11-9-2012

Flexural Rigidity Measurements of Biopolymers Using Gliding Assays

Douglas S. Martin
Lawrence University

Lu Yu
Lawrence University

Brian L. Van Hoozen Jr.
Lawrence University, bvanhooz@nova.edu

Video Article

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Douglas S. Martin, Lu Yu, Brian L. Van Hoozen
Department of Physics, Lawrence University

Correspondence to: Douglas S. Martin at douglas.s.martin@lawrence.edu

URL: http://www.jove.com/video/50117
DOI: doi:10.3791/50117

Keywords: Biophysics, Issue 69, Bioengineering, Physics, Molecular Biology, Cellular Biology, microtubule, persistence length, flexural rigidity, gliding assay, mechanics, cytoskeleton, actin

Date Published: 11/9/2012

Citation: Martin, D.S., Yu, L., Van Hoozen, B.L. Flexural Rigidity Measurements of Biopolymers Using Gliding Assays. *J. Vis. Exp.* (69), e50117, doi:10.3791/50117 (2012).

Abstract

Microtubules are cytoskeletal polymers which play a role in cell division, cell mechanics, and intracellular transport. Each of these functions requires microtubules that are stiff and straight enough to span a significant fraction of the cell diameter. As a result, the microtubule persistence length, a measure of stiffness, has been actively studied for the past two decades[1]. Nonetheless, open questions remain: short microtubules are 10-50 times less stiff than long microtubules[2-4], and even long microtubules have measured persistence lengths which vary by an order of magnitude[5-9].

Here, we present a method to measure microtubule persistence length. The method is based on a kinesin-driven microtubule gliding assay[10]. By combining sparse fluorescent labeling of individual microtubules with single particle tracking of individual fluorophores attached to the microtubule, the gliding trajectories of single microtubules are tracked with nanometer-level precision. The persistence length of the trajectories is the same as the persistence length of the microtubule under the conditions used[11]. An automated tracking routine is used to create microtubule trajectories from fluorophores attached to individual microtubules, and the persistence length of this trajectory is calculated using routines written in IDL.

This technique is rapidly implementable, and capable of measuring the persistence length of 100 microtubules in one day of experimentation. The method can be extended to measure persistence length under a variety of conditions, including persistence length as a function of length along microtubules. Moreover, the analysis routines used can be extended to myosin-based acting gliding assays, to measure the persistence length of actin filaments as well.

Video Link

The video component of this article can be found at http://www.jove.com/video/50117/

Introduction

The cytoskeleton, a network of biopolymers found in most eukaryotic cells, plays a role in cellular organization, intracellular transport, and cell mechanics. The mechanical characteristics of the biopolymers of the cytoskeleton (primarily actin and microtubules) play a significant role in determining the mechanical characteristics of the cell as a whole[12]. Since whole cell mechanics can characterize healthy and diseased cells[13,14] and is involved in cellular motility[15], the mechanical properties of the underlying cytoskeletal components have been an active area of study for the past two decades[1].

The flexibility (or stiffness) of biopolymers is characterized by the persistence length, the length of polymer which bends by approximately one radian under thermal fluctuations at ambient temperature. A number of techniques have been developed to measure persistence length[16], for example active techniques which involve bending the polymer using hydrodynamic flow, optical traps, or electric fields[4,17,18] , and passive techniques which measure the fluctuations of free polymers in solution[5,6] . The active measurements, however, require specialized setups to implement known forces on the micrometer scale, and the free-fluctuation measurements can be challenging due to diffusion out of the plane of focus of the microscope used.

In this article, we describe a complementary, passive, technique to measure the persistence length of microtubules, a cytoskeletal polymer. The technique involves gliding assays, which ensure that the polymer always remains in the focal plane[19]. Moreover, it involves tracking single fluorophores attached permanently to the polymer of interest, so that specific locations along the polymer are well characterized.

A cartoon of the method is shown in **Figure 1**. Kinesin moves specifically toward the + end of microtubules, so the microtubules in a gliding assay are propelled unidirectionally. The leading end of the microtubule, beyond the last kinesin attached, is free to fluctuate under the thermal forces of the surrounding solution. As the microtubule is propelled forward, the end fluctuates until binding to a new kinesin molecule further along the glass slide freezes in a given fluctuation. Because kinesin attaches microtubules very strongly, the microtubule is constrained to follow

the path of the leading end. Therefore, the statistical fluctuations frozen into the microtubule trajectory are the same as the statistical fluctuations of the free end of microtubules[11], and can therefore be used to calculate the persistence length according to[20]

$$\langle \cos\theta_s \rangle = e^{-s/l_p}$$

where l_p is the persistence length of the microtubule, θ_s is the angle between tangents to the trajectory separated by a contour length s, and <> denotes an average over all pairs of positions separated by a contour length s.

The gliding assay itself uses kinesin biotinylated at the coiled-coil[21] specifically bound to the glass slide via a streptavidin-biotin linkage. This attachment ensures that the motor domains are free to bind to and propel microtubules. In order to follow microtubule trajectories, microtubules are sparsely labeled with organic fluorophores[22,23] - the labels must be sparse enough that single fluorophores are resolvable using single molecule fluorescence microscopy. Single fluorophores are tracked using image analysis routines written in IDL. The trajectories of each fluorophore bound to a given microtubule are combined into a composite microtubule trajectory automatically[24]. The tangent angles θ to each point along a trajectory are calculated; from these tangent angles the $<cos\theta_s>$ value is calculated for each contour length s. Finally, these data are fit to Eq. 1 in order to extract a persistence length for a given microtubule, or for many microtubules in the same gliding assay.

The method is robust enough to work with microtubules prepared in a wide variety of conditions (with different stabilizing agents or other small molecules bound to the microtubule, with bound microtubule associated proteins (MAPs), or with a variety of viscous solutions). In our lab, the technique has been used to characterize the persistence length of microtubules as a function of length along the microtubules and microtubules with different stabilizing agents. The main restriction is that the microtubules must still support kinesin motility. Since kinesin is a robust motor enzyme, this is a fairly loose restriction. By replacing microtubules with actin and kinesin with a myosin family enzyme, the persistence length of actin can be measured using the same technique.

Protocol

1. Microtubule Gliding Assay Stock Solutions

Prepare ahead of gliding assay.

1. Polymerize 0.5 mg microtubules sparsely labeled with bright organic fluorophore [22]. The target label concentration is 1 fluorophore per micrometer of microtubule, or a labeling density of approximately 1 fluorophore per 1,500 tubulin dimers. Store at room temperature, light protected with aluminum, foil for up to two weeks.
2. Purify biotin-kinesin[21] at approximately 1 µM. Store at -80 °C in 5 µl aliquots for use in individual experiments.
3. Prepare the assay buffer (AB) of 50 mM imidiazole, 50 mM potassium chloride (KCl), 4 mM magnesium chloride ($MgCl_2$), 2 mM ethylene glycol tetraacetic acid (EGTA), pH 6.7. Sterile filter and store at 4 °C.
4. Dissolve biotinylated bovine serum albumin (biotin-BSA) to 2 mg/ml in AB. Filter with 0.2 µm syringe filter. Store at -80 °C in 100 µl aliquots for long periods, at 4 °C for up to one month.
5. Dissolve streptavidin (SA) to 10 mg/ml in AB. Filter with 0.2 µm syringe filter. Store at -80 °C in 20 µl aliquots for long periods, at 4 °C for up to two weeks.
6. Dissolve α-casein to 5 mg/ml in AB. Filter with 0.2 µm syringe filter. Store at -80 °C in 100 µl aliquots for long periods, at 4 °C for up to two weeks.
7. Dissolve dithiothreitol (DTT) in deionized water at 200 mM. Store at -20 °C in 100 µl aliquots, use within 8 hr of thawing. Can be refrozen.
8. Dissolve paclitaxel (PT) in HPLC-grade dimethyl sulfoxide (DMSO) at 4 mM. Store in 10 µl aliquots at -80 °C long term, -20 °C short-term. Use within 8 hours of thawing. Can be refrozen.
9. Dissolve glucose in deionized water at 120 mg/ml. Store in 100 µl aliquots at -20 °C. Can be refrozen.
10. Dissolve adenosine triphosphate (ATP) in deionized water at 150 mM, pH 7.0. Store in 5 µl aliquots at -80 °C, use within 8 hr of thawing.
11. Prepare 100x oxygen scavenging stock[25] by dissolving 10,000 units glucose oxidase and 156,000 units catalase into 600 µl total assay buffer. Centrifuge briefly in a microcentrifuge to pellet solids; filter supernatant with 0.2 µm syringe filter. Store at -80 °C in 10 µl aliquots for long periods, at 4 °C for up to one week.

2. Microtubule Gliding Assay, Same Day Solution Preparation

Prepare the solutions in 2.1-2.6 on the day of the experiment. With practice, solutions 2.2-2.6 can be prepared during flow-cell washing. Unless otherwise noted, keep stock solutions on ice.

1. Prepare AB, 1 ml of assay buffer with 10 µl of DTT stock (AB with 2 mM DTT). Keep at room temperature.
2. Prepare bio-BSA buffer, 40 µl of a 1:1 mixture of the biotin-BSA stock and AB. Keep at room temperature.
3. Prepare BSA buffer, mixing 645 µl of AB with 5.5 µl BSA (1 mg/ml BSA in AB). Keep at room temperature.
4. Prepare SA buffer, mixing 57 µl of AB with 3 µl streptavidin stock (0.5 mg/ml streptavidin in AB). Keep at room temperature.
5. Prepare α-casein buffer, mixing 200 µl of BSA buffer, 50 µl casein stock, and 0.8 µl of 15 µM ATP (1 mg/ml α-casein, 0.8 mg/ml BSA, 50 nM ATP in AB). Keep at room temperature.
6. Prepare fluorescence anti-bleach buffer, mixing 95 µl α-casein buffer, 2.5 µl glucose stock, 1 µl 100x oxygen scavenging mix, 1 µl paclitaxel stock, 1 µl 2-mercaptoethanol (βME). Add βME under fume hood. Use fluorescence anti-bleach buffer within 1 hr of preparation. *CAUTION*:

βME is highly toxic and smells awful. Be sure to only open under the fume hood. Store bottle in the absence of light; light degrades βME and its ability to reduce photobleaching.

3. Microtubule Gliding Assay

1. Construct about four flow lanes between a 24 x 60 mm coverslip and 22 x 22 mm coverslip using vacuum grease, extruded from a syringe through a pipette tip, as a spacer. Each flow lane should be approximately 10 µl in volume (scale subsequent volumes accordingly).
2. Wash 10 µl of bio-BSA buffer into each lane. Incubate 5 min to allow BSA to coat the glass surfaces.
3. Wash each lane three times with 15 µl BSA buffer to remove free biotin-BSA, while continuing to block the slide surface. Use a Kimwipe or filter paper to remove buffer from opposite side of flow chamber, *being sure not to allow air bubbles to go through the flow chamber*.
4. Wash 15 µl SA-buffer into each lane. Wait 10 min, or up to 2 hr. The streptavidin will bind to the surface-bound biotin-BSA.
5. Wash each lane three times with 15 µl BSA buffer to remove free SA while continuing to block the slide surface.
6. Wash each lane with 15 µl α-casein buffer. α-casein helps further block non-specific binding of kinesin and microtubules to the glass.
7. Dilute kinesin to 10 nM in α-casein buffer and wash each lane with 15 µl of this kinesin - α-casein solution. Wait 15 min (or up to an hour) for the biotinylated kinesin to bind specifically to the surface-bound streptavidin. The kinesin motor domains will remain free to bind microtubules.
8. Dilute paclitaxel to 40 µM in *room-temperature* α-casein buffer, and wash each lane with 15 µl of this solution to wash out free kinesin and to pre-load each flow chamber with a paclitaxel solution to prevent microtubules from depolymerizing. Cold also depolymerizes microtubules, so make sure these steps occur at room temperature with room temperature solutions.
9. Dilute fluorescently labeled microtubules 1:100-1:1,000 in fluorescence anti-bleach buffer with 1 mM ATP. Wash 15 µl into one lane and observe within 30 min.

4. Data Collection

1. Observe the microtubules gliding using fluorescence microscopy; a microscope capable of resolving single fluorophores such as a commercial or home-build TIRF setup is required[26] (**Figure 2**).

   The microtubules should be propelled by the kinesin motors over the substrate at approximately 0.5 µm/s, depending on temperature. If the field-of-view of the microscope is 50 µm, individual microtubules should be visible for approximately 100 sec. Set illumination intensity so that single fluorophores do not photobleach more quickly than 100 sec. If using laser excitation, a power setting of approximately 3-5 mW is appropriate.
2. Collect sequences of images for analysis. 600 images at 5 Hz (2 min total) works well. Use sequences long enough that microtubules traverse the entire field-of-view.

5. Data Analysis

An IDL routine, get_lp.pro, is attached. This routine returns a persistence length value based on all microtubules gliding in a given image sequence. Either run this routine on each image sequence, modifying intensity parameters depending on your particular microscope setup, or do the following:

1. Track each fluorophore attached to a microtubule to create fluorophore trajectories.
2. Combine all the trajectories of fluorophores on a given microtubule into an overall microtubule trajectory; repeat for each microtubule in the image sequence (**Figure 3**).
3. Calculate the tangent angle to each point along the trajectory (**Figure 4**), and calculate $\langle\cos\theta_s\rangle$ in Eq. 1 by averaging the cosine of the angle difference for every pair of points separated by a path-length s (**Figure 5**). Find the persistence length for a single microtubule or group of microtubules by fitting $\langle\cos\theta_s\rangle$ to Eq. 1, weighting the individual data points in the fit by the number of independent values of $\cos\theta_s$ that are used to compute the average.

Representative Results

A snapshot from a gliding assay is shown in **Figure 2**. A good microtubule density is 1-10 microtubules per field of view; substantially more will result in mistracking as microtubules cross each other. A plot of the 11 microtubule trajectories from the gliding assay in **Figure 2** is shown in **Figure 3**. Typical trajectories are 10 to 30 µm long; some trajectories have gaps where one microtubule crosses another. These trajectories may be discarded from analysis.

A single, long microtubule trajectory is shown in **Figure 4A** with example tangent angles at two positions along the trajectory. The difference between tangent angles separated by a fixed distance is related to the persistence length; the tangent angle as a function of position along the microtubule trajectory is shown in **Figure 4B**.

The tangent angle data from many microtubule trajectories is combined to calculate a single persistence length for all microtubules in a given experiment. **Figure 5** shows a plot of $\langle\cos\theta_s\rangle$ versus contour length s for the gliding assay of **Figure 2**. At large contour length values, very few values of $\cos\theta$ are measured; hence the average is highly variable. The weighted fit conforms to the short s, high-precision data more closely than the long s, low-precision data.

The value of persistence length from these 11 trajectories, 500 ± 40 µm (± standard error of the mean), is representative of persistence lengths for relatively short microtubules[2]. Similar experiments give a range of persistence lengths between 300 and 1,000 µm.

Troubleshooting

If microtubules are completely absent, increase the concentration of microtubules in step 3.9 to 0.5 mg/ml. If microtubules are still missing, re-polymerize microtubules. Make sure paclitaxel is present in each solution microtubules are diluted into, otherwise microtubules will depolymerize.

If microtubules are freely diffusing in solution but not binding to surface, increase the kinesin concentration in step 3.7 and use AMP-PNP instead of ATP to ensure kinesin binds microtubules irreversibly. If microtubules still do not bind, make new streptavidin stock and buffer, followed by new biotin-BSA buffer. Finally, purify new biotinylated kinesin.

If microtubules bind but do not move, replace ATP stock solution with fresh ATP.

If fluorophores photobleach too quickly, reduce illumination intensity. If fluorophores still photobleach too quickly, replace oxygen scavenging system with fresh stock.

If fluorophores are too dim, increase illumination intensity.

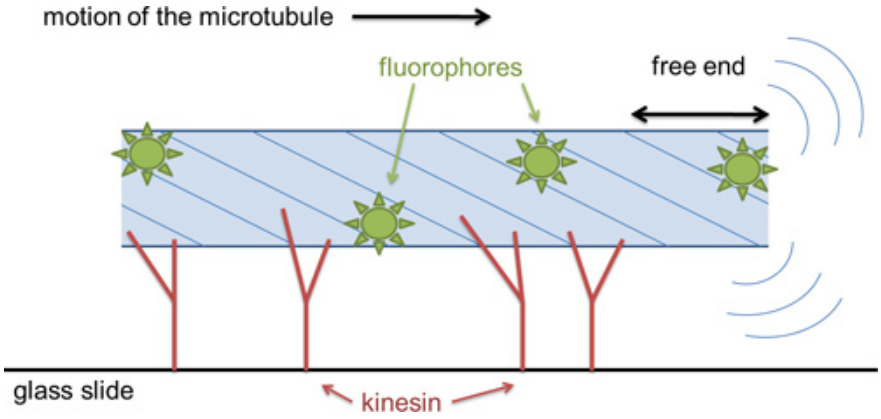

Figure 1. Cartoon of the microtubule gliding assay. Kinesin enzymes are specifically bound to a coverslip by a biotin-streptavidin linkage. Microtubules are sparsely labeled with organic fluorophores. Upon addition of ATP, microtubules are pushed by the kinesin motors. The leading free end of the microtubule fluctuates due to thermal forces in solution; these fluctuations are used to calculate the microtubule persistence length. The length-scale of the microtubule probed by these experiments is the length of the free end.

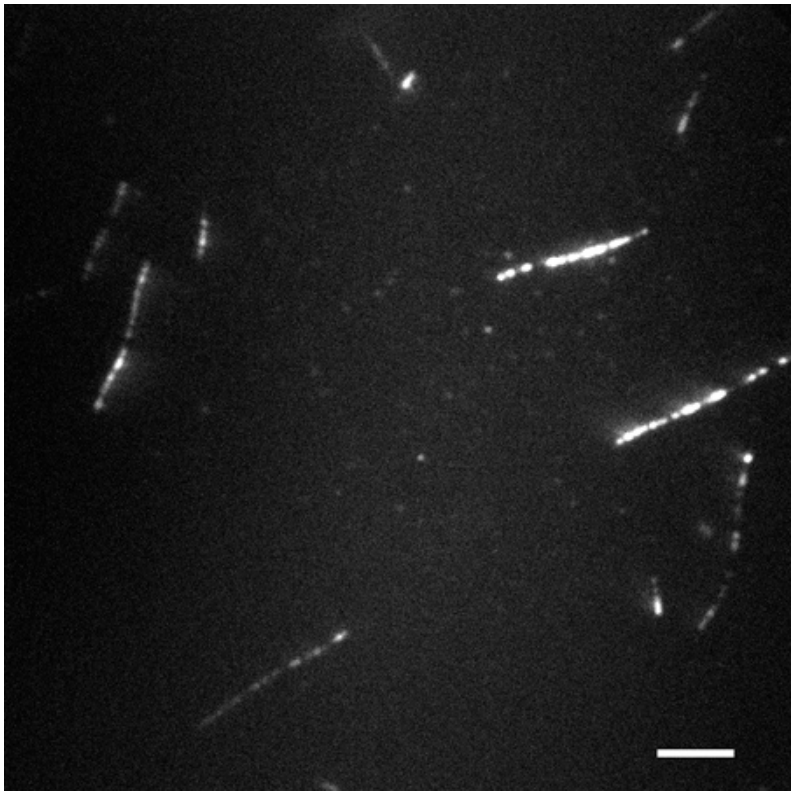

Figure 2. Typical microscope snapshot of a gliding assay, taken via TIRF microscopy. Microtubules are sparsely decorated with single fluorophores. Scale bar is 5 μm.

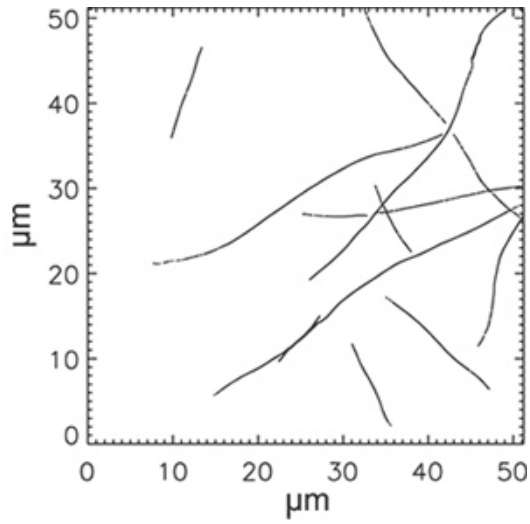

Figure 3. Microtubule trajectories from the image sequence shown in **Figure 2**. Each microtubule trajectory combines many single fluorophore trajectories (about 10 on average), and have been thinned to one point per 100 nm.

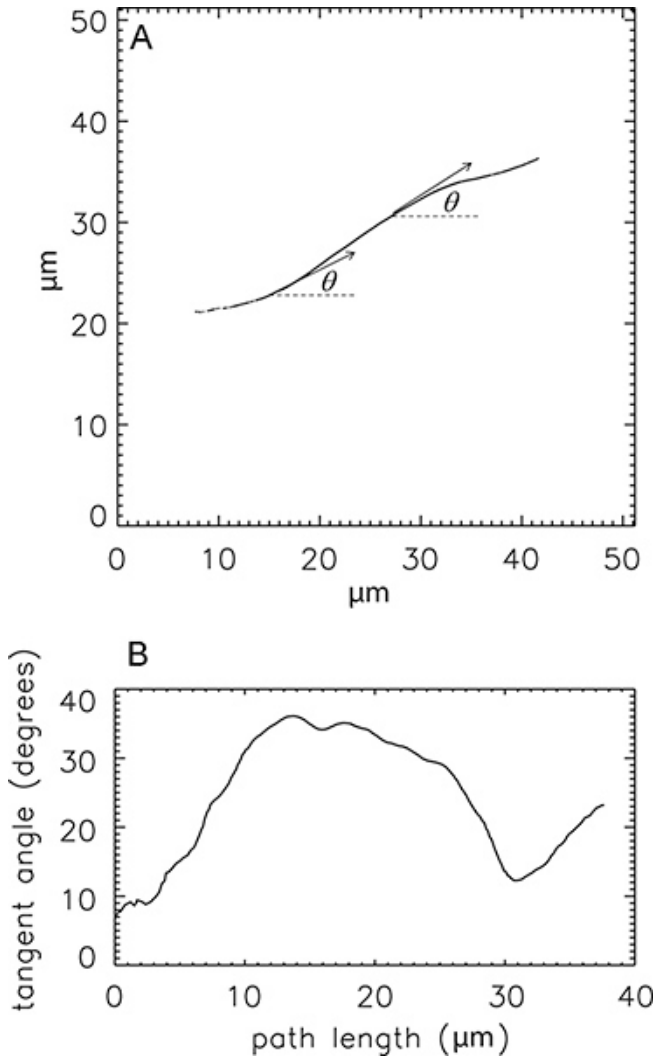

Figure 4. Calculating the tangent angles to a trajectory. (A) Trajectory of one microtubule, with example tangent angles (θ) shown. (B) Tangent angle as a function of position along the microtubule trajectory. These data are used to calculate the average angles used in Eq. 1.

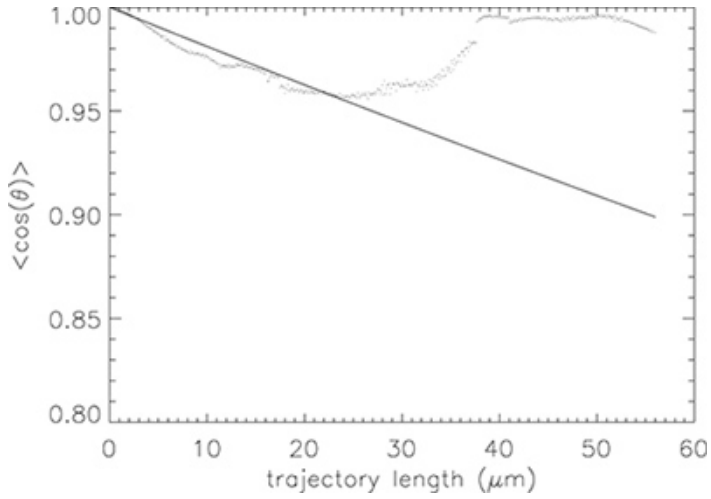

Figure 5. Calculating persistence length from tangent angles. (*dots*) Plot of $\langle\cos\theta_s\rangle$ versus contour length s for the 11 trajectories shown in **Figure 3**. (*solid line*) Fit to Eq. 1. For this group of microtubules, the persistence length is 500 ± 40 μm. For long contour lengths (above 10 μm or so), the data are highly variable due to limited statistics.

Discussion

Persistence length measurements are a good characterization of the mechanical properties of individual biopolymers. In this article, we have described a method of measuring the persistence length of microtubules. As noted in the introduction, this method is readily extended to examining microtubule mechanical properties in a variety of conditions simply by varying the reagents, temperature, or viscosity in the final step of the gliding assay, 3.9, or by polymerizing microtubules, step 1.1, under different conditions.

The technique itself is quick to perform. Once stock solutions have been prepared ahead of time (step 1), a gliding assay experiment can be performed in 2-3 hr, including up to four variations in conditions on a single microscope slide (the four lanes in step 3.1). Many microtubules can be examined in each experiment; typical experiments in our lab examine 100 microtubules per condition (about 10 image sequences per lane, with 10 microtubules per sequence). The analysis of large data sets is likewise fairly quick. With practice, 10 image sequences can be analyzed for persistence length in an afternoon or so with a modern PC. The rate-limiting step in the analysis is the time to track individual fluorophores; newer tracking methods promise to increase this speed substantially[27].

Because the method involves tracking single fluorophores attached to a given microtubule, the precision of tracking is only limited by photon collection, and can be on the scale of nanometers[22,25] . By including data from many fluorophores, the precision is further improved. Potentially, even higher precision could be reached by attaching very bright fluorescent objects, such as quantum dots, to the microtubule.

While the precision of individual trajectories is quite good, the uncertainty in persistence lengths measured with this technique (on the scale of ± 50% for a single microtubule) is still significant. The key limitation in precision of the persistence length measurements is the statistics involved in averaging $\cos\theta_s$. As the contour length between pairs of points, s, increases, the number of independent measurements of θ_s decreases as L/s, where L is the length of the microtubule trajectory studied. Increasing the length of the microtubule trajectories would allow for improved precision in persistence length measurements.

A second limitation to this method is the requirement that biotinylated kinesin-1 be used for specific attachment of kinesin to microscope slides. Biotinylated kinesin must be purified (from *E. Coli* in our case), and cannot simply be purchased off the shelf. However, a modified gliding assay using unbiotinylated kinesin may be used[28]; kinesin heavy chain protein which might be suitable for this technique is commercially available from Cytoskeleton (KR01). Modifying the gliding assay to use this alternate kinesin would not affect microtubule flexural rigidity in any way, and would thus enable groups without access to recombinant kinesin protein to use this method.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

We thank Melissa Klocke for assistance preparing **Figure 1** and Anna Ratliff for demonstrating the protocol. This work was supported by the Research Corporation for Science Advancement.

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