

2010

Chapter 27 – Studying Plus-End Tracking at Single Molecule Resolution Using TIRF Microscopy

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Dixit, Ram and Ross, Jennifer L., "Chapter 27 – Studying Plus-End Tracking at Single Molecule Resolution Using TIRF Microscopy" (2010). *Biology Faculty Publications & Presentations*. Paper 71.
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Studying plus-end tracking at single molecule resolution using TIRF microscopy

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Abstract

The highly dynamic microtubule plus-ends are key sites of regulation that impact the organization and function of the microtubule cytoskeleton. Much of this regulation is performed by the microtubule plus-end tracking (+TIPs) family of proteins. +TIPs are a structurally diverse group of proteins that bind to and track with growing microtubule plus-ends in cells. +TIPs regulate microtubule dynamics as well as mediate interactions between microtubule tips and other cellular structures. Most +TIPs can directly bind to microtubules *in vitro*; however the mechanisms for their plus-end specificity are not fully understood. Cellular studies of +TIP activity are complicated by the fact that members of the +TIP family of proteins interact with each other to form higher-order protein assemblies. Development of an *in vitro* system, using minimal components, to study +TIP activity is therefore critical to unequivocally understand the behavior of individual +TIP proteins. Coupled with single molecule imaging, this system provides a powerful tool to study the molecular properties that are important for +TIP function. In this chapter, we describe a detailed protocol for *in vitro* reconstitution of +TIP activity at single molecule resolution using total internal reflection fluorescence (TIRF) microscopy.

I. Introduction

Microtubules are built up of α - β tubulin dimers that polymerize in a head-to-tail fashion to give rise to a tubular structure with intrinsic structural polarity. The structural polarity of microtubules contributes to a kinetic polarity: one end of the microtubule polymerizes more rapidly and is called the plus-end, and the other end grows more slowly and is called the minus-end. In animal cells, the minus-ends of microtubules are typically stably capped and anchored at the centrosome (Dammermann et al., 2003). In contrast, the plus-ends of microtubules are free

and dynamically probe the cellular space, switching between periods of growth and shortening, until they encounter cellular components with which to interact (Kirschner and Mitchison, 1986; Mitchison and Kirschner, 1984). As a result, the plus-ends play a particularly important role in regulating the dynamics and architecture of the microtubule cytoskeleton in the cell.

Microtubule plus-end tracking proteins (+TIPs) are a specialized class of microtubule-associated proteins (MAPs) that are distinguished by their ability to specifically localize to and track with growing microtubule plus-ends. The mammalian cytoplasmic linker protein-170 (CLIP170) was the first bonafide +TIP identified (Perez et al., 1999; Rickard and Kreis, 1990). Subsequently, other +TIPs have been identified, many of which are evolutionarily conserved from yeast to man. One of these conserved members is end-binding 1 (EB1) (Tirnauer and Bierer, 2000). +TIPs function in a wide array of cellular processes by controlling microtubule dynamics, coupling microtubules to other cellular structures and delivery of signaling molecules (Akhmanova and Steinmetz, 2008).

From a mechanistic perspective, it has been challenging to come up with a clear model for how +TIPs specifically target growing microtubule ends. In part, this is because the +TIP family is made up of structurally unrelated proteins (Akhmanova and Steinmetz, 2008; Carvalho et al., 2003) that do not share significant sequence homology or a conserved domain that can be studied to reveal a shared mechanism. In addition, many +TIPs directly interact with each other and are thought to function as part of a dynamic +TIP complex (Akhmanova and Hoogenraad, 2005). In particular, EB1 interacts directly with many other +TIPs and is thought to function as the master regulator of +TIP activity (Lansbergen and Akhmanova, 2006; Vaughan, 2005). This network of protein interactions complicates the study of +TIP activity of a particular protein in cells. Two fundamentally different models have been proposed for the mechanism of plus-end

binding specificity. The co-polymerization model suggests that +TIPs bind to free tubulin subunits, become incorporated at microtubule ends during polymerization and subsequently unbind from the microtubule lattice. Alternatively, the end-recognition model proposes that +TIPs preferentially bind to microtubule plus-ends due to a higher affinity for the distinctive structure and/or biochemical state of the growing plus-end.

