTA agarose (macherry-Nagel Corp.), washed, and eluted with lysis buffer with 250 m*M* imidazole.
6. A second affinity step is performed by binding protein from the nickel elution to Amylose resin (New England Biolabs), washed extensively in lysis buffer to remove insect cell contaminants, and eluted with lysis buffer with 25 m*M* maltose.
7. Purified his-MBP-Alp14 protein is then treated with TEV protease (Sun et al., 2011) to remove his-MBP tags for 24–48 h and then passed onto Ni-NTA agarose beads to remove tags or uncleaved protein. Purified cleaved protein is collected in the flow through.
8. Alp14 is concentrated using Amicon concentrators and injected onto a Superdex 200 or Superose-6 (GE Healthcare) using a FPLC system equilibrated with lysis buffers.
9. The eluted protein is then analyzed using SDS-PAGE to determine the degree of purity and then utilized for TIRF experiments.

2.2. Fluorescent labeling of high-quality soluble tubulin for TIRF microscopy

2.2.1 Rationale

Reconstitution of dynamic MT polymerization requires high-quality, polymerization-competent tubulin. Soluble tubulin is extracted from bulk tissues through GTP-dependent MT polymerization and cold-driven

depolymerization cycles, as described more than three decades ago (Kirschner, Williams, Weingarten, & Gerhart, 1974). A recently modified method produces higher quality polymerization-competent soluble tubulin (Castoldi & Popov, 2003). Fluorescent soluble tubulins, produced by amine-reactive labeling reactions and purified through MT polymerization and depolymerization cycles, are used to monitor soluble tubulin and dynamic MTs by TIRF microscopy. In this method, fluorescent dyes are covalently attached to amine residues in polymerized tubulin which can be either purified in the lab or purchased commercially (Cytoskeleton, Inc.), and active tubulin is selected by two additional GTP-dependent polymerization cycles. High-quality soluble tubulin with moderate (10–50%) labeling is extremely critical for studies using dynamic MTs in TIRF with MT regulators, described here. Below, I present a method that was initially described by the Hyman et al. (Hyman et al., 1991). I recommend performing tubulin fluorescent labeling in your laboratory for successful and reproducible TIRF experiments. I also recommend producing multiple fluorescent dye color-labeled soluble tubulin pools and to determine the experimental dye/tubulin-labeling ratio for each pool. The chosen dyes must be compatible with excitation wavelength for the lasers used and the particular emission and dichroic filter properties of your TIRF microscope (Gell et al., 2010). The fluorescent dyes must have very little or no overlap of their excitation or emission spectra to prevent channel cross-signal contamination in these experiments. An extensive discussion of dye choices is previously described (Gell et al., 2010). Common dyes used for tubulin labeling include Cy5, Cy3, and Texas-Red-amine-reactive dyes. A variety of enhanced chemically related dyes with higher fluorescence intensity are termed Alexa-Fluors, which are sold and marketed by Invitrogen Corp.

2.2.2 Method

1. Dilute 10–20 mg of purified tubulin in BRB-80 (80 m*M* pipes, 1 m*M* EGTA pH 6.8) with 3.5 m*M* GTP to 2 mg/mL and transferred to 37 °C and 50% glycerol is added, and this mixture is incubated at 37 °C. This step promotes MT polymerization.
2. Layer the polymerized mix on a cushion, or a dense solution of 50 m*M* Hepes (pH 8.6), 40% glycerol, 1 m*M* $MgCl_2$. Centrifuge at 80 K × *g* for 45 min at 35 °C. The goal of this step is to transfer the polymerized MTs to a high pH buffer in preparation for labeling reaction.
3. Aspirate the supernatant slowly, with 37 °C warm 50 m*M* Hepes (pH 8.6), 5 m*M* $MgCl_2$, 40% glycerol. The polymerized MTs must

remain warm during resuspension process and should be thoroughly resuspended to ensure full labeling.

