CHAPTER

Single-molecule imaging of Tau dynamics on the microtubule surface

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CHAPTER OUTLINE

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Abstract

The microtubule-associated protein Tau is primarily expressed in neurons and plays an integral role in the regulation of multiple functions within the axon. In the adult brain, the six Tau isoforms are expressed allowing for a complex system of control. Despite Tau's central role, the mechanisms by which Tau acts are not fully understood. We have used single-molecule total internal reflection fluorescence (TIRF) microscopy and the methods described in this chapter to further our knowledge of Tau's behavior and function. We have demonstrated that Tau's dynamic binding behavior allows for regulation of motor protein motility and microtubule dynamics in an isoform-specific manner. The continued use and refinement of the single-molecule techniques detailed here can only further our knowledge of Tau and other proteins integral to the maintenance of axonal transport.

Tau is a microtubule-associated protein (MAP) primarily expressed in neurons that performs multiple functions within the axon, most notably promoting microtubule stability and regulating axonal transport. The diversity of Tau's functions arise partly from its structural diversity—six isoforms are expressed in adult neurons that are conformationally hyperdynamic and undergo multiple posttranslational modifications. However, the mechanisms by which Tau carries out its various functions remain unclear. Single-molecule imaging techniques have provided new insights into Tau's interaction with the microtubule surface and its ability to carry out specific functions, including regulation of motor protein motility (Dixit, Ross, Goldman, & Holzbaur, 2008; Hoeprich, Mickolajczyk, Nelson, Hancock, & Berger, 2017; Hoeprich, Thompson, McVicker, Hancock, & Berger, 2014; McVicker, Chrin, & Berger, 2011; McVicker, Hoeprich, Thompson, & Berger, 2014; Stern, Lessard, Hoeprich, Morfini, & Berger, 2017; Vershinin, Carter, Razafsky, King, & Gross, 2007; Vershinin, Xu, Razafsky, King, & Gross, 2008; Yu et al., 2014). Our lab has successfully used single-molecule imaging with TIRF (total internal reflection fluorescence) microscopy to elucidate the dynamic behavior of Tau on the microtubule surface (McVicker et al., 2014; Stern et al., 2017), as seen in Fig. 1, and assess the impact of different Tau states on the motility of axonal motor proteins such as kinesin-1 (McVicker et al., 2011; Stern et al., 2017) and kinesin-2 (Hoeprich et al., 2017, 2014). Of particular importance has been the discovery that Tau exists in an equilibrium on the microtubule surface between static and diffusive states that is isoform specific and can be regulated by site-specific phosphorylation events (Hinrichs et al., 2012; McVicker et al., 2014; Stern et al., 2017). Furthermore, the distribution between these states differentially affects different motor proteins such as kinesin-1, kinesin-2, and cytoplasmic dynein.

In the following sections we detail how TIRF experiments can be performed to study the dynamics of Tau on the microtubule surface (Section 1), the affinity of Tau for microtubules (Section 2), as well as functional assays to assess the effects of different Tau constructs on microtubule dynamics (Section 3) and motor protein motility (Section 4).

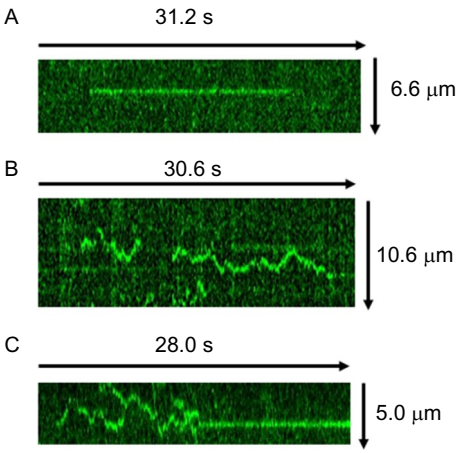

FIG. 1

Representative kymographs of Tau dynamics on the microtubule surface, demonstrating (A) static behavior, (B) diffusive behavior, and (C) switching between diffusive and static behavior.

1 TAU DYNAMICS IMAGING ASSAY

All protein work and experiments were done in BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9 at room temperature) unless otherwise noted.

1.1 COVERSLIP AND FLOW-CELL CONSTRUCTION

All single-molecule imaging experiments described below require the use of 24 mm × 60 mm No. 1 coverslips coated with PEG-Silane (2-[methoxy (polyethyleneoxy)-propyl]trimethoxysilane) to prevent nonspecific protein binding. *Note*: Our imaging system is an inverted TIRF microscope; therefore, the smaller top coverslips (22 mm × 22 mm No. 1) used to construct the imaging flow cells do not need to be PEG-Silane coated. The PEG-Silane coating procedure as previously described (Stern et al., 2017) is as follows:

1.1.1 Cleaning coverslips

1. Place glass slides in a holder and incubate in 100% methanol for 2 h with gentle shaking. *Note*: From this point forward during the coverslip-coating procedure use clean forceps, rinsed with 100% methanol, to transfer coverslips between holders.
2. Remove each coverslip one at a time from the slide holder and dry using nitrogen gas. We use rubber tubing attached to the outlet nozzle of the nitrogen gas cylinder to flow nitrogen gas at 10–15 PSI directly onto the coverslip.

3. Plasma clean methanol-washed coverslips using a plasma cleaner for 2–5 min. *Note*: The coverslips are placed face up in the plasma cleaner and only the upward facing side of the coverslip is plasma-cleaned.
4. Transfer the plasma-cleaned coverslips to a slide holder. *Note*: Keep track of the plasma-cleaned side from this point forward as this is the side that must be used for imaging. We do this by placing a piece of tape on the side of the slide holder and place the plasma-cleaned side of the coverslips facing the taped side of the slide holder.