Recently, we and others have developed total internal reflection fluorescence (TIRF) microscopy based *in vitro* reconstitution assays to study the mechanisms of +TIP activity at single molecule resolution (Bieling et al., 2008; Bieling et al., 2007; Brouhard et al., 2008; Dixit et al., 2009; Helenius et al., 2006; Honnappa et al., 2009; Komarova et al., 2009). In these assays, microtubule polymerization is initiated with preformed microtubule seeds immobilized on a glass surface and microtubule assembly is monitored by the incorporation of rhodamine-labeled tubulin subunits in the presence of green fluorescent protein (GFP) or Alexa 488-tagged +TIPs. In this chapter, we describe a detailed method to reconstitute +TIP activity of mammalian EB1 and CLIP170 proteins at single molecule resolution. Because this chapter focuses on techniques, we do not discuss the structural and functional properties of these +TIPs, for which the reader is referred to several excellent review articles (Akhmanova and Steinmetz, 2008; Carvalho et al., 2003; Lansbergen and Akhmanova, 2006).

II. Rationale

It is difficult to study the autonomous properties of individual +TIPs in cells due to the myriad interactions between different +TIPs in the cellular environment. Indeed, it is theoretically possible that +TIP activity emerges only in the context of multiple +TIPs interacting with each other and that purified +TIPs lack this property. Therefore, a functional *in*

in vitro reconstitution system is a powerful method to study the minimal components required for +TIP activity. Another benefit of an *in vitro* approach is that it can be readily combined with TIRF microscopy to study the molecular mechanisms of +TIP activity at single molecule resolution. Using this approach, we and others have revealed that some +TIPs, like EB1, are inherently able to plus-end track, whereas other +TIPs, like CLIP170, plus-end track in an EB1-dependent manner (Bieling et al., 2008; Dixit et al., 2009). In addition, single +TIP molecules show rapid on-off kinetics at growing microtubule plus-ends (Bieling et al., 2008; Bieling et al., 2007; Dixit et al., 2009) and loss of plus-end specificity when microtubules are polymerized in the presence of GMPCPP (Dixit et al., 2009). Together, the data argue against the copolymerization model for +TIP activity and suggest that +TIPs bind preferentially to a distinct conformation at growing microtubule plus-ends. Thus, single molecule *in vitro* reconstitution offers fundamental new insight into the molecular mechanisms of +TIP activity and introduces a new and powerful means to study the function of the +TIP complex of proteins.

The basic *in vitro* reconstitution assay for +TIPs consists of three main components: (1) flow chamber; (2) purified +TIPs; and (3) dynamic microtubules. We describe each of the components below followed by a detailed protocol for the reconstitution assay. Technical aspects of TIRF microscopy instrumentation and use are discussed in chapter 27 of this volume and will not be covered here.

III. Methods

A. Flow chamber

The *in vitro* reconstitution assay is conducted in a flow chamber that is assembled by attaching a glass cover slip to a glass slide using double-sided sticky tape (Fig. 1). The flow

chamber provides a path through which different reagents can be sequentially added to prepare the sample for TIRF imaging. It is important to clean the glass surfaces thoroughly to ensure even coating of the surfaces with solutions and to reduce background during imaging. The protocol described below is adapted from Helenius et al. (2006).

1. Preparation of the glass surface

Glass cover slips and slides are cleaned in coplin jars. The jars are convenient for coating batches of slides and cover slips and also help minimize direct handling of the slides and cover slips between steps. Dedicated jars are used for different solutions to avoid mixing of solutions. It is advisable to avoid getting air bubbles stuck on the glass surfaces during this procedure as this prevents even cleaning of the glass. Slow exchange of solutions usually prevents air bubbles. However, if air bubbles are formed, they can be easily removed by brief sonication or shaking of the slides. Take care to use number 1.5 cover slips (average thickness of 0.17 mm) since most microscope objectives are optimized for this thickness.