4. Add 10- to 20-fold molar excess of Succimidyl-ester Alexa-Fluor-dye (such as Alexa-Fluor-488, Invitrogen) dissolved in DMSO to the polymerized MTs. Each Succimidyl-ester dye is added in two portions over 60 min during the labeling reaction at 37 °C. The labeling reaction is then quenched with 1 m*M* amine to the labeling reaction and mixed well.
5. Layer the quenched labeling reaction onto a cushion, or dense buffer solution, of 50 m*M* pipes pH 6.8, 1 m*M* $MgCl_2$, 40% glycerol and then centrifuged at 80K × g for 20 min at 37 °C.
6. Remove the supernatant and the cushion slowly and wash the MT pellet with 37 °C warmed disassembly buffer (50 m*M* K-glutamate $MgCl_2$, 1 m*M* EGTA, pH 7.0). Resuspend the MT pellet using a dounce homogenizer at 0 °C and let sit on ice for 30 min. Glutamate improves MT depolymerization at 0 °C.
7. Clarify the depolymerized tubulin by centrifugation at 100 K × *g* at 0 °C for 20 min.
8. Repolymerize depolymerized tubulin (supernatant from step 8) by diluting in 80 m*M* pipes 4 m*M* $MgCl_2$ and 1 m*M* GTP with 33% glycerol at 37 °C for 30 min.
9. Layer the MT polymerization reaction on a 1 mL of 50 m*M* pipes pH 6.8, 1 m*M* $MgCl_2$, 40% glycerol in a TLA100.3 tube and pellet MTs at 80 K in a TLA100.3 rotor for 20 min at 37 °C.
10. Aspirate the supernatant slowly, with 37 °C warmed disassembly buffer. The MT pellets are depolymerized by careful resuspension at 0 °C using a 2-mL dounce homogenizer.
11. Clarify the depolymerized tubulin at 80 K × g for 10 min at 0 °C. Recover the supernatant, which consists of depolymerized tubulin dimer. Estimate the tubulin dimer concentration and the degree of labeling using protein and dye absorbance at 280 nm and the unique dye emission at that wavelength.
12. The tubulin is aliquoted in 1–2 μL portions, frozen in liquid nitrogen, and stored at −80 °C. Freshly thawed tubulin aliquots are used in all the experiments described below.

2.3. Preparing MT polymerization dynamics flow chambers for TIRF microscopy

2.3.1 Rationale

TIRF microscopy has become a powerful tool to image dynamic MT polymerization. For these studies, short and stable MTs (termed MT seeds) are used to

nucleate polymerization, while coupled in close proximity to the glass coverslip. Thus, high-quality glass surfaces and precise functional coupling of MT to glass are both critical for the success of this approach. Glass surfaces must be clean, neutral, with little height variation to prevent soluble αβ-tubulin or MT regulator MAPs from aggregating in flow chambers. The MT seeds are polymerized using nonhydrolysable GTP analog, GMPCPP. The methods described below are modifications of earlier studies (Bieling et al., 2010; Gell et al., 2010). MT seeds are coupled to functionalized glass, which retains them a short distance away from the glass surface, which is important for MT polymerization reconstitution studies. Although many MT attachment strategies are described, I prefer using antibodies against unique chemical moieties incorporated into the MT seeds. Here, I will describe the methods of preparing glass surfaces and treatments to attach microtubules along these surfaces. I will also describe how flow chambers are assembled and treated in preparation for dynamic MT polymerization studies.

2.3.2 Cleaning and neutralizing glass surfaces

1. Load no 1.5 coverslips onto porcelain racks and wash them in a sonic bath for 20–25 min in the following solutions (in order): 10% commercial dishwasher detergent, 1 *M* KOH, acetone, and 100% ethanol. After each sonic wash step, rinse coverslips four times with distilled water.
2. Dry coverslips along with the porcelain rack in a clean 110–120 °C oven for about 1 h. Also dry a glass vessel to be used in step 4.
3. Plasma etch the dry coverslips in an 100% oxygen environment at 100 W/h for 10 min using an oxygen gas enabled plasma etching device.
4. Silanize coverslips in 0.1–0.2% dimethyldichlolorsilane diluted in ultra-dry tricholorethylene (Sigma-Aldrich, with less than 1 ppm H_2O) and react for 2 h.
5. Rinse twice in 100% methanol for 3 min each.
6. Dip and slowly remove coverslip rack in deionized water to observe wicking effect upon slow removal. When properly silanized, the glass coverslips must be hydrophobic and retain no water drops as they are removed out of the deionized water.
7. Glass must be stored between sheets of lens paper under vacuum at all times. Vacuum storage decreases the dust accumulation on the coverslips.