1.1.2 PEG-Silane coating

1. Prepare the silanization solution in a slide holder in a fume hood. The reaction solution is 97.6% (V/V) toluene, 1.76% (V/V) PEG-Silane, and 0.64% (V/V) *n*-butylamine. Make 50 mL of silanization solution by mixing 48.8 mL toluene, 0.866 mL PEG-Silane, and 0.311 mL butylamine. *Note*: All subsequent steps involving the silanization solution and toluene rinses should be performed in a fume hood.
2. Transfer the plasma-cleaned coverslips to a slide holder containing the silanization solution and incubate for 2 h under flowing (10–15 PSI) nitrogen gas. We use a custom-built enclosed chamber with a gas inlet at the bottom of one side and a gas outlet at the top of the opposite side for this purpose.
3. Wash coated coverslips one at a time in 100% toluene. Dip the coated coverslips 5× each in two successive toluene washes. Dry the toluene-washed coverslips using a gentle flow (10–15 PSI) of nitrogen gas.
4. Place dry coated coverslips on dry rack in the nitrogen gas chamber and cure them with flowing nitrogen gas (10–15 PSI) for 30 min.
5. Store the coated coverslips under nitrogen in the sealed chamber after turning off the nitrogen flow.

1.1.3 Flow cell construction

1. Cut spacers to 1 mm × 60 mm from a full ARTUS shim sheet.
2. Coat three 1 × 60 shims on both sides with Norland optical adhesive 61 to adhere the spacers to the silanized 24 × 60 coverslips. Three equally spaced spacers are placed on a coverslip to create two ~20 μL chambers (shown in Fig. 2A). The two chambers can be used to image both control and experimental samples side-by-side. Complete chambers by placing a 22 × 22 top uncoated coverslip over the spacers. *Note*: Ensure that the adhesive completely coats the area under the top coverslip to ensure that chambers do not leak, but wipe excess adhesive from the spacer edges.
3. Set adhesive with 15 min of UV irradiation. We use a UVP White/UV Transilluminator.

Materials

24 mm × 60 mm No. 1 Coverslips (ThermoFisher, Waltham, MA).
22 mm × 22 mm No. 1 Coverslips (ThermoFisher, Waltham, MA)

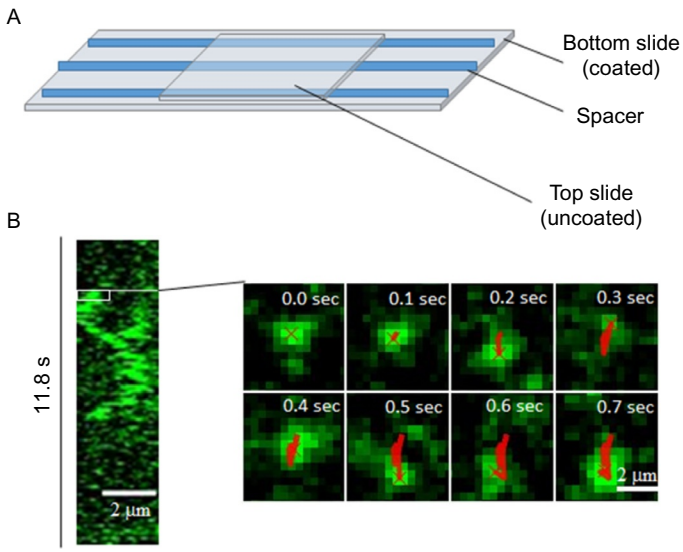

FIG. 2

(A) Flow cell chambers constructed with PEG-Silane-coated bottom slide, ARTUS shim spacers sealed with UV-treated adhesive, and an uncoated top coverslip. Slide is shown in the imaging orientation. (B) Kymograph and corresponding eight-frame track for Alexa 488-labeled 3RS-Tau imaged by TIRF microscopy and analyzed using ImageJ. Data collected at 10 fps.

100% Methanol (Fisher Chemical, Hampton, NH)
Toluene (Sigma-Aldrich, St. Louis, MO)
2-Methoxy(polyethyleneoxy)propyltrimethoxysilane (Gelest Inc., Morrisville, PA)
Butylamine (Acros Organics, Plains, NJ)
Norland optical adhesive (Norland Products, Cranbury, NJ)
ARTUS shims (ARTUS, Eaglewood, NJ)
Nitrogen gas (Airgas, Radnor, PA)
UVP White/UV Transilluminator (UVP LLC, Upland, CA)
Harrick Plasma Cleaner (Harrick Plasma, Ithaca, NY)
Custom nitrogen chamber

1.2 TAU PROTEIN EXPRESSION

All of our Tau protein constructs are expressed in BL21-CodonPlus(DE3)-RP *Escherichia coli* cells and purified using Q and SP Sepharose (Sigma-Aldrich, St. Louis, MO) affinity column chromatography as previously described (McVicker et al., 2011, 2014). Following purification, samples are dialyzed against BRB80, and protein concentrations are determined with the bicinchoninic acid (BCA) protein

assay (Pierce, Rockford, IL) using known Tau protein standards. The concentrations of our Tau protein standards are validated against known concentrations of bovine serum albumin (BSA) using SDS-PAGE.