Procedure for cleaning of glass slides and cover slips:

1. Add 100% acetone to the coplin jars to completely immerse the glass slides and cover slips. Treat for 1 hr to remove any oils and organic residues from the glass surface.
2. Transfer the slides and cover slips to fresh coplin jars containing 100% ethanol for 10 min to rinse out the acetone.
3. Rinse off the ethanol by two washes with milliQ-water for 5 min each.
4. Soak the slides and cover slips in 0.1 M potassium hydroxide for 15 min. Prepare this solution just before use.
5. Rinse off the alkali by three washes with milliQ-water for 5 min each.

6. Air-dry the slides and cover slips. Cleaned slides can be stored in a slide holder for a few weeks but are routinely used in a few days after preparation.

Procedure for silanization of the cleaned cover slips:

The cleaned, air-dried cover slips are treated with dimethyldichlorosilane to render the surface hydrophobic. This treatment is necessary for subsequent use of the Pluronic polymer to block the exposed glass surface (see below).

1. Submerge dry cover slips in a 2% solution of dimethyldichlorosilane for 5 min. We use PlusOne Repel-Silane ES (GE Healthcare) which is supplied as a 2% solution of dimethyldichlorosilane in octamethylcyclooctasilane. Do this treatment in the safety hood.
2. Rinse off the excess silane with two washes of 100% ethanol for 5 min each. In our experience, best coating is obtained by removing the cover slips as slowly as possible from the silane-containing jar.
3. Wash the cover slips thrice with milliQ-water for 5 min each. After the last wash, slowly remove each cover slip with a flat, unserrated forceps (e.g., Millipore filter forceps). If the silanization has worked well, the cover slips will emerge with little or no water droplets adhered to the surface. At this point, handle the cover slips only from the edges. Silanized cover slips are stored in a dedicated cover slip box and can be used for at least one month without significant loss of performance.

2. Preparation of Pluronic as a blocking agent

Traditionally, researchers have used bovine serum albumin or casein as blocking agents in an effort to minimize unwanted signal, due to nonspecific adsorption of fluorescent proteins to

glass, during fluorescence imaging. An alternative to this approach is to use triblock copolymers called Pluronics, which result in superior surface blocking and lower nonspecific protein binding compared to bovine serum albumin or casein (Fig. 2). Pluronics are amphiphilic molecules whose general structure is $(\text{PEO})_x\text{-(PPO)}_y\text{-(PEO)}_x$, where polypropylene oxide (PPO) forms a hydrophobic core flanked by hydrophilic polyethylene oxide (PEO) chains. The mechanism by which Pluronic prevents protein adsorption is not fully understood, but it is generally thought that the PPO core adheres to a hydrophobic substrate and that the exposed hydrophilic PEO chains, by virtue of their flexibility and high steric exclusion volume in aqueous solution, resist protein adsorption (Schroen et al., 1995). Both Pluronic F-108 ($(\text{PEO})_{129}\text{-(PPO)}_{56}\text{-(PEO)}_{129}$) and Pluronic F-127 ($(\text{PEO})_{99}\text{-(PPO)}_{65}\text{-(PEO)}_{99}$) show equivalent efficacy in our hands and we routinely use Pluronic-F127 obtained from Sigma. A 5% stock solution of Pluronic F-127 is prepared in BRB80 buffer (80 mM PIPES, 1 mM MgCl_2 , 1 mM EGTA, pH 6.8). It is best to prepare this stock solution overnight because it takes several hours to dissolve Pluronic F-127 completely. The stock solution is then centrifuged at 10,000g for 10 min in a microfuge to remove any residual clumps that otherwise show up as unsightly dark patches during TIRF microscopy. The Pluronic stock solution is stored at room temperature and is stable for one month, but is routinely used within two weeks of preparation.

B. Purification and fluorescent tagging of +TIPs

1. Purification of recombinantly expressed +TIPs

Mammalian EB1 and CLIP170 proteins can be efficiently expressed in BL21(DE3) Rosetta E. coli cells using standard techniques which we do not detail here. To avoid autoinhibition of full-length CLIP170, it is necessary to use the H2 fragment of CLIP-170 for the

+TIP assays (Lansbergen et al., 2004). The H2 fragment is truncated at the carboxy terminus but forms dimers and directly binds microtubules like the full-length CLIP170 molecule (Scheel et al., 1999). It is worthwhile to point out that while the H2 fragment can be successfully purified using a 6x histidine tag and nickel or cobalt affinity chromatography, histidine-tagged EB1 tends to form inactive aggregates after purification. Therefore, it is advisable to use a GST tag to purify recombinant EB1 protein. It is necessary to cleave the GST tag before using EB1 for experiments. Purified proteins should be desalted and exchanged into BRB80 buffer supplemented with 50 mM NaCl (to reduce protein aggregation) using gel filtration or other techniques such as dialysis. Protein aliquots are flash frozen and kept at -80°C for long-term storage. It is best to avoid freeze-thaw cycles to retain full protein activity.

2. Chemically labeling +TIPs

+TIPs need to be fluorescently tagged to visualize them using TIRF microscopy. In some cases, like the CLIP170(H2) fragment, a green fluorescent protein (GFP) tag can be used without significantly perturbing its microtubule binding and +TIP activity. However, in the case of other proteins like EB1, a GFP tag can significantly affect its ability to interact with microtubules and with other +TIPs (Skube et al., 2009) and therefore it is advisable to use smaller fluorophores like Alexa dyes to tag EB1.

There are many different chemistries that can be used to conjugate fluorescent dyes to protein molecules. These are readily available through Molecular Probes (now Invitrogen) and other commercial vendors. In many cases, these suppliers have developed water-soluble forms of dye conjugates, which allow protein labeling to be conducted in the absence of organic solvents. One popular approach is to use amine-reactive dyes which target primary amine groups such as the ϵ -amino group of lysine or the amine at the N-terminus of proteins. This method can be used

to label virtually any protein. However, drawbacks of amine-based chemistry are that some proteins may not withstand the basic pH needed for maintaining the ϵ -amino group of lysine in the non-protonated form and it may be difficult to optimize conditions that reproducibly give 1 or a few dye molecules per protein molecule, which is desirable to retain protein activity as well as optimal for bleaching analysis of single molecules. A good alternative is to use thiol-reactive dyes that predominantly target cysteine residues. Since most proteins bear few cysteines, this approach typically results in fewer dye molecules per protein molecule. In our lab, purified EB1 is fluorescently labeled at cysteine residues using Alexa Fluor-488 maleimide. Maleimides are water-soluble and most reactive at pH 7.0; therefore dye conjugation of EB1 can be carried out in BRB80 buffer according to the manufacturer's protocol. We describe only the salient parts of the protocol here.

Procedure for tagging EB1 protein using Alexa Fluor-488 C₅ Maleimide:

1. Add drop wise an appropriate volume of 10 mM stock of Alexa Fluor-488 C₅ maleimide freshly prepared in BRB80 buffer supplemented with 50 mM NaCl to 250 μ l of 50 μ M EB1 protein to yield a 15x molar excess of dye to protein. Allow the dye-conjugate to react with the protein at room temperature for 2 hrs in the dark.
2. Stop the reaction by adding 10x molar excess of reduced glutathione and incubate for 10 min at room temperature to quench the unused reactive dye.
3. Separate the protein conjugate from free dye using gel filtration. A Sephadex G-25 column works well for this purpose.
4. Determine the concentration of the recovered protein conjugate using a colorimetric assay such as Bradford or Lowry. Also, measure the absorbance of the protein

conjugate at the peak absorption wavelength for Alexa Fluor-488 (494 nm). Use dilutions if necessary. The degree of labeling can then be calculated:

$$\frac{\text{Moles of dye}}{\text{Mole of protein}} = \frac{A_{494}}{\epsilon \cdot C}$$

Where,

A_{494} = absorbance of the protein conjugate at 494 nm

ϵ = molar extinction coefficient of Alexa Fluor-488 at 494 nm (71,000 $\text{cm}^{-1}\text{M}^{-1}$)

C = protein concentration (M)

Regardless of the type of fluorescent tag used to visualize +TIPs, it is important to test that the tag does not disrupt normal protein activity. One such test is to compare the microtubule binding affinity of labeled versus unlabeled +TIPs using microtubule sedimentation assays.

C. Assembling dynamic microtubules

Reconstituting +TIP activity warrants assembly and visualization of individual dynamic microtubules. Axoneme seeds have been used to nucleate microtubule growth *in vitro*. However, microtubules spawned from these seeds grow as a bundle of up to 9 parallel growing microtubules that can make it hard to easily track individual dynamic plus-ends. Therefore, single microtubule seeds work better for these experiments. These seeds are assembled in the presence of the slowly hydrolyzing GTP analog guanosine 5'-[α,β -methylene] triphosphate (GMPCPP) to stabilize them. New growth is subsequently initiated by the addition of soluble tubulin subunits to the seeds.

1. Preparation of microtubule seeds

It is important to freshly prepare microtubule seeds as they noticeably lose nucleation activity 1 day after preparation. The seeds are brightly labeled in order to distinguish them from

the dimly labeled new growth. A small fraction of biotinylated-tubulin (obtained from Cytoskeleton, Inc., Denver, CO) is also incorporated into the seeds to facilitate their attachment to the flow chamber cover slip.

Procedure for assembling GMPCPP-stabilized microtubule seeds:

1. In an ultracentrifuge tube, mix 44 μ l of unlabeled tubulin with 4 μ l of rhodamine-labeled tubulin and 2 μ l of biotinylated-tubulin. Each of the tubulin components is at 5 mg/ml prepared in BRB80 buffer. This composition yields a ~1:12 ratio of rhodamine-labeled tubulin to non-fluorescent tubulin.
2. Centrifuge the mix at 290,000 g at 4 °C for 10 min to remove tubulin aggregates.
3. Add 5 μ l of 10 mM GMPCPP to the supernatant (1 mM final GMPCPP concentration) and polymerize the tubulin at 37 °C for 30 min.
4. Harvest the microtubule polymers by centrifugation at 30,000 g at 25 °C for 20 min.
5. Resuspend the microtubule pellet in 50 μ l of warm (25 °C) BRB80 buffer supplemented with 1 mM GMPCPP by pipetting up and down using a p20 pipettor. Avoid introducing air bubbles while pipetting.
6. Shear the microtubules to obtain short seeds by passage through a 50 μ l Hamilton syringe fitted with a 22 gauge small bore needle. Typically, 6 up-down passes are necessary so that the majority of seeds in the population are only a few microns long. Store the seeds at room temperature in the dark.

2. Preparation of soluble tubulin mix

The soluble tubulin mix is prepared just before use because of significant loss in its ability to polymerize after ~ 3 hours on ice. Depending on the sensitivity of the TIRF system, ratios ranging from 1:30 to 1:50 of rhodamine-labeled tubulin:unlabeled tubulin can be used. In

our experience, microtubules assembled with a labeling ratio of 1:25 or higher showed significantly decreased ability to bind to MAPs such as +TIPs and tau.

Procedure for preparation of soluble tubulin:

1. Mix rhodamine-labeled tubulin and unlabeled tubulin at the desired ratio. The final tubulin concentration is 5 mg/ml.
2. Centrifuge the mix at 290,000 g at 4 °C for 10 min to remove tubulin aggregates.
3. Store the supernatant on ice. Preferably use within 2 hrs of preparation.

D. Imaging +TIP activity at the single molecule level

The basic configuration for reconstituting +TIP activity is shown in Fig. 3. A key aspect of this protocol not shown in the figure is the presence of methylcellulose (4000 cP, Sigma) in the final polymerization mix. Methylcellulose is used to dampen the random thermal motion of growing microtubule ends. This feature is necessary to keep the new microtubule growth in focus since it is not anchored to the cover slip surface, unlike the microtubule seeds. It is thought that methylcellulose forms a loose hydrophilic meshwork that inhibits movement of microtubules in directions perpendicular to the microtubule growth axis but not along directions parallel to the microtubule growth axis (Uyeda et al., 1990; Young et al., 1998). Importantly, our tests showed that methylcellulose has no significant effect on microtubule growth and shortening velocities at concentrations ranging from 0.15-0.5% as compared to controls with no methylcellulose. It is also worth noting that streptavidin is not as effective as the anti-biotin antibody in attaching the microtubule seeds to the cover slip surface. This result is unexpected because the streptavidin-biotin bond is much stronger compared to the antibody-biotin bond. One possible reason for this observation is that the expected 6-9 nm thick brush-like layer of Pluronic

F-127 on the cover slip surface (Nejadnik et al., 2009) prevents access to the smaller streptavidin molecule as opposed to a much larger IgG molecule.