2.3.3 Polymerizing and isolating stabilized MT seeds

1. Mix freshly thawed 4 μ*M* tubulin dimer mixture containing 80% unlabeled tubulin, 10% fluorescently labeled, and 10% biotin-labeled,

freshly thawed tubulin dimer (prepared using the labeling protocol described above) in 10–20 μL of BRB-80 buffer supplemented with 1 m*M* GMPCPP (Jena Biosciences). The mixture is then warmed up to 37 °C for 90–120 min to polymerize MTs with an average length of 3–6 μm (suitable as seeds from which to grow extensions).

2. The mixture is then diluted with 100 μL of warm BRB-80 and then pipetted into 200-μL tube and centrifuged in a TLA100.3 rotor at 18 K rpm using a Beckmann table top-ultracentrifuge, or equivalent for 20 min.
3. The supernatant is removed and mixture is resuspended in 80 μL of BRB-80 buffer.

2.3.4 Assembling flow chambers and preparation for dynamic MT polymerization

1. Flow chambers are assembled from three layers (Fig. 8.1A): (A) top layer is a 2-cm thick, 20 × 20 cm Quartz Glass Adaptor with four holes positioned in the corners. (B) 20 × 20 mm double adhesive sheets (Grace Biolabs, Inc.) with 20 × 4 mm two rectangular channels cut into each side of the square size. (C) 22 × 22 mm salinized-treated glass, prepared as described above (Fig. 8.1A). The channels are placed in line with two holes in the quartz, to generate two rectangular sealed channels along sides of square. The adhesive should be pressed to ensure no air pockets, aside from the entry (Fig. 8.1B).
2. Inject 200 μL of BRB-80 through each flow channel through entry holes, while keeping a weak house-vacuum stream near the exit hole of that flow channel. This effectively cleans and washes the flow cell from any small particles.
3. Using the above approach pipetting approach, inject the following solutions through the flow chamber for the following periods of time. The protocol described below will attach antibodies through hydrophobic surface interaction to the glass, after which the glass is neutralized with a detergent to form a polar layer along the glass surface to prevent protein aggregation and inject the following solutions into the flow channel in this order, which is summarized in Fig. 8.1C:
   (A) 50 μL of BRB-80 and wait for 1 min.
   (B) 50 μL of 50-fold diluted antibiotin mouse monoclonal antibody (Invitrogen) in BRB-80 and wait for 4 min.
   (C) 50 μL of BRB-80 for 1 min.

