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Use of Single Molecule Fluorescence Polarization Microscopy to Study Protein Conformation and Dynamics of Kinesin–Microtubule Complexes

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Abstract

Single molecule fluorescence polarization microscopy (smFPM) is a technique that enables to monitor changes in the orientation of a single labeled protein domain. Here we describe a smFPM microscope set-up and protocols to investigate conformational changes associated with the movement of motor proteins along cytoskeletal tracks.

Keywords

Kinesin; Microtubule; Molecular motor; Fluorescence polarization; Dichroism; Single molecule; BSR

1 Introduction

A variety of single molecules techniques have been used to elucidate the mechanism of action of biological motors at the molecular level with a high level of detail [1–3]. Fluorescence polarization microscopy methods in general (ensemble and single molecule) allow to investigate the orientation and reorientation (angular mobility) of macromolecules in space and time. They have been used to monitor conformational changes in a variety of biological systems (e.g. [4–8]) and are particularly well suited to the study of cytoskeletal motors where key conformational changes usually involve reorientation of protein domains relative to the associated cytoskeletal filament track [1]. We describe a microscope set-up and experimental protocols to measure the orientation and angular mobility of single kinesin molecules interacting and moving along microtubules, as well as complementary ensemble FPM measurements. In the experimental set-up used, the projected angle on the microscope stage plane and angular mobility of the transition dipole moment of a fluorophore (hereinafter referred simply as the dipole) labeled protein domain is determined from the relative absorption of light polarized in various directions. With this system we have detected and characterized conformational changes associated with movement along microtubules of motile [9–12] and microtubule depolymerizing kinesins [13].

2 Material

2.1 Solutions

All solutions should be prepared using ultra pure water (resistance of 18 MΩ cm at 25 °C) and analytical grade reagents.

2.1.1 Buffers

1. BRB80: 80 mM K-PIPES, 2 mM MgCl₂, 1 mM EGTA, pH 6.8 adjusted at 25 °C by titration with a 10 M KOH solution (*see Note 1*).
2. BRB12: 12 mM K-PIPES, 2 mM MgCl₂, 1 mM EGTA, pH 6.8 adjusted at 25 °C by titration with a 10 M KOH solution (*see Note 2*).
3. Purification buffer: BRB80 supplemented with 250 mM KCl, 5 mM β-mercaptoethanol, 50 μM ATP.
4. Labeling buffer: BRB80 (pH adjusted to 7.4), 250 mM KCl, 50 μM ATP (*see Note 3*).

2.1.2 Stock Solutions

1. 75 mg/ml Bovine Serum Albumin (BSA) in BRB12 (*see Note 4*).
2. 2 mM Paclitaxel (Taxol) solution in DMSO.
3. Nucleotide stock solutions: 100 mM nucleotide (e.g. AMPPNP, ADP, ATP), 100 mM MgCl₂, in BRB80 (*see Note 5*).
4. 80% w/v Sucrose in purification buffer.
5. 1 μM Protocatechuate-3,4-dioxygenase (PCD) solution in 100 mM Tris-HCl pH 8, 50% (v/v) glycerol, 50 mM KCl.
6. 100 mM Protocatechuic acid (PCA, the PCD substrate) in BRB80.
7. 100 mM Trolox in BRB80.

All stock solutions are aliquoted, flash frozen in liquid nitrogen and stored at -80 °C.

2.1.3 Others

1. Imaging solution: BRB12 supplemented with 7.5 mg/ml BSA, 20 μM paclitaxel, 2.5 mM protocatechuic acid (PCA), 10 nM protocatechuate-3,4-dioxygenase (PCD), 1 mM Trolox, nucleotides and kinesin at the desired experimental concentrations. The imaging solution is prepared fresh for each experimental session by mixing the appropriate stock solution to the indicated final concentrations. PCA, PCD, and Trolox are used to reduce fluorophore bleaching [14].
2. DETA solution: 0.1% (v/v) of DETA (N-(3-Trimethoxysilyl-propyl) diethylenetriamine) in water with 1 mM acetic acid.