1. Protocol for reconstituting +TIP tracking

Note: all steps are carried out at room temperature.

1. Assemble a flow chamber with a chamber volume of about 15-20 μ l.
2. Flow in a 20% solution of anti-biotin monoclonal antibody (Sigma, clone BN-34) prepared in BRB80 buffer and incubate for 5 min.
3. Block the exposed cover slip surface by introducing 5% Pluronic F-127 into the flow chamber and incubate for 5 min.
4. Prepare a 1:400 dilution of microtubule seeds in warm (25 °C) BRB80 buffer.
Introduce the diluted seeds into the flow chamber and incubate for 5 min to allow seeds to bind to the antibody.
5. Prepare a 20 μ l polymerization mix as follows:
 - 8 μ l soluble tubulin mix (final concentration of 20 μ M)
 - 4 μ l +TIP protein (final concentration of 250 nM for EB1-Alexa Fluor488 and 25 nM for CLIP170(H2)-GFP)
 - 3 μ l 1% methylcellulose in BRB80 (final concentration ~ 0.15%)
 - 1 μ l 1M DTT (final concentration of 50 mM)
 - 1 μ l Deoxy enzyme mix (3.5 mg/ml catalase and 25 mg/ml glucose oxidase)
 - 1 μ l 450 mg/ml glucose prepared in BRB80
 - 1 μ l 20 mM Mg-GTP (final concentration of 1mM)
 - 1 μ l BRB80

Flow in the polymerization mix and visualize the sample using time-lapse TIRF microscopy. Initially, microtubules will predominantly polymerize and subsequently, as the system reaches steady-state, individual microtubules will exhibit dynamic instability. Plus-end tracking activity is evident by a comet-like localization pattern of +TIPs at growing microtubule ends. EB1 is an autonomous +TIP and specifically decorates the microtubule plus-ends during phases of polymerization and not during phases of depolymerization (Fig. 4A). In contrast, CLIP170(H2) by itself does not plus-end track. Rather, it binds along the length of microtubules and shows 1-dimensional diffusive movement (Fig. 4B). However, in the presence of unlabeled EB1 (250 nM), CLIP170(H2)-GFP shows robust +TIP activity (Fig. 4C).

In the above example, multiple unresolved EB1-Alexa Fluor488 and CLIP170(H2)-GFP molecules are present at growing microtubule plus-ends. To visualize single +TIP molecules, it is necessary to use a lower concentration of the fluorescently labeled +TIPs as well as faster frame rates (10-30 fps) to capture the binding and unbinding of individual +TIP molecules at microtubule ends. One approach is to conduct the experiment by simply using low concentrations of EB1-Alexa Fluor488 (10 nM) and CLIP170(H2)-GFP (5 nM) in the polymerization mix. An alternate approach is to dilute EB1-Alexa Fluor488 (10 nM) and CLIP170(H2)-GFP (5 nM) in unlabeled EB1 (250 nM) and CLIP170(H2) (25 nM) in order to keep the total concentration of EB1 and CLIP170(H2) the same as for the basic +TIP experiment. The latter approach is preferred because the behavior of single +TIP molecules is assessed within the context of the normal +TIP concentration and decoration pattern at microtubule plus-ends.

2. Image analysis

A popular way to analyze images from time-lapse movies is to generate kymographs. Using kymographs, one can quickly measure features such as growth rates and bound times for large numbers of individual microtubules and +TIP molecules. A detailed description of how measurements are made using kymographs can be found in chapter 27 of this volume.

V. DISCUSSION

In vitro reconstitution assays are gaining popularity as a powerful means to dissect complex biological processes. Here we have provided a detailed protocol for reconstituting +TIP activity at the single molecule level and highlighted how this method provides unique insight into the fundamental molecular properties of +TIPs. While we have emphasized the ability of this assay to deconstruct the +TIP complex in this chapter, it is important to note that this technique provides the foundation to reconstruct more complicated scenarios in the future. For example, the interactions between different +TIPs at dynamic microtubule ends can be studied using multi-color TIRF microscopy to probe the dynamics, hierarchy and function of +TIP assemblies. Another exciting future prospect is the possibility of reconstituting +TIP-mediated microtubule capture by incorporating +TIP-binding proteins in these assays. These and other future applications will benefit by combining multi-color, high-resolution single molecule imaging with microfluidics and nanofabrication technologies to more closely emulate the complex cellular environment.