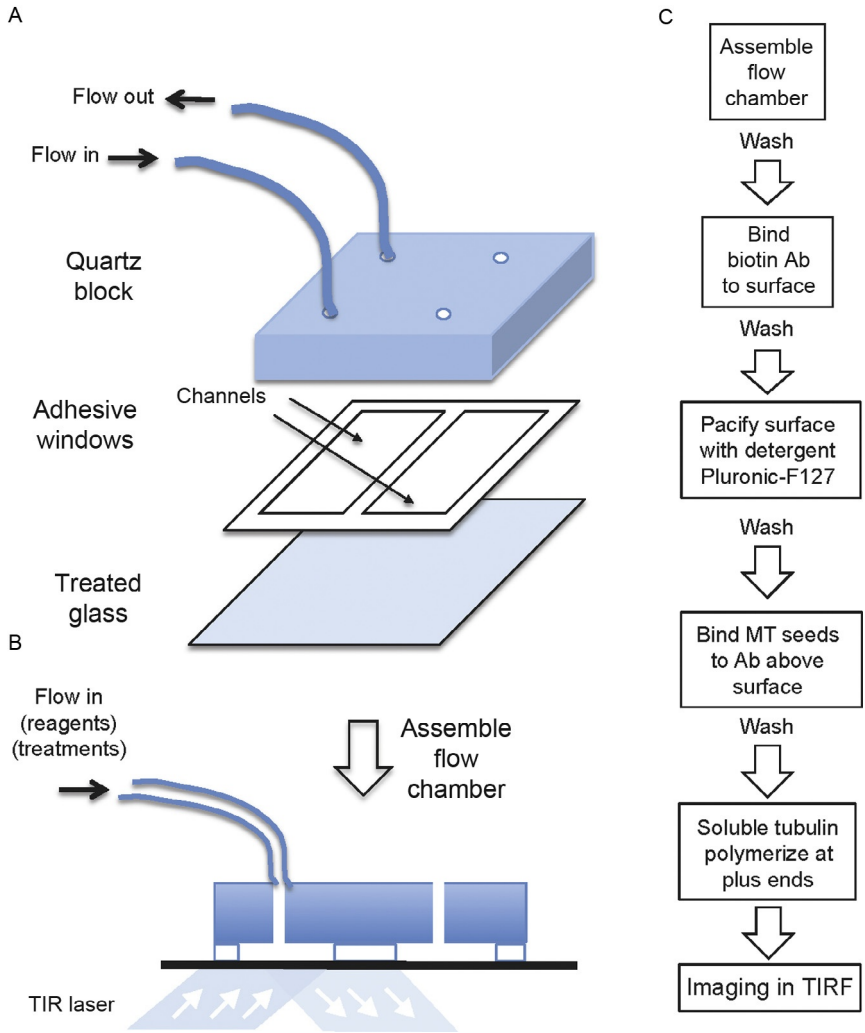

Figure 8.1 Reconstituting MT regulator dynamic MT polymerization using TIRF microscopy. (A) Top panel, composition of Flow chamber components: 20 × 20 mm Quartz block, 20 × 20 mm adhesive window with dual cut 4 × 22 mm channels, and 22 × 22 mm clean, silianized glass, treated as described in Section 2.3.2. Lower panel shows the assembled flow chamber showing the imaging surface. (B) Flow diagram describing a summary of the treatments to reconstitute dynamic MT polymerization along glass-surface; this diagram summarizes Sections 2.3.4, 2.4, and 2.5 in this chapter.

(D) 50 μL of 1% pluronic F127 in BRB-80 (Sigma-Aldrich), prefiltered through 0.2 μm and wait for 4 min to neutralize the glass surface.

(E) 200 μL of BRB-80 through the flow channel to remove residual Pluronic F127.

(F) 60 μL of 40-fold diluted GMPCPP MT seeds from the original stock described above and wait for 10 min.

(G) 50 μL of BRB-80 to remove excess MT seeds.

(H) 100 μL of imaging buffer (contents of imaging buffer are described below).

2.4. Reconstituting dynamic MT polymerization with MT regulators using TIRF microscopy

2.4.1 Rationale

Surface-attached fluorescent and biotin-containing MT seeds are bound by antibiotin antibodies above the glass surface, within the evanescent field for TIRF microscopy (Fig. 8.2A). Dynamic MT polymerization is nucleated by MT seeds from fluorescently labeled tubulin with a dye color distinct from those in the MT seeds. The newly formed dynamic MTs are observed in a different fluorescent channel from MT seeds, as shown in Fig. 8.2B (Al-Bassam et al., 2010, 2012). I first optimize biochemical conditions, such as MT regulators concentration and solubility, in an appropriate imaging buffer at 37 °C. I recommend that dynamic MTs polymerization assays are performed in a variety of conditions, including (A) varying soluble αβ-tubulin from 6–12 μ*M* in chemical conditions compatible with MT regulator. (B) Increasing MT regulator concentrations and observing MT dynamic parameters at each soluble tubulin concentration (such as 6 or 8 μ*M*). I generally recommend starting at 6 μ*M* αβ-tubulin where MT dynamic polymerization rate is slow and MT catastrophe transitions are frequent, leading the average MT length to be very short. The approach described here was used in prior studies that found that fission yeast Alp14 is a MT polymerase accelerates MT polymerization by threefold, while Cls1 is a MT rescue factor that decreases MT catastrophe frequency and increases MT rescue frequency (Al-Bassam et al., 2010, 2012).

2.4.2 Dynamic MT reconstitution and TIRF microscopy imaging

1. Prepare flow chambers as described above in Section 2.3.4. This method starts at the point after which the MT seeds are added to the coverslip.