Materials

BL21-CodonPlus(DE3)-RP *E. coli* cells (Stratagene, La Jolla, CA)
Q and SP Sepharose affinity columns (Sigma-Aldrich, St. Louis, MO)
BCA protein assay (Pierce, Rockford, IL)
BioRad MiniProtean Precast Gels (BioRad, Hercules, CA)

1.3 LABELING TAU PROTEIN

We image single Tau molecules fluorescently labeled with maleimide-conjugated dyes at single cysteine residues. 3R (3-repeat) Tau isoforms have one cysteine (C322) which makes this the best site for single site-specific labeling (McVicker et al., 2014; Stern et al., 2017). 4R Tau isoforms have two cysteines. We therefore typically mutate the other cysteine residue (C291I—isoleucine is the residue found at the analogous position in the other microtubule-binding repeats) to achieve single site-specific labeling at C322 (McVicker et al., 2014). Mutations in our Tau constructs are generated using the QuikChange II XL Site-Directed Mutagenesis Kit following the manufacturer's instructions.

1. Incubate purified Tau protein in 10-fold molar excess dithiothreitol (DTT) at room temperature for 2 h.
2. Equilibrate a 2-mL 7K MWCO Zeba spin desalting column with 1 × BRB80 at room temperature. One spin to remove storage buffer and three spins to equilibrate with 1 × BRB80. All spins: 2 min at 1000 × *g*.
3. Apply samples to equilibrated column and spin for 2 min at 1000 × *g* at room temperature to remove DTT.
4. Incubate samples with 10-fold molar excess of the appropriate wavelength Alexa Fluor probe with a Maleimide linkage for 2 h in the dark at room temperature.
5. Remove excess fluorophore using an equilibrated Zeba desalting column (as in step 2).
6. Determine the concentration of fluorophore conjugated to Tau using a standard spectrophotometer. Use the appropriate extinction coefficient (ϵ) for the fluorophore to calculate concentration (C) using Beer–Lambert's Law:

$$A = \epsilon l C$$

where A, absorbance at the appropriate wavelength for the extinction coefficient (ϵ) used and l, the path length of the cuvette. *Note*: Labeling in the presence of 10 × DTT can improve the labeling efficiency of some fluorophores.

7. Determine final concentration of Tau protein using the BCA assay and known Tau protein standards. A Modified Lowry Assay may also be used.

Note: Regardless of the protein assay used, it is important to use Tau protein standards of known concentration, which can be validated against known BSA concentrations using SDS-PAGE.

8. Determine the ratio of fluorophore to Tau labeling by dividing the fluorophore concentration determined in Section 1.3, step 6 by the Tau protein concentration determined in Section 1.3, step 7. We strive to have our Tau preparations labeled at 60%–90%.

Materials

1 × BRB80: 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9 at room temperature (constituents all from Sigma-Aldrich, St. Louis, MO)
Purified Tau protein
DTT (Sigma-Aldrich, St. Louis, MO)
2 mL 7K MWCO Zeba spin desalting columns (Peirce, Rockford, IL)
Alexa Flour probes with maleimide linkage—wavelength specific (Invitrogen Molecular Probes, Carlsbad, CA)
BCA assay (Pierce, Rockford, IL)
Modified Lowry Assay (Sigma-Aldrich, St. Louis, MO)
BioRad MiniProtean Precast Gels (BioRad, Hercules, CA)
QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA)
Beckman DU640 Spectrophotometer (Beckman, Pasadena, CA)

1.4 POLYMERIZING PACLITAXEL-STABILIZED MICROTUBULES

We use bovine brain tubulin purified following the protocol detailed by Castoldi and Popov (2003).

1. Clarify purified bovine brain tubulin by ultracentrifugation (20 min, 95,000 rpm, 4°C) in an OptimaTM TLX Ultracentrifuge using a TLA100 rotor.
2. Save the supernatant and discard the pellet. *Note*: The supernatant contains the clarified tubulin and the pellet contains unwanted debris.
3. Calculate postclarification tubulin concentration with the spectrophotometer using Beer–Lambert law (see Section 1.3, step 6) and a tubulin extinction coefficient of 115,000 $cm^{-1}M^{-1}$ at 280 nm.
4. Combine 30–40 μM of clarified bovine brain tubulin with 0.5% rhodamine-labeled tubulin (or other fluorophore if required) and 1 mM guanosine-5′-triphosphate (GTP).
5. Mix thoroughly and incubate at 37°C for 20 min.
6. Stabilize microtubules with 20 μM paclitaxel. *Note*: Paclitaxel should be initially dissolved at a concentration of 10 mM in DMSO and then diluted in buffer of choice such as 1 × BRB80.
7. Incubate paclitaxel-stabilized microtubules 15 min at 37°C.

Materials

1 × BRB80: 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9 at room temperature (constituents all from Sigma-Aldrich, St. Louis, MO)
Purified bovine brain tubulin
Labeled tubulin (e.g., porcine labeled with rhodamine) (Cytoskeleton Inc., Denver, CO)
Paclitaxel (Sigma-Aldrich, St. Louis, MO)
DMSO (Sigma-Aldrich, St. Louis, MO)
Optima TLX Ultracentrifuge with TLA100 rotor (Beckman Coulter Life Sciences, Indianapolis, IN)

1.5 CHAMBER PREPARATION FOR IMAGING

1. Incubate paclitaxel-stabilized microtubules with the appropriate concentration of labeled Tau for a 1:3000 (Tau:tubulin) molar ratio at 37°C, for 30 min prior to imaging.
2. Perform all chamber washes and incubations in BRB80+OS (oxygen scavengers—10 mM DTT, 0.1 mg/mL glucose oxidase, 0.15 mg/mL catalase, 3.0 mg/mL glucose).

Prepare BRB80+OS while Tau is incubating with microtubules. Keep BRB80+OS on ice.