2.2 Fluorescent Probe

1. A bifunctional fluorescence probe is used to cross-link two cysteines so that it restrains the direction of the dipole relative to the targeted protein domain's 3D structure. We have used a sulfhydryl reactive fluorescent probe to specifically label cysteine residues on the target protein, bis-((N-iodoacetyl)piperazinyl)

sulfonerhodamine (BSR also called BSR-I₂). This probe has the direction of the dipole collinear to the main axis of the xanthene (Fig. 1, [15]). BSR was commercially available until recently from ThermoFisher/Molecular Probes, but this is no longer the case at the time of writing. A probe similar to BSR, but with more flexible linkers, *N,N'*-Bis[2-(iodoacetamido) ethyl]-*N,N'*-dimethylrhodamine (BR-I₂), available from Santa Cruz Biotechnology, has also been effectively used in several fluorescence polarization studies [16, 17]. In our experience, and consistent with other reports [16, 17], BR-I₂ is less reactive than BSR resulting in a lower yield of labeled protein for similar labeling conditions. For the protocols and results described here the use of the BSR probe is assumed.

2. Prepare BSR aliquots of known concentration. The rhodamines and especially the iodoacetamide groups in solution are light sensitive, so all the manipulations with the BSR are performed under subdued light. Resuspend the BSR in DMSO. Estimate its concentration assuming an extinction coefficient of 88,000 l/mol/cm at 549 nm. Adjust the concentration to no less than 5 mM to avoid adding significant amount of DMSO during the incubation with the kinesin. Aliquot the BSR solution and store at -20 °C protected from light.

2.3 Proteins

1. Kinesin: *Homo sapiens* (KF5B) and *Drosophila melanogaster* (KLP10A) recombinant kinesins proteins are expressed in *Escherichia coli* (BL21) cells and purified according to standard protocols.
2. The strategy to site-specific label kinesin proteins with a bifunctional fluorophore (Subheading 3.1) involves: (a) The mutation of all of the exposed cysteines to either alanines or another residue based on substitutions found in homologous sequences (cysteine to serine is a common one) to create a cys-lite version. (b) The mutation of the two residues to cysteines in the target region. We have effectively cross-linked with BSR cysteines with a predicted separation between C β carbons of 14–17 Å. For BR-I₂ a distance range of 10–16 Å has been reported [16].
3. Tubulin: We either purified tubulin from bovine brain following the protocol of [18] or obtained it from a commercial supplier (Cytoskeleton, porcine brain tubulin). In both cases, the purified tubulin is eventually aliquoted at 5 mg/ml (45 μ M) in BRB80 pH_{25°C} 6.8, flash frozen in liquid nitrogen and stored at -80 °C.

2.4 Microscope

For FPM we used a custom modified wide field inverted epifluorescence microscope [19, 20]. All the instrumentation necessary to control the direction of polarization of the light is kept outside the microscope so the system can be adapted to most microscopes with minimal or no modifications (Fig. 2).

1. The source of excitation light is a laser with a wavelength of 532 nm close to the excitation maximum of the BSR probe (549 nm).

2. Adjustable mirrors M1, M2, and M3 are used to reorient and align the beam path.
3. A filter wheel with a variety of neutral density filter enables the regulation of the light intensity.
4. An electro-optic modulator (EOM) followed by a quarter wave plate ($\lambda/4$) modulate the laser linear polarization in four distinct directions ($0^\circ, 45^\circ, 90^\circ$ and 135°) in the microscope stage plane.
5. A filter wheel is used to alternatively block the laser path (beam shutter), pass the beam through a linear polarizer (to test the polarization direction of the beam), or open the beam. The linear polarizer is inserted temporarily at the beginning of each movie to determine the correspondence between frame number and beam polarization direction.
6. To compensate for phase distortions introduced by a single dichroic mirror, two identical dichroic mirrors (DM1 and DM2) with reflection planes perpendicular to each other are used. The first dichroic (DM1) is outside the microscope and the second one (DM2) is located in one of the microscope filter cubes.
7. The beam is expanded by a beam expander (BE).
8. A defocusing lens (DL) provides wide field illumination of the sample which is directed to the epifluorescence port of an inverted microscope.
9. The beam is directed towards the objective (100 $\times$, oil immersion, 1.45 NA) and the sample by reflection in a dichroic mirror (DM2).
10. The fluorescence light emitted by the sample passes through the dichroic mirror and is filtered by additional emission filters (EF) to further eliminate unwanted background fluorescent and scattered light.
11. Images of the emitted light are recorded on a high sensitivity digital camera (Andor iXon +897 back-illuminated EMCCD). The resulting image pixel size in this set up is 48 nm.
12. Movie data acquisition is synchronized by the waveform generators with the electro-optic modulator so that every four frames images corresponding to each of the four excitation polarization directions are recorded.

2.5 Protein Preparation

1. Desalting column: Biorad, Econo-Pac 10DG.
2. AKTA Basic Fast Performance Liquid Chromatography (FPