Chapter 19

In Vitro Microtubule Severing Assays

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Abstract

Microtubules are rigid and highly dynamic cellular polymers essential for intracellular transport, cell division and differentiation. Their stability is tightly regulated by a vast array of cellular factors. In vitro microtubule assays have proven to be powerful tools for deciphering the mechanism of microtubule dynamics regulators such as molecular motors and microtubule associated proteins. In this chapter we focus on microtubule severing enzymes that use the energy of ATP hydrolysis to introduce internal breaks in the microtubule lattice. We present a detailed protocol for a light microscopy based in vitro microtubule severing assay that was instrumental in the identification and characterization of these enzymes.

Key words Cytoskeleton, Microtubule, Tubulin, Severing, Depolymerization, Spastin, Katanin, AAA ATPase

1 Introduction

The microtubule cytoskeleton is a highly dynamic structure that undergoes constant restructuring in response to environmental and developmental cues. Microtubules display a polymerization characteristic called "dynamic instability," i.e., they undergo growth (rescue) and shrinkage (catastrophe) of their polymer ends and can switch rapidly between these two states in a stochastic manner [1]. In addition to their intrinsic dynamic properties, microtubule stability is regulated by a vast array of molecular motors and microtubule associated proteins (MAPs). Microtubules can be destabilized by cellular factors *via* depolymerization from their ends (reviewed in [2]) or severing along their length (reviewed in [3, 4]). Microtubule severing was first observed in metaphase-like *Xenopus* egg extracts [5]. The protein responsible for this activity was later purified and named katanin after the Japanese for sword "katana" [6]. Since this initial discovery, several other enzymes that can sever microtubules have been identified: spastin [7, 8], fidgetin [9, 10], and the katanin-like proteins [9, 11]. These

Amanda S. Coutts (ed.), *Adhesion Protein Protocols*, Methods in Molecular Biology, vol. 1046,
DOI 10.1007/978-1-62703-538-5_19,

enzymes belong to the large family of AAA ATPases (ATPases associated with various cellular activities) and use the energy of ATP hydrolysis to destabilize the microtubule lattice. Microtubule severing enzymes are involved in basic cellular processes ranging from cell division [9–15] and cilia biogenesis [16, 17] to neurogenesis [18–21], axonal maintenance and regeneration [22, 23]. Even though katanin was discovered more than 25 years ago, the mechanism of action of microtubule severing proteins is still poorly understood. In vitro microtubule severing assays are powerful tools for deciphering the biophysical mechanism of microtubule severing enzymes as well as for the identification and characterization of cellular factors that regulate their activity.

Here we describe the recombinant expression and purification of the microtubule severing enzyme spastin and illustrate in detail a fluorescence microscopy based in vitro microtubule severing assay. This assay is an adaptation of earlier microtubule severing assays that were used to identify katanin and spastin as microtubule severing enzymes [7, 24]. Direct observation by light microscopy was key to being able to establish that these proteins were indeed severing microtubules along their length and not depolymerizing them from their ends [5].

2 Materials

2.1 Expression and Purification of Recombinant Spastin

2.1.1 Materials and Equipment

1. pDEST15 Gateway destination vector expressing *Drosophila melanogaster* spastin (residues 220—C-terminus [25]).
2. *Escherichia coli* Rosetta 2(DE3)pLysS competent cells (Novagen #71403-4).
3. LB Broth, Miller.
4. Ampicillin sodium salt: 200 mg/mL stock solution in water. Store at −20 °C.
5. Chloramphenicol: 34 mg/mL stock solution in ethanol. Store at −20 °C.
6. Isopropylthiogalactoside (IPTG): 1 M stock solution in water. Store at −20 °C.
7. Dithiothreitol (DTT): 1 M stock solution in water. Store at −20 °C.
8. Phenylmethylsulfonyl fluoride (PMSF): 1 M stock solution in DMSO. Store at −20 °C.
9. 5 M NaCl.
10. DNase I (Roche Applied Science #10104159001).
11. PreScission protease (GE Healthcare # 27-0843-01). Store at −80 °C.
12. Bottle-top filters, 500 mL, pore size 0.22 μm.

13. Glutathione Sepharose 4 Fast Flow (GE Healthcare).
14. Econo-Column Chromatography Columns (Bio-Rad #737-2512).
15. HiTrap Capto S column: 5 mL (GE Healthcare #17-5441-23) or Mono S 10/100 GL, 8 mL (GE Healthcare #17-5169-01) for higher resolution separation.
16. Millex—GV Syringe Driven Filter Units, PVDF, low protein binding, pore size 0.22 μm (Millipore #SLGV033NB).
17. Amicon Ultra Centrifugal Filters, 10K MWCO (Millipore).
18. Dialysis tubing with 10K MWCO or Slide-A-Lyzer Dialysis Cassettes, 10K MWCO, 12 mL (Thermo Scientific).
19. EmulsiFlex C5 homogenizer (Avestin).
20. FPLC system (AKTA, GE Healthcare).

2.1.2 Solutions and Buffers

Prepare all buffer solutions with ultrapure water (>18 MΩ cm at 25 °C) and filter using 0.22 μm bottle-top filters.

1. Lysis buffer: Phosphate buffered saline (PBS) supplemented with 10 mM $MgCl_2$, 1 mM PMSF, protease inhibitor cocktail (0.001 mg/mL aprotinin, 0.002 mg/mL leupeptin, 0.001 mg/mL pepstatin). The EDTA free inhibitor cocktail tablets from Roche Applied Science can also be used.
2. Wash buffer: 20 mM Tris–HCl pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT.
3. Elution buffer: 50 mM Tris–HCl pH 8.8, 300 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 20 mM reduced glutathione. Glutathione lowers the pH of the solution, thus the use of Tris–HCl pH 8.8. The final pH of the elution buffer is 8.0.
4. Ion exchange buffer A: 50 mM MES pH 6.5, 10 mM $MgCl_2$, 5 mM DTT, 10 % glycerol.
5. Ion exchange buffer B: 50 mM MES pH 6.5, 10 mM $MgCl_2$, 2 M NaCl, 5 mM DTT, 10 % glycerol.
6. Dialysis buffer: 20 mM HEPES pH 7.0, 300 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 15 % glycerol.

2.2 Microtubule Severing Assay

2.2.1 Microscope

This assay requires a fluorescence microscope equipped with filters to detect rhodamine and suitable for time lapse image capturing. For example in our lab we use the following:

1. Motorized inverted microscope (e.g., Nikon Ti-E 2000 with perfect focus).
2. TIRF objective (e.g., CFI Apochromat TIRF 100× Oil, NA 1.49, Nikon). A high NA TIRF objective is not essential.
3. 532 nm solid-state laser (Sapphire, Coherent). Microtubules can also be imaged in epifluorescence using a Xe-Hg lamp.

4. Appropriate single-band filters and dichroic mirrors for TMR fluorescence imaging.
5. Cooled charged-coupled device (CCD) camera (e.g., iXON 897, Andor).
6. Controller software: μManager (100× Imaging).

2.2.2 Flow Chamber Preparation

1. Frosted microslides (3 × 1 in.).
2. Cover glass (22 × 40 mm).
3. Polytetrafluoroethylene racks for holding cover glass and slides (custom made in our workshop). Polytetrafluoroethylene (also known as Teflon®) is resistant to the highly corrosive Piranha solution (see below).
4. 500 mL glass beakers to hold the racks.
5. Hot plate.
6. Sonicating water bath.
7. Scotch permanent double-sided tape.
8. Parafilm.
9. Chemical fume hood.
10. Lab coat and safety glasses.

2.2.3 Solutions and Buffers for Slide and Cover Glass Preparation

1. Mucasol: 2 % solution (dilute in water).
2. Sulfuric acid.
3. Hydrogen peroxide (30 %).
4. 0.1 M KOH.
5. Trichloroethylene (TCE) (≥99.5 %).
6. Dichlorodimethylsilane (DDS) (≥99.5 %). Store DDS in a desiccator at 4 °C.
7. Methanol.
8. Ethanol, 200 proof.

2.2.4 Equipment, Solutions and Buffers for Flow Chamber Assembly and Severing Assays

1. TLA 100 rotor (Beckman).
2. 0.1 μm low protein binding filters (Millipore # UFC30VV00).
3. Anti-Tetramethylrhodamine (TMR) antibody (Life Technologies #A-6397).
4. Casein: 32 mg/mL stock solution in 20 mM Tris–HCl, pH 8.0.
5. Guanosine 5′-triphosphate (GTP): 100 mM stock solution in water. Store at −80 °C.
6. Adenosine 5′-triphosphate disodium salt (ATP): 100 mM stock in water and adjusted to pH 7.0 with 5 M NaOH. Store at −80 °C.

7. Dimethyl sulfoxide (DMSO) (≥99.7 %).
8. DTT: 1 M stock solution in water. Store at −20 °C.
9. Taxol, commercially available under the trade name "Paclitaxel" (Acros Organics #328420050): 10 mM stock solution in DMSO. Store at −20 °C.
10. BRB80: 80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$ (*see* **Note 1**).
11. BRB80-D: 80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM DTT.
12. BRB80-DT: 80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM DTT, 20 μM taxol.
13. 2× Polymix: 80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 2 mM GTP, 20 % DMSO. Store at −80 °C.
14. D-(+)-Glucose: 2 M stock solution in water. Store at −80 °C.
15. Glucose oxidase (Sigma-Aldrich #G-7016). Store as powder at −20 °C.
16. Catalase (Roche Applied Science #106810). Store at 4 °C.
17. Oxygen scavenger mix (100× stock solution): mix 200 μL of BRB80, 50 μL catalase (65,000 units), and 30 mg glucose oxidase (~6,000 units). Mix gently and incubate for 5 min at 4 °C. The solution will be cloudy. Spin at 20,000 × *g* in a tabletop centrifuge and retain the supernatant. Filter the supernatant using a 0.1 μm filter (Millipore). Prepare 10 μL aliquots, snap-freeze in liquid nitrogen, and store at −80 °C.

2.2.5 Tubulin

1. Unlabeled tubulin purified from porcine brain (Cytoskeleton, Inc. #T240).
2. TMR-labeled tubulin (Cytoskeleton, Inc. #TL447M). Resuspend the lyophilized pellet in BRB80, aliquot and freeze immediately in liquid nitrogen. Use one aliquot *per* experiment and discard any unused material (*see* **Note 2**).

2.3 Data Analysis

ImageJ (NIH) for basic image processing. http://rsb.info.nih.gov/ij/

3 Methods

3.1 Spastin Purification

1. Transform N-terminal glutathione S-transferase (GST) fusion spastin (residues 220—C-terminus) expression vector into *E. coli* strain Rosetta2(DE3)pLysS. Grow cells to an OD_{600} of 0.8 in LB broth containing 0.2 mg/mL ampicillin and 0.034 mg/mL chloramphenicol at 37 °C. Use beveled culture flasks for maximal aeration. Induce expression with 0.5 mM IPTG and harvest cells after 16 h of induction at 16 °C.

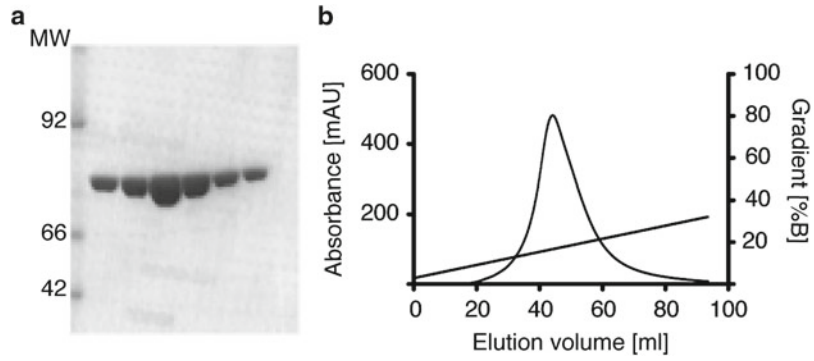

Fig. 1 (**a**) SDS-PAGE gel showing spastin purity after the ion exchange chromatography step. (**b**) Elution profile of spastin from a HiTrap Capto S ion exchange chromatography column

2. Resuspend cells in lysis buffer and disrupt using the EmulsiFlex C5 (three passes at 10–12 Kpsi while making sure that the sample is kept constantly chilled on ice).
3. Supplement the lysate with 0.02 mg/mL DNAse I and incubate on ice for 15 min while mixing gently. Add 400 mM NaCl and incubate on ice for an additional 15 min.
4. Pack a GST-affinity column using the glutathione sepharose 4 fast flow resin (10 mL of resin are sufficient for 15 g of wet cell pellet).
5. Collect supernatant after centrifugation in SS-34 rotor at 30,000 × *g* for 50 min. Supplement supernatant with 5 mM DTT and load on the GST-affinity column equilibrated in wash buffer.
6. Wash the GST-affinity column with 20 column volumes (CV) of wash buffer and elute the fusion protein with the elution buffer. Run a SDS-PAGE gel to estimate the purity of the fusion protein.
7. Cleave the GST fusion tag by incubating with PreScission protease (1 unit will cleave >90 % of 100 μg of a GST-fusion protein) at 4 °C overnight.
8. Run SDS-PAGE gel the next morning to examine whether cleavage is complete (*see* **Note 3**).
9. If cleavage is successful, dilute the protein sample 1:2 in ion exchange buffer A. Filter sample through a PVDF low protein binding filter with pore size 0.22 μm and load on a HiTrap Capto S column equilibrated in 5 % ion exchange buffer B. Wash the column with 20 CV of 5 % ion exchange buffer B. Elute the protein using a continuous gradient of 5–50 % ion exchange buffer B over 20 CV.
10. Collect peak fractions and run a SDS-PAGE gel to determine the purity of the eluted sample (Fig. 1).

11. Dialyze overnight against dialysis buffer using Slide-A-Lyzer Dialysis Cassettes 10K MWCO.
12. Prepare 20 μL aliquots of the purified protein, snap-freeze in liquid nitrogen, and store at −80 °C (*see* **Note 4**).

3.2 Light Microscopy Based Microtubule Severing Assay

3.2.1 Cover Glass and Slide Preparation

Proteins adsorb nonspecifically to untreated glass. Thus, preparation of clean, silanized cover glass that can be efficiently passivated by a blocking agent is essential for these assays. In the absence of this treatment, most of the spastin protein adsorbs to the glass and is rendered inactive. This results either in failure to see robust activity or the need to use large concentrations of the severing enzyme in these assays. We detail below a procedure we adapted from [26] and use in our laboratory to prepare slides and cover glass for microtubule severing assays.

1. Sonicate cover glass and slides for ~1 h in 2 % mucasol.
2. Rinse cover glass and slides five times with ultrapure water.
3. Prepare "Piranha" solution by mixing sulfuric acid and hydrogen peroxide in a 2:1 ratio. Make sure to add the peroxide to the acid and not the other way around! The reaction is exothermic. Having an excess of hydrogen peroxide to sulfuric acid can cause an explosion (*see* **Note 5**). Prepare the Piranha solution just before use as it loses its efficacy after ~1 h.
4. Place polytetrafluoroethylene racks with the cover glass and slides in a beaker with Piranha solution for 1 h. Make sure that the slides and cover glass are completely submerged in the solution. Maintain the temperature at ~60 °C by placing the beaker on a hot plate.
5. Rinse cover glass four times with ultrapure water.
6. Transfer the rack to a beaker with 0.1 M KOH solution and incubate at room temperature for 15 min.
7. Rinse glass three times with ultrapure water.
8. Place glass in ethanol for ~1 min and air dry.
9. Prepare silanizing solution just before usage by mixing 150 mL TCE with 75 μL DDS. Withdraw the appropriate volume of DDS using a syringe with a long needle. Seal the bottle well with parafilm when finished and store in a desiccator.
10. Submerge racks with the cover glass in silanizing solution at room temperature for 1 h.
11. Transfer the rack to a beaker with methanol and sonicate for 5 min. Exchange the methanol and sonicate again for 10 min.
12. Transfer to ethanol.
13. Dry cover glass and slides. Store in sealed containers. Cleaned cover glass and slides can also be stored for longer periods of time in ethanol and dried before use.

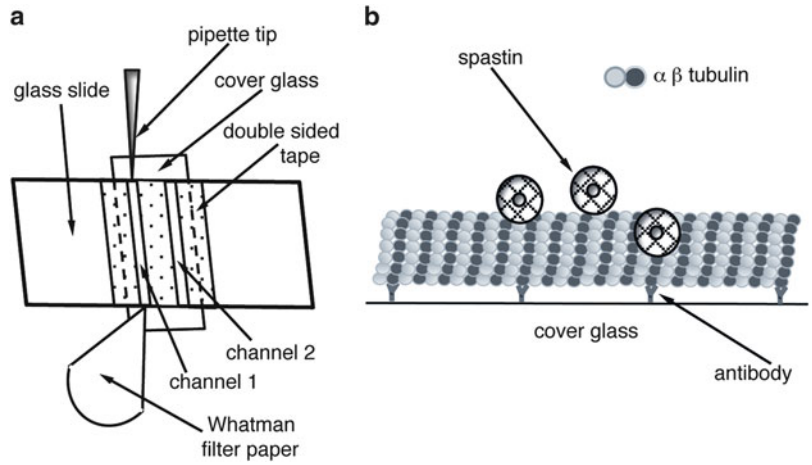

Fig. 2 (**a**) Schematic of the flow chamber used in microtubule severing assays. (**b**) Cartoon of the microtubule severing assay. A TMR-labeled microtubule is immobilized on the glass surface using anti-TMR antibodies. The microtubule severing enzyme is perfused into the chamber in the presence of ATP and oxygen scavengers

3.2.2 Preparation of TMR Labeled Taxol Stabilized Microtubules

1. Mix 2 μL of 2 mg/mL TMR-tubulin with 3 μL of 10 mg/mL unlabeled tubulin and incubate on ice for 2 min. This results in microtubules that have ~11 % of tubulin fluorescently labeled.
2. Add 5 μL of 2× Polymix and incubate in a water bath at 37 °C for 40 min.
3. Add 10 μL of BRB80-DT (prewarmed to 37 °C) and continue to incubate in the water bath at 37 °C for another 10 min.
4. Spin down in TLA 100 rotor (prewarmed to 30 °C) for 7 min at 109,000 × *g* (50,000 rpm) to remove unpolymerized tubulin.
5. Discard supernatant and resuspend pelleted microtubules in 50 μL of BRB80-DT. Because microtubules shear easily, use a cut pipette tip to resuspend the microtubule pellet. Store the TMR-labeled microtubules on your bench covered with aluminum foil (*see* **Note 6**). They are suitable for assays for several days.

3.2.3 Flow Chamber Assembly and Severing Assay

1. Attach pieces of double-sided tape to a slide forming two 2-mm wide chambers and cover with a cover glass. This creates a thin "channel" between the strips of tape and the two pieces of glass with a volume of ~5 μL (Fig. 2a).
2. Perfuse the flow chamber by adding 10 μL of 50 μg/mL anti-TMR antibody to one end of the chamber while fluid is wicked on the other end with a filter paper strip. Incubate at room temperature for 20 min (*see* **Note 7**).
3. Wash the flow chamber with 20 μL of BRB80-D supplemented with 2 mg/mL casein. In order to exchange the solution in the

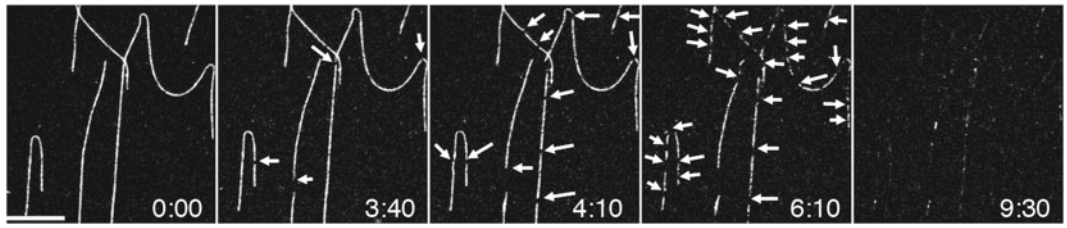

Fig. 3 TMR-labeled microtubules severed by spastin. Severing sites along the microtubules are indicated by *arrows*. The last time point shows the almost complete disassembly of the microtubules. Time is in minutes. Scale bar is 10 μm

channel completely, it is necessary to flow at least two channel volumes of solution.

4. Dilute TMR-labeled microtubule stock (typically a 25× dilution gives a suitable microtubule density) in BRB80-DT supplemented with 2 mg/mL casein. Perfuse 20 μL into the flow chamber and incubate at room temperature for 10 min. Microtubules are attached to the silanized cover glass surface by the anti-TMR antibodies that adsorbed nonspecifically to the glass (Fig. 2b; *see* **Note 8**). It might be necessary to adjust the dilution of the microtubule stock depending on the preparation. For a new microtubule stock we quickly test a series of dilutions by assembling slides with multiple channels (Fig. 2a). A typical chamber with an optimal density of microtubules is shown in Fig. 3. The antibodies act as spacers between the glass and the microtubule and prevent a collapse of the microtubule structure through strong nonspecific interactions with the glass surface. This configuration also allows better access to the microtubule of the severing enzyme.
5. Wash the flow chamber with 30 μL of BRB80-DT supplemented with 2 mg/mL casein, oxygen scavenger mix (1:100 dilution of stock), 20 mM glucose.
6. Prepare 20 μL of a solution containing 50 nM spastin, 2 mg/mL casein, 1 mM ATP, oxygen scavenger mix, 20 mM glucose in BRB80-DT and perfuse in the flow chamber while trying to maintain the focal plane unperturbed. Start imaging shortly before perfusion. After data acquisition is completed, move to a different field of view that was not illuminated and examine the integrity of the microtubules in that area (*see* **Note 9**).

3.3 Data Analysis

Microtubule severing activity can be quantified using two basic methods: (a) counting the number of severing events observed *per* unit time *per* total microtubule length or (b) by measuring the rate of tubulin removal, i.e., the extension rate of the microtubule gap after the initial severing event (*see* **Notes 10** and **11**). These analyses can be performed using basic functions available in ImageJ (NIH).

3.4 Conclusions

The microtubule severing assay described here can be very powerful as it allows the severing reaction to be reconstituted entirely from purified components and thus can be used to investigate the basic biophysical mechanism of severing enzymes (*see* **Note 12**) as well as to test the effects of cellular factors on severing activity. These can influence severing activity by directly binding and/or post-translationally modifying the microtubule severing enzyme itself [15, 24] or indirectly by binding or post-translationally modifying the microtubule track [27–30]. Through the gradual addition of purified components this basic assay can be used to investigate mechanisms of microtubule severing enzyme regulation and identify novel cellular cofactors that regulate microtubule severing enzyme activity.

4 Notes

1. This buffer is commonly used in microtubule assays.
2. Tubulin denatures easily and needs to be stored at −80 °C in single use aliquots flash frozen in liquid nitrogen. When thawing a new aliquot, spin it in a precooled rotor in the ultracentrifuge at 440,000 × *g* (100,000 rpm in a TLA 100 rotor) at 4 °C to remove any aggregates formed during the freezing and thawing process.
3. The cleavage is usually 80 % complete after overnight incubation. The two fragment sizes expected (spastin and GST) are 54 and 26 kDa, respectively.
4. Spastin activity decreases if the protein is stored for more than a day at 4 °C. Discard any unused protein at the end of the day and start with a fresh aliquot for a new set of assays. When thawing a new aliquot, spin it in a precooled rotor in the ultracentrifuge at 440,000 × *g* (100,000 rpm in a TLA 100 rotor) at 4 °C to remove any aggregates formed during the freezing and thawing process.
5. Cover glass cleaning and silanization must be performed in a chemical fume hood with caution and using protective equipment. Familiarize yourself with the appropriate protocols for chemical spill handling.
6. Unpolymerized tubulin should be kept on ice. Microtubules should not be placed on ice as they will immediately depolymerize.
7. The anti-TMR antibody denatures and loses its activity over time if stored at 4 °C. It can be flash frozen in liquid nitrogen in single use aliquots without loss of activity.
8. An anti-tubulin antibody (clone TUB 2.1) (Sigma-Aldrich #T4026) [31] or a rigor kinesin construct [7, 32] can also be used to immobilize microtubules on the cover glass.

9. TMR-labeled microtubules can break if photodamaged [33]. To minimize photodamage, decrease light exposure as much as possible and use fresh oxygen scavengers. Always perform your controls judiciously.
10. Severing rates measured in these assays vary as a function of the density of anti-TMR antibodies used to immobilize the microtubules on the glass. Thus, when comparing severing activities between samples it is important that the same antibody concentration and glass treatment be used.
11. The microtubule severing reaction is dependent on the electrostatic interaction between the enzyme and the negatively charged C-terminal tubulin tails [7, 8, 24]. Thus, the ionic strength of the buffer used in severing assays is important and should be kept as low as protein stability allows in order to observe maximal activity.
12. The assay described here can be adapted to also observe the dynamics of spastin molecules on the microtubule by using a fluorescently labeled variant of the enzyme and total internal reflection fluorescence microscopy.

Acknowledgments

A. Roll-Mecak is supported by the National Institutes of Health Intramural Program. N. E. Ziółkowska is supported by a Searle Scholar Award to A. Roll-Mecak.

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