3. Once BRB80+OS has been made, make a few 1 mL aliquots of 20 μM paclitaxel diluted with BRB80+OS (now called BRB80+OS/taxol). Warm these aliquots at 37°C. This is the buffer in which your final microtubule/Tau dilutions and washes are to be done.
4. Place the slide at an angle (~10–15 degrees) from the lab bench. This will allow solutions to flow through the chamber assisted by gravity. *Note*: Place a Q-tip at the lower end of the slide away from the end of the chamber to soak up the fluid that flows through the chamber.
5. Flow in monoclonal anti-β III (neuronal) tubulin antibodies diluted to 33 μg/mL with cold BRB80+OS and incubate for 5 min.
6. Block chamber with 2 × chamber volume wash of 2 mg/mL BSA (room temperature, dilute from stock with cold BRB80+OS) for 5 min.

   Alternatively chambers can be incubated for 5 min with a 2.5% Pluronic F-127 solution for further coating. Chambers can then be blocked with BSA (10 × chamber volume) for 5 min to remove excess Pluronic® F-127.
7. Dilute microtubule/Tau mixture to a final imaging concentration of 0.5–1 μM (tubulin) in warm BRB80+OS/taxol. Flow in one chamber volume of diluted microtubule/Tau mixture and incubate for 12 min.
8. Wash chambers with Tau (at the same 1:3000 imaging concentration as in the microtubule/Tau mixture, diluted with warm BRB80+OS/taxol) for 2–4 min to remove nonadherent microtubules.

Materials

Glucose (Sigma-Aldrich, St. Louis, MO)
Catalase (Sigma-Aldrich, St. Louis, MO) Prod. # C1345-1G
Glucose oxidase (Sigma-Aldrich, St. Louis, MO)
Paclitaxel (Sigma-Aldrich, St. Louis, MO)
Monoclonal anti-β III (neuronal) tubulin antibodies (Sigma-Aldrich, St. Louis, MO) Prod. # T857
Pluronic F-127 (Sigma-Aldrich, St. Louis, MO)
BSA (Sigma-Aldrich, St. Louis, MO)

1.6 IMAGING TAU DYNAMICS ON THE MICROTUBULE

For imaging we use an inverted Eclipse Ti TIRF Microscope (Nikon, Melville, NY) with a 100× PlanApo objective lens (1.49 NA) and auxiliary 1.5× magnification and two iXON Ultra EMCCD cameras (Andor, Belfast, N-IRL) running NIS Elements v4.51 (Nikon, Melville, NY).

1. Once the slide has been prepared for imaging, it can be mounted on the microscope stage). *Note*: There is the risk of the chamber drying out so imaging should take no more than half an hour. To prevent the slide from drying out, the chamber can be sealed at each end with immersion oil.
2. To find Tau-binding events that occur on the microtubule surface, one microtubule field must be imaged for every corresponding Tau field. To ensure that the Tau data is of use, the microtubule field should be chosen and imaged first to ensure that most of the microtubules are properly attached, the field is not crowded and the microtubules are *not* bundled. We generally collect 50–100 frames at 10 fps.
3. Once an acceptable microtubule field has been collected, the corresponding Tau field should be imaged. We collect 500–1000 frames at 10 fps.

1.7 ANALYZING TAU DYNAMICS

To quantify the dynamics of tau on the microtubule surface we generate kymographs using ImageJ (NIH, Bethesda, MD) for individual microtubules. Kymographs are analyzed using a custom MATLAB (Mathworks Inc., Natick, MA) script. The following analysis can also be manually performed using ImageJ. Briefly, the ImageJ MTrackJ plugin is used to track individual events (Fig. 2B), and the ImageJ MultipleKymograph plugin is used to generate kymographs (see Fig. 1) to visualize all the Tau-binding events that occur on a microtubule.

1.7.1 MTrackJ analysis

1. Tau fields can be broken up into four quadrants to make tracking easier.
2. The ImageJ MTrackJ plugin allows you to track an event from frame to frame. Using the MTrackJ plugin, track events that last more than two frames and have

a signal that remains within a snap range of 2.08 μm from one frame to the next (the "snap" feature of this plugin automatically moves the position of the cursor to a nearby image feature; the snap range here is chosen based on maximum diffusive range of a Tau protein). Save tracks as they are completed.

3. Load tracks onto the corresponding microtubule field. Delete any tracks that do not begin, remain, and end on the same microtubule.
4. When the tracks are loaded on the correct Tau movie, they can be measured using the MTrackJ plugin. Measuring the tracks will generate two windows, *Points* and *Tracks*. *Points* has information for each point in a track, and *Tracks* has information for the entire track. Export the Tracks information to a spreadsheet such as Microsoft Excel.
5. We use GraphPad Prism to calculate average dwell times. Dwell times can be found in the Duration column of the exported spreadsheet. To separate static dwell times from dynamic dwell times, a filter can be applied to the Max distance to start (M2DS) column for the data set (static: ≤ 0.5 μm and dynamic: > 0.5 μm) (Stern et al., 2017). The cumulative distributions are then fit to a one or two phase exponential function (depending on the goodness of the fit) (Stern et al., 2017). The average dwell time is the rate constant (τ). We report the average dwell time with the goodness of the fit (R^2) and 95%–99% confidence bands. Statistical significance is determined using Fisher's Exact Test.

1.7.2 MultipleKymograph

The MultipleKymograph plugin in ImageJ can be used to generate images representative of Tau's-binding behavior on the microtubule surface.

1. Use the segmented line tool in ImageJ to trace a microtubule in the microtubule field.
2. Copy the trace to the corresponding Tau field and use the MultipleKymograph plugin to generate a kymograph using a line width of 3, which includes a pixel on either side of the trace.

1.8 BRIGHTNESS ANALYSIS

This assay is used to determine the number of Tau molecules in a given binding event (McVicker et al., 2014). Images of the free fluorophore are collected and used to calculate the mean intensity of a single fluorophore relative to the corresponding background intensity. This method is especially helpful if the laser intensity is variable during imaging, since measurements for each event are internally controlled.

1. Dilute the fluorophore used to label Tau (e.g., Alexa 488) to 50 pM in BRB80 + OS.
2. Block the flow chamber with 2 mg/mL BSA as described earlier.
3. Flow in one chamber volume of the 50 pM fluorophore solution in BRB80 + OS and image using the same conditions that were used for imaging Tau (Section 1.6). Collect 20 frames at 10 fps.

4. Make kymographs of the free fluorophore using the ImageJ MultipleKymograph plugin (Section 1.7.2) and measure the intensity of the first five frames. This is done by drawing a line segment in ImageJ through the first five frames of the kymograph and measuring the intensity. The background intensity can be measured by drawing a line on the background of the kymograph beside the event but five pixels away from the event.
5. Export the intensity values for events and their corresponding backgrounds to a spreadsheet such as Microsoft Excel.
6. The corrected mean single fluorophore intensity is calculated by subtracting the mean background intensity for all the events from the mean fluorophore intensity for all events.
7. Perform analogous measurements on kymographs of Tau-binding events, measuring the intensity of each event and its corresponding background to obtain a corrected event intensity. To determine how many Tau molecules are present in an event, divide the corrected event intensity by the mean corrected single fluorophore intensity.

2 SINGLE-MOLECULE TAU-BINDING ASSAY

This assay was developed to determine the effect of isoform differences and mutations on Tau's affinity for the microtubule surface.

2.1 IMAGING

1. For this assay prepare slides with 10 chambers each. In this case we create chambers using $3 \times 1''$ Gold Seal Plain Microscope slides as the top coverslip and 24 mm $\times$ 60 mm No. 1 coated slides as the bottom coverslip (relative to the chamber position on our inverted TIRF setup).
2. Prepare paclitaxel-stabilized microtubules as described earlier with two changes:
   a. The microtubule labeling ratio should be low (1:400). This is sufficient to find the image plane in the first few movies, while reducing the risk of microtubule fluorescence bleed-through into the Tau channel.
   b. Do not incubate microtubules with Tau before imaging.
3. Prepare Tau dilutions for 10 concentrations from an initial stock, e.g., 50, 100, 150, 200, 250 nM, etc. (final concentrations).

   Note: Imaging concentrations may vary depending on the imaging system. You must determine the optimal concentrations for imaging on your system.
4. Prepare imaging chambers as you would for the Tau Dynamics Assay (Section 1.5). The final wash should be BRB80 + OS/taxol before flowing in the appropriate Tau dilution for imaging, one concentration for each chamber. *Note*: Microtubules should have an imaging concentration of 1–1.5 μM to ensure that there are at least three microtubules in each field.
5. Use the microtubule field to find your imaging plane. For the first two Tau concentrations you may need to collect microtubule images for use in analysis.

Note: It is important to keep within the center of the slide while imaging so that your fields are similar.

6. We collect 20–50 frames of Tau at 10 fps for each concentration. This limits potential photobleaching events.
7. Laser power and camera gain must be consistent for all assays.

2.2 ANALYSIS

For imaging analysis, you will need the MultiMeasure (MM) plugin for ImageJ. This plugin behaves like the Measure feature of ImageJ but you can measure the intensity of all the frames in a movie.

1. Open the 50 nM Tau movie and make an Average Intensity *Z* projection. You will use this image to find tracks and save this image so that you know which microtubules you used.
2. Microtubules must be chosen from the center of the field where the laser intensity is uniform. Ensure that chosen microtubules have not moved during imaging as this will affect intensity values. Using the segmented line tool in ImageJ, trace your chosen microtubule (length does not matter) on the *Z*-projected image.
3. Move your selection to the Tau movie and then add the selection to MM.
4. Within MM, select measure. Measure all frames. A separate window will open with intensity measurements, for each microtubule. The column you need is the Mean column, which is the average intensity measured along the microtubule in each frame. You can save the track on the *Z*-projected image should you need to reanalyze the data.

   Note: You can use any number of microtubules from each movie as long as it is consistent for all the movies in the assay. We do not use less than three.
5. Repeat steps 2–4 for the number of microtubules you have chosen.
6. Copy and paste the measurements you have generated to into a spreadsheet such as Microsoft Excel. In the spreadsheet, determine the overall average intensity from the sum of the average intensity (which in ImageJ is the Mean column) values for all individual microtubules at each Tau concentration.
7. Repeat steps 1–6 for all Tau concentrations.
8. Export average intensity values, which are normalized in ImageJ for the length of the trace (i.e., microtubule), to software capable of graphing and fitting data such as GraphPad Prism. Plot [Tau] vs the Avg I/Unit Length, where Avg I/Unit length is the average intensity value exported from ImageJ.
9. Fit data to one site-specific-binding equation:

$$\frac{\text{AvgI}}{\text{Unit Length}} = \frac{A_{\max}[\text{Tau}]^h}{K_D{}^h + [\text{Tau}]^h}$$

This will give you a value for K_D. You can normalize to each data set to its A_{max}, the maximum fit value of Avg I/Unit Length. *Note*: For a further test of cooperativity, data can be fit to a sigmoidal curve.

Materials

24 mm × 60 mm No. 1 coverslips (ThermoFisher, Waltham, MA)
3 × 1″ Gold Seal Plain Microscope slides (ThermoFisher, Waltham, MA)

3 DYNAMIC MICROTUBULE IMAGING ASSAY

3.1 PREPARATION OF GMPCPP-STABILIZED MICROTUBULE SEEDS

We make 1 μL aliquots of 20 μM guanosine-5′-[(α,β)-methyleno]triphosphate (GMPCPP)-stabilized microtubule seeds and store them at −80°C. The protocol we follow is a modified version of Gell et al. (2010). Briefly, two cycles of microtubule polymerization and one cycle of microtubule depolymerization is used to generate GMPCPP-stabilized microtubule seeds.