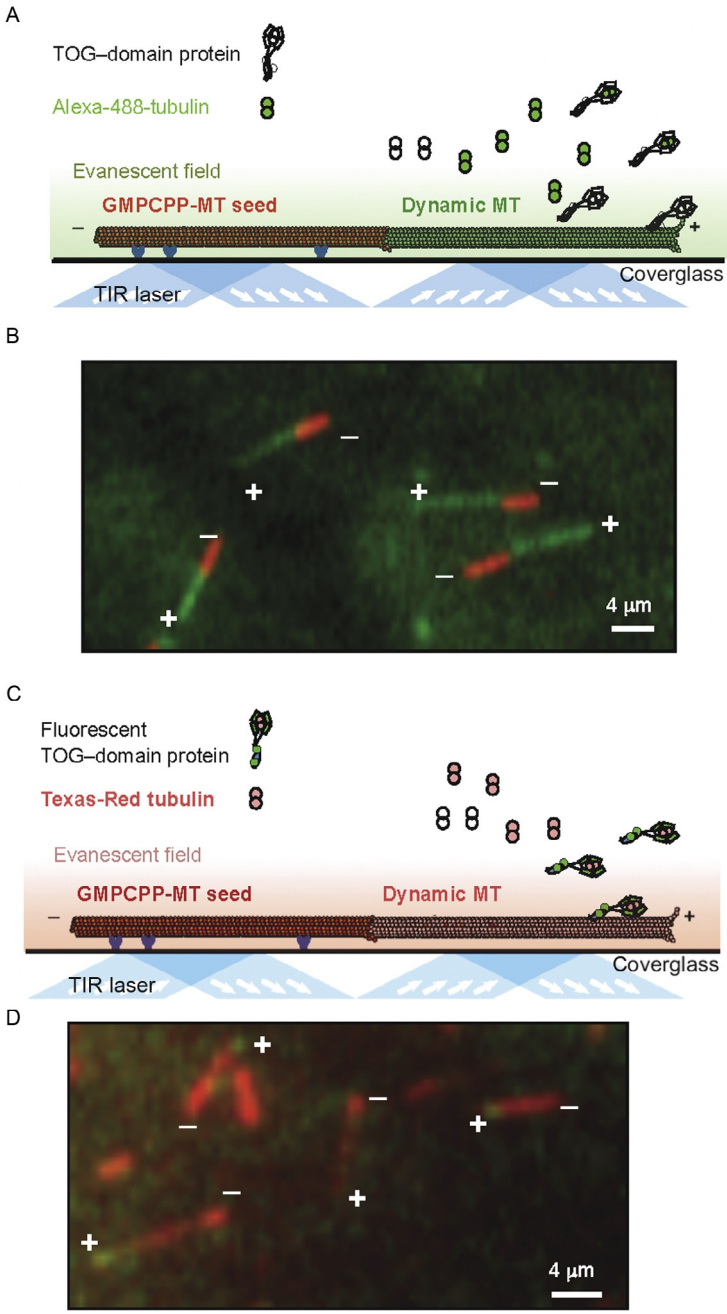

Figure 8.2 Schematic representation of dynamic MT polymerization reconstitution studies. (A) Scheme described in Section 2.4, for dual fluorescent MTs to measure MT

2. Prepare 40 μL of imaging buffer solution containing 50 m*M* buffer such at BRB-80 or others and
   (A) 6–10 μ*M* soluble tubulin with 10% fluorescently labeled of a different dye than the MT seeds attached to the glass surface; for example, Alexa-488-labeled soluble tubulin is prepared in the mix, if MT seeds are labeled with Texas Red (Fig. 8.2A).
   (B) 1–2 m*M* GTP diluted from a 100-m*M* GTP Stock.
   (C) Additives like salt (200 m*M* KCl for Alp14 or 70 m*M* KCl for Cls1)
   (D) Adding MT regulator (such as 0–200 n*M* Alp14 or Cls1 proteins), which should be added last.
3. Filter the mixture through a 0.2-μm spin filter (Amicon) to remove small particles.
4. Inject the imaging mixture in flow channel as described previously.
5. Warm flow chamber to 35–37 °C at the imaging TIRF objective lens.
6. Focus microscope imaging objective TIRF lens to identify the MT seeds, while waiting 5–10 min for tubulin/dynamic MTs to reach 35–37 °C. Apply autofocusing strategy, if available on your microscope. Autofocusing is extremely useful for keeping the sample in focus in the TIRF field.
7. Begin collecting MT polymerization image stacks data for each of the two channels (dynamic MT and static MT seeds). Typically, I acquire every 2–4 s for 10–30 min.

2.4.3 Analysis of dynamic MT image data to determine MT polymerization parameters

The stacks of images (termed movies) collected for each channel can then be corrected and analyzed in parallel using the ImageJ (Rasband, 1997) collection, as previously described (Al-Bassam et al., 2010).

polymerization dynamics. Dynamic MTs, shown in green, grow from Alexa-Fluor-488-labeled tubulin, while stabilized GMPCPP MT-seeds, shown in red, are polymerized from Texas-Red-labeled tubulin. MT regulators are nonfluorescent. (B) Example data of reconstituted, dynamic MTs, in the scheme described in Section 2.4. (C) Scheme for dynamic MT polymerization reconstitution using fluorescent MT regulator and fluorescent MTs, as described in Section 2.5. Dynamic MTs are shown in faint red grown 10% Texas-Red tubulin, while stabilized MT seeds are shown in red, polymerized from a higher ratio of Texas-Red-labeled tubulin. MT regulators, shown in green, are labeled with GFP-tags. (D) Example data of reconstituted MT dynamics with fluorescent MT regulators at. All images in this figure are generated with permission from publisher of these references (Al-Bassam et al., 2010, 2012). *(B) Reproduced with permission from Al-Bassam et al. (2010). (D) Reproduced with permission from publisher of reference Al-Bassam et al. (2010).*

1. Image stacks are adjusted for photobleaching by applying an average total fluorescence correction.
2. Image linear stage drift is corrected in image stacks by calculating translation parameters in the static MT seed channel and applying these onto each image in the stack.
3. Identify dynamic MT polymerization events and produce kymograph images using imaging tracking algorithms, or manual kymograph functions using the multikymograph plug-in as previously described (Al-Bassam et al., 2010, 2012) to determine the rate and time for MT polymerization and MT depolymerization.
4. Determine average parameters from a collection of kymographs in each data set (Fig. 8.3A), using histogram analysis to determine average numerical values for the dynamic MT polymerization parameters: assembly rate

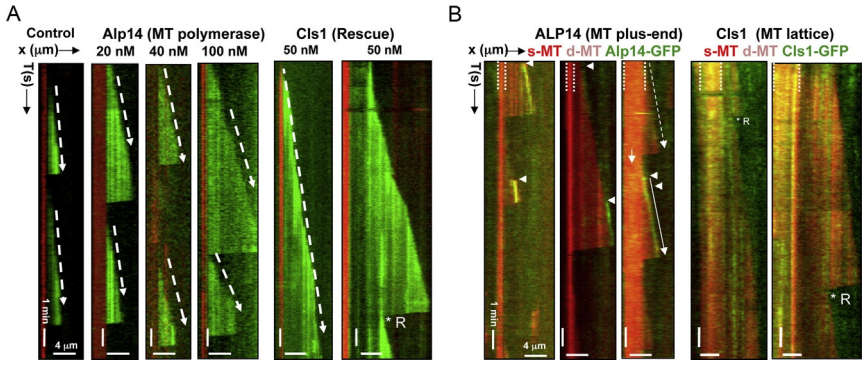

Figure 8.3 Example MT dynamic polymerization kymographs based on studies described. (A) Kymographs of dynamic MTs produced using ImageJ, using methods described in Section 2.4. Dynamic MTs are shown in green, while stabilized MT seeds are shown in red. Left panel, MTs grow slowly at 6 μ*M* tubulin dimer and depolymerize in frequent catastrophe events. Middle panels, increasing ALp14 concentration incrementally increases MT polymerization, without influencing MT catastrophe frequency (Al-Bassam et al., 2012). Right panels, Cls1 decreases in the frequency of MT catastrophe, and occurrence of MT rescues, labeled R* (Al-Bassam et al., 2010). (B) Kymographs of dynamic MTs produced using ImageJ, using methods described in Section 2.5. Dynamic MTs are shown in light color red, while stabilized MT seeds are shown in intense red. Left panels, Alp14-GFP, shown in green, binds at MT plus ends to increase MT polymerization rate (Al-Bassam et al., 2012). Right panels, Cls1-GFP binds along MT lattices without tracking MT plus ends, where it correlates with absence of MT catastrophes, and its presence coincides with MT rescues, labeled R* (Al-Bassam et al., 2010).All images in this figure are generated with permission from publisher of these references (Al-Bassam et al., 2010, 2012).