Acknowledgements

We apologize to colleagues whose work we could not discuss in detail due to space restrictions.

We thank both Drs. Erika L. F. Holzbaaur and Yale E. Goldman in whose labs much of this work was initiated and performed. We also thank Mariko Tokito for her expert advice on purification of recombinantly expressed +TIPs.

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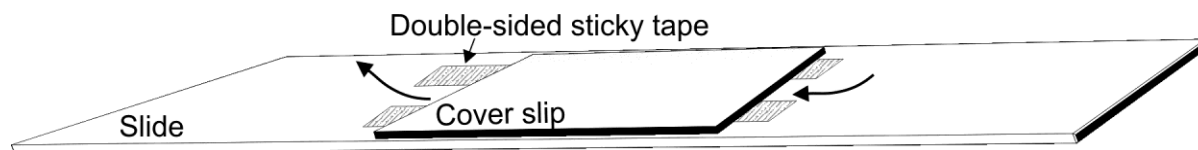


Fig. 1: Flow chamber

A flow chamber for *in vitro* imaging is constructed by adhering a cover slip to a glass slide using double-sided sticky tape. Solutions are flowed through the channel for sample preparation.

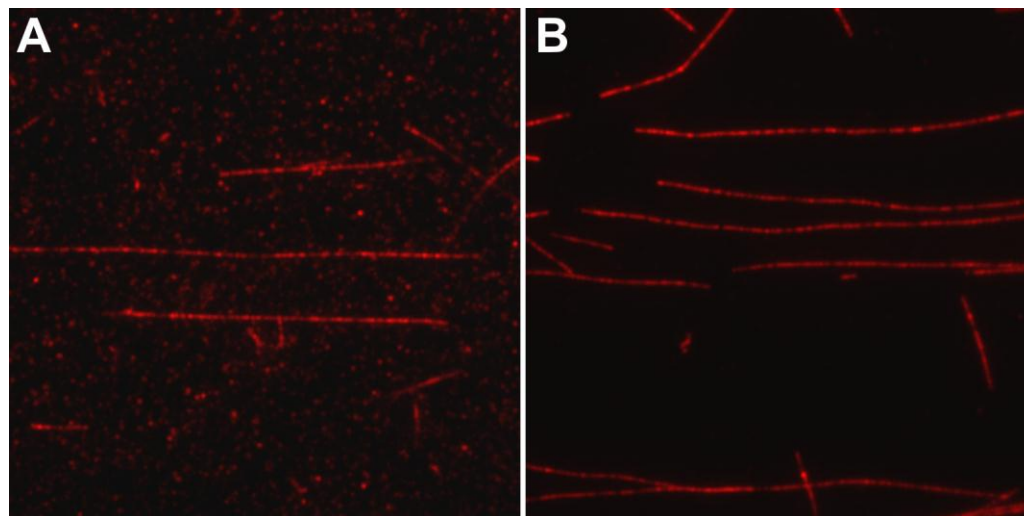


Fig. 2: Comparison of BSA and Pluronic as blocking agents

Flow chambers assembled using silanized glass cover slips were treated with 0.1 % Tub 2.1 anti- β -tubulin antibody for 5 min and subsequently blocked with either 10 mg/ml bovine serum albumin (A) or 5% Pluronic F-127 (B) for 5 min. Taxol-stabilized rhodamine-labeled microtubules were allowed to bind to the antibody for 5 min and then washed once with BRB80 to remove any unbound microtubules/tubulin. Images were captured using TIRF microscopy with the same settings.

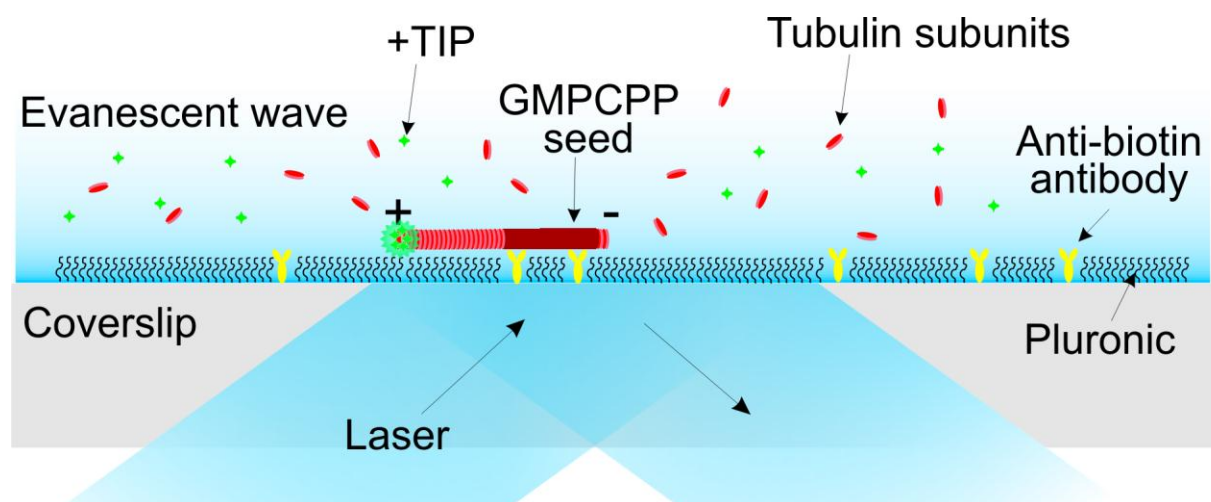


Fig. 3: Basic configuration for reconstituting +TIP activity

GMPCPP-stabilized rhodamine-labeled and biotinylated microtubule seeds are affixed to the cover slip surface using anti-biotin antibodies. 5% Pluronic F-127 is used to block the glass surface and then a polymerization mix consisting of soluble tubulin and +TIPs flowed in to initiate microtubule growth. Fluorescently labeled +TIPs that bind to growing microtubule ends are excited by the evanescent wave during TIRF microscopy.

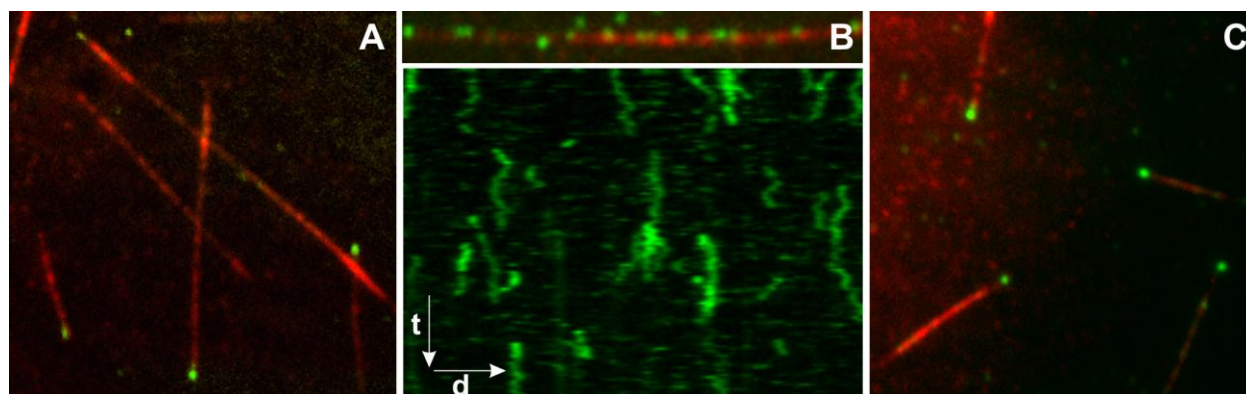


Fig. 4: +TIP tracking of EB1 and CLIP170 proteins

(A) Alexa Fluor-488-labeled EB1 (bright green spots) specifically localizes to the ends of growing microtubules (in red). (B) CLIP-170(H2)-GFP by itself binds along the microtubule length and shows diffusive movement along the microtubule lattice (shown in the kymograph below). (C) CLIP-170(H2)-GFP in the presence of unlabeled EB1 specifically localizes to the ends of growing microtubules.