1. Dilute unlabeled bovine tubulin (the method for purifying unlabeled tubulin is detailed in Castoldi & Popov, 2003) to 20 μM by adding 1 × BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9 at room temperature).
2. Add GMPCPP to a final concentration of 1 mM.
3. Add rhodamine (or a different fluorophore depending on your microscopy needs) labeled bovine tubulin at a 1:10 (rhodamine-labeled tubulin:unlabeled tubulin) ratio.
4. Place the reaction mixture from step 3 on ice for 5 min.
5. Transfer the reaction mixture to a 37-C water bath and incubate for 30 min.
6. Centrifuge the reaction mixture at 80,000 rpm in an Optima TLX Ultracentrifuge with TLA100 rotor equilibrated at 37°C for 5 min.
7. Remove the supernatant and discard it.
8. Resuspend the pellet in 1 × BRB80. The volume of 1 × BRB80 should be 0.8 times the volume of 1 × BRB80 used in step 1. In our experience ∼80% of the tubulin is recovered after the centrifugation step; therefore, 0.8 times the volume of 1 × BRB80 used in step 1 is added to keep the concentration of tubulin at ∼20 μM.
9. Put the resuspended pellet on ice for 20 min.
10. Add GMPCPP to a final concentration of 1 mM.
11. Repeat steps 4–8.
12. Resuspend the pellet in warm (room temperature) 1 × BRB80. Assuming 80% tubulin recovery add 0.8 times the volume of 1 × BRB80 used in step 8. This is the final GMPCPP-stabilized microtubule seed solution as a concentration of ∼20 μM.
13. Make 1 μL aliquots of the GMPCPP-stabilized microtubule seed solution and store at −80°C.

3.2 POLYMERIZING DYNAMIC MICROTUBULES

1. Dilute antitubulin antibody by putting 12 volumes of 1 × BRB80 in 1 volume of antitubulin antibody.

2. Flow 20 μL of diluted antitubulin antibody into the flow cell using a P200 pipette tip. *Note*: Use a cotton Q-tip on the opposite end of the chamber to help the liquid flow through.
3. Let the antitubulin antibody sit in the chamber for 5 min at room temperature.
4. Flow in 100 μL of 1 × BRB80 to flush out unbound antitubulin antibody.
5. Flow 50 μL of 1% Pluronic F-127 into the flow cell and let it sit for 5 min. *Note*: We use 1% Pluronic F-127 because in our experience it helps to prevent nonspecific protein binding to the coverslip.
6. Flow in 100 μL of 1 × BRB80 to flush out unbound 1% Pluronic F-127.
7. Dilute 1 aliquot of the GMPCPP-stabilized microtubule seeds prepared in Section 3.1, step 13 in the following manner:
   - Add 500 μL of warm 1 × BRB80 in 1 aliquot and slowly pipette up and down to mix the stabilized seeds and BRB80. This is solution A.
   - Add 100 μL of solution A to 600 μL of warm 1 × BRB80 and slowly pipette up and down to mix the stabilized seeds and BRB80. This is solution B.
   - Add 40 μL of solution B to 160 μL of warm 1 × BRB80 and slowly pipette up and down to mix the stabilized seeds and BRB80. This is solution C.
   - Solution C will be flowed into the flow cell. The final concentration of GMPCPP-stabilized seeds in solution C is ∼1 nM. This is an optimized concentration to give the maximum number of seeds per image field while minimizing microtubules crossing over each other.
8. Flow 50 μL of solution C from step 7 into the flow cell and let it sit for 15 min.
9. Flow in 100 μL of 1 × BRB80 to flush out unbound GMPCPP microtubule seeds.
10. Prepare the dynamic microtubule solution with the following constituents:
    - 4.5 μM unlabeled bovine tubulin.
    - 0.5 μM HiLyte 488-labeled tubulin (or a different fluorophore depending on your microscopy needs).
    - 1% methyl cellulose.
    - 1 × BRB80.
    - 0.06 M DTT.
    - 0.5 mg/mL BSA.
    - 40 mM glucose.
    - 64 nM catalase.
    - 25 nM glucose oxidase.
    - 1 mM GTP.
    - Tau or other MAPs that affect microtubule dynamics, depending on the needs of a specific experiment, can be added to the reaction mixture just before it is flowed into the imaging chamber.
11. Flow 50 μL of the dynamic reaction solution into the flow cell. *Note*: Our dynamic reaction solution has a tubulin concentration of 5 μM, which we find optimal to promote microtubule assembly but minimizes microtubules crossing over each other. Based on the requirements of the experiment the tubulin concentration in the dynamic reaction mixture can be varied.

12. Use a drop of immersion oil to seal the two ends of the flow chamber to prevent evaporation of dynamic reaction solution. The flow chamber is now ready to be imaged.

3.3 IMAGING DYNAMIC MICROTUBULES

We use Total Internal Reflection (TIRF) Microscopy to image dynamic microtubules. For imaging we use an inverted Eclipse Ti Microscope (Nikon, Melville, NY) with a 100× PlanApo objective lens (1.49 NA) and auxiliary 1.5× magnification and two iXON Ultra EMCCD cameras (Andor, Belfast, Northern Ireland) running NIS Elements v4.51 (Nikon, Melville, NY). Images are taken at a rate of 0.5 frames/s for 10 min and an exposure time of 300 ms. Imaging takes place at 37°C. One flow chamber (Fig. 3) preparation is imaged for no more than 40 min. Three different fields are imaged per flow chamber. Imaging starts 10 min after the flow chamber is sealed to let the polymerization reaction reach an equilibrium.