(μm/min), disassembly rate (μm/min), catastrophe frequency (event/min assembly time), or rescue frequency (event/min disassembly time).

2.5. Tracking fluorescent MT regulators along dynamic MTs using TIRF microscopy

2.5.1 Rationale

Visualizing dynamic localization of MT regulators along MTs or at their ends during dynamic MT polymerization requires active and fluorescently tagged MT regulators. Many types of fluorescent tags can be genetically or chemically fused to MT regulators for these studies such as fusions of green fluorescent protein (GFP) variants (Ilagan et al., 2010), fusions with domains for specific covalent fluorescent dye attachment, such as CLIP, SNAP, or Halo tags (Gautier et al., 2008), and or short sequences for covalent attachment to biarsenate fluorescent dyes like FlAsH and ReAsH (Machleidt et al., 2007). It is critical to determine that the fluorescently tagged MT regulator behaves similar to native (nonfluorescent) MT regulator using the method described in Section 2.4. Mixtures of fluorescently tagged to native MT regulator may be utilized to decrease average number of labeled molecules without decreasing MT regulatory activity. The fluorescent MT regulator activity must be analyzed the approach described in Section 2.4. These experiments do not necessarily require three channels and can be studied using two channels only, by using two different ratios of a single tubulin color polymerized into the MT seeds (higher ratio) compared to those added in solution and polymerizing into dynamic MTs (low ratio). This leads dynamic MTs to be fainter than the MT seeds in these experiments (Fig. 8.2B). These studies can be performed as described in Section 2.3.4.

2.5.2 Method

1. Prepare flow chambers as described in Section 2.3.4. This method starts at the point after which the MT seeds are added. MT seeds are polymerized with a higher ratio of dye (40%) compared to previously described protocol. This helps distinguish them from the dynamic MTs polymerized from 10% dye-labeled tubulin in the same color.
2. Prepare 40 μL of imaging buffer solution containing
   (A) 6–10 μ*M* freshly thawed soluble tubulin with 10% fluorescently labeled tubulin.
   (B) 1–2 m*M* GTP diluted from a 100-m*M* GTP Stock.
   (C) Additives like salt (200 m*M* for Alp14).

(D) 1–200 n*M* fluorescent MT regulator. The concentration depends on activity range. Single-molecule experiments with MT regulators require low concentration of fluorescent molecules 1–10 n*M*.

(E) Antioxidants and/or oxygen scavengers such as Trolox or the glucose oxidase-catalase system, as previously described (Gell et al., 2010), which maintain fluorescence intensity and prevent photodamage through long exposure periods.

3. Inject the imaging mixture containing items in A–D into flow channel as described.
4. Warm the flow chamber to 35–37 °C at the imaging TIRF objective lens.
5. Focus microscope imaging objective TIRF lens to identify the MT seeds, while waiting 5–10 min for tubulin/dynamic MTs to reach 35–37 °C.
6. Begin collecting MT polymerization image data stacks for each of the two channels (dynamic MT and static MT seeds) every 2–4 s for 10–30 min.

The data in these experiments are processed in the same manner as described above in Section 2.4. The residence time of MT regulators can be determined using particle tracking approaches as previously described. Determining the localization and residence time for a fluorescent MT regulator, such as Alp14 and Cls1, along MT plus ends or MT lattices, respectively, is critical to understand their mechanism in regulating MT polymerization rates or influencing rates of MT dynamic transitions such as activating MT rescues (Fig. 8.3B).

3. CONCLUSION

TIRF microscopy has emerged as a powerful approach to study the mechanisms of MT polymerization regulators or MT organizing proteins determine their unique effects on MT dynamic polymerization and organization. The approach described here can be used to study a variety of MT regulators or complexes of MT regulators with dynamic MTs. The reconstitution of complex MT dynamic regulation or organization activities with multiple MAPs and motors is critical to understand the mechanisms of MT regulation. In the future, I expect MT dynamic polymerization reconstitution and imaging with TIRF microscopy to become the standard approach in studying the biochemistry and mechanisms of MT motor, regulators, and

organizer proteins in regulating complex MT assemblies observed in cellular phenomena such as stages of cell division.

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