3.4 ANALYZING DYNAMIC MICROTUBULES

To quantify the dynamics of microtubules we generate kymographs using ImageJ (NIH, Bethesda, MD) for individual microtubules. Kymographs of dynamic microtubules (see Fig. 4) are analyzed using a custom MATLAB (Mathworks Inc., Natick, MA) script. The following analysis can also be manually performed using ImageJ. Briefly, for every microtubule the total length grown, the total length shortened, the total time spent in the growth phase, the total time spent in the shortening phase, the number of transitions toward the shortening phase (catastrophes), and the number of transitions from the shortening phase (rescues) are calculated and recorded. The value of average growth velocity is calculated for each microtubule by dividing the total length grown by the total time spent in the growth phase. Similarly, average shortening velocity for a microtubule is calculated by dividing the total length shortened by the total time spent in the shortening phase. The total number of catastrophes (for all the microtubules) is divided by the total time that all the microtubules spend in the growth phase to calculate the catastrophe frequency. Similarly, the

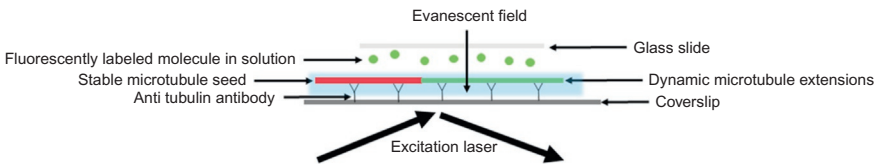

FIG. 3

Schematic of flow cell chamber mounted on the inverted TIRF microscope stage. Fluorescently labeled molecules in solution can be Tau, molecular motors such as kinesin, or free tubulin dimers, depending on the assay (i.e., Tau dynamics, motor protein motility, or dynamic microtubule assays, respectively).

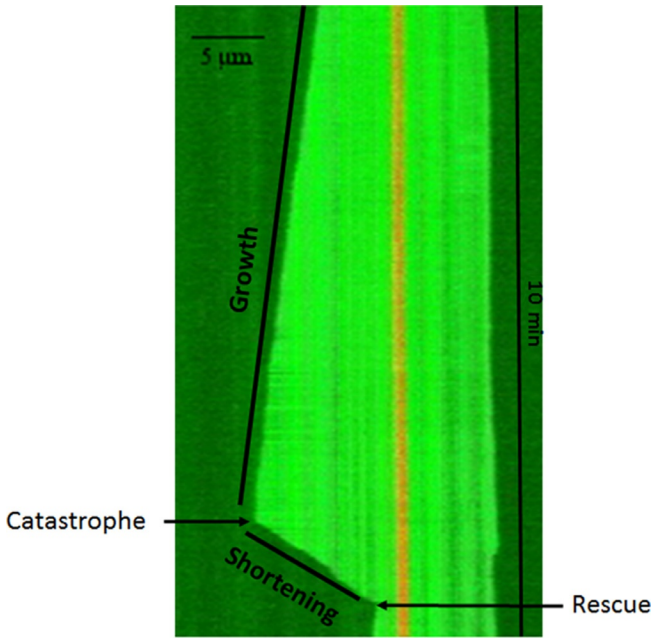

FIG. 4

Kymograph of a fluorescently labeled (*green*) dynamic microtubule undergoing growth and shortening behaviors, as well as rescue and catastrophe events. The *red band* denotes the stable GMPCPP microtubule seed.

number of rescues is divided by the total time spent in the shortening phase to calculate the rescue frequency. These parameters completely define the dynamics of the microtubule.

Materials

Monoclonal anti-β III (neuronal) tubulin antibodies (Sigma-Aldrich, St. Louis, MO) Prod. # T857
Purified bovine brain tubulin
TRITC Rhodamine-labeled (Porcine) tubulin (Cytoskeleton Inc., Denver, CO)
HiLyte Fluor 488-labeled (Porcine) tubulin, (Cytoskeleton Inc., Denver, CO)
Guanosine-5′-[(α,β)-methyleno]triphosphate (GMPCPP) (Jena Bioscience, Jena, Germany)
GTP, (Sigma-Aldrich, St. Louis, MO)
Catalase (Sigma-Aldrich, St. Louis, MO) Prod. # C1345-1G
Glucose (Sigma-Aldrich, St. Louis, MO)
Glucose oxidase (Sigma-Aldrich, St. Louis, MO)
Pluronic F-127 (Sigma-Aldrich, St. Louis, MO)
DTT(Sigma-Aldrich, St. Louis, MO)

BSA (Sigma-Aldrich, St. Louis, MO)
Ultracentrifuge, Beckman Optima TLX with TLA100 rotor (Beckman Coulter Life Sciences, Indianapolis, IN)
Water Bath (VWR Scientific, Radnor, PA)
TIRF microscope
ImageJ software (NIH, Bethesda, MD)
MATLAB software (Mathworks Inc., Natick, MA)

4 MOLECULAR MOTOR MOTILITY ASSAYS

4.1 MOTOR EXPRESSION

Molecular motors can be expressed in a variety of systems, each with their own benefits and disadvantages. To obtain high concentration, purified motor preps, bacterial, or baculovirus/Sf9 expression systems may be utilized. Bacterial protein preps offer a comparatively low-cost motor expression option (Hoeprich et al., 2017). However, protein products may become insoluble, aggregated, or incorrectly folded. In contrast, a baculovirus/Sf9 expression system is able to synthesize proteins of unlimited size with a much higher success rate of protein solubility and functionality (Sladewski, Krementsova, & Trybus, 2016). Certain mammalian variants of motor proteins are extremely vulnerable to active site inactivation and may benefit from a mammalian cell line expression system. While a cell lysate prep sacrifices yield, detergent-based cell lysis is much gentler on synthesized motors than ultrasonic or mechanical cell lysis, and gives the added benefit of motors being synthesized under physiological conditions (Soppina & Verhey, 2014).

4.2 MICROTUBULE POLYMERIZATION AND FLOW CHAMBER PREPARATION

1. Paclitaxel-stabilized microtubules are prepared as described in Section 1.4 except motility assay buffer (MAB) is used in place of 1 × BRB80 in all steps. MAB consists of 10 mM PIPES, 50 mM potassium acetate, 4 mM magnesium acetate, 1 mM EGTA, pH 7.4 at room temperature.
2. Labeled tubulin for visualizing microtubules should use a different color fluorophore than that of the molecular motor being visualized. For example, if the molecular motor being assessed is a GFP-tagged kinesin construct, rhodamine-tubulin should be used to visualize the microtubules.
3. Flow chambers are prepared as described in Section 1.5 except;
   a. MAB is used in place of 1 × BRB80 in all steps and
   b. Dilute motors (50–100 pM final concentration) in MAB + 20 μM paclitaxel + 1 mM ATP (adenosine triphosphate) are added to the flow chamber prior to sealing the ends of the flow chamber with immersion oil.

4.3 SINGLE-MOLECULE MOTILITY ASSAY ANALYSIS

Imaging kinesin motility using TIRF microscopy is performed in an identical manner to that of Tau dynamics described in Section 1.6.

4.4 ANALYSIS OF MOLECULAR MOTOR MOTILITY

The processive run length (distance moved along a microtubule for an isolated motility event) and velocity of individual kinesin motor proteins along a microtubule are determined using the MTrackJ plugin for ImageJ (NIH, Bethesda, MD) (Figs. 2 and 5) as follows:

1. Import image stack for a single movie into ImageJ.
2. Adjust brightness/contrast, if necessary.
3. Measure microtubule lengths using ImageJ. We calculate the average intensity of the *Z*-projection for each stack of the microtubule field and then measure each microtubule length from this image in ImageJ. This *Z*-projection image is also used with the corresponding MTrackJ kinesin tracks to make sure that the kinesins are not running into other motors, off of the ends of microtubules, or into microtubule intersections.
4. Load movies of motor motility and disregard any events are not on microtubules, which run off the end of a microtubule, or run into other motors or microtubule intersections.
5. Compute processive run lengths and velocities for motility of selected events using the MTrackJ plugin.

Motility assay data analysis should be conducted in a careful manner to account for all of the intricacies of motor behavior. For example, single-molecule kinesin motility experiments show that run lengths of kinesin motors are exponentially distributed. Without a Gaussian distribution, this makes standard deviation of the mean

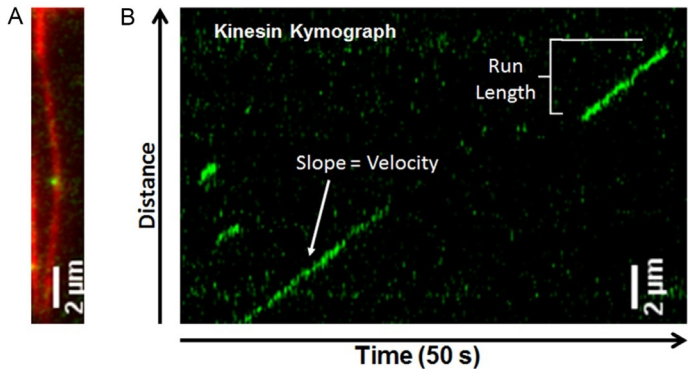

FIG. 5

Kymograph (B) of GFP-kinesin-1 motility on a rhodamine-labeled microtubule (A), from which the processive run length and velocity of an individual motor protein can be measured.

a poor tool to assess the statistical significance between populations. In other data sets, a standard error of fit could address this issue; however, this fit can overemphasize the differences between populations. Nonparametric statistical alternatives are commonly used in to address nonnormally distributed data sets; however, these tests account for limited assumptions. This presents a problem when one also considers that a molecular motor's processive run length is additionally inherently limited by the length of the track itself, depending on landing position. Additionally, the sample size needed to increase the power of data sets is often difficult to collect, specifically in in vivo motility experiments.

We have developed a bootstrapping resampling technique as a powerful tool to account for many limitations of single-molecule motility data analysis. In this technique, implemented in MATLAB (Mathworks Inc., Natick, MA), the data set is resampled with replacement to yield a characteristic run length. This resampling technique is then repeated with high repetition (e.g., 10,000 times) to yield a histogram of the measured characteristic run lengths. To determine the statistical relationship between two resampled populations, a Monte Carlo permutation resampling scheme can be utilized (Thompson, Hoeprich, & Berger, 2013).

Materials

Purified expressed molecular motors (fluorescent)
Purified bovine brain tubulin
Purified expressed Tau
MAB—10 mM PIPES, 50 mM potassium acetate, 4 mM magnesium acetate, 1 mM EGTA, pH 7.4 at room temperature (all constituents from Sigma-Aldrich, St. Louis, MO)
ATP (Sigma-Aldrich, St. Louis, MO)
Paclitaxel (Sigma-Aldrich, St. Louis, MO)
TIRF microscope
ImageJ software (NIH, Bethesda, MD)
MATLAB software (Mathworks Inc., Natick, MA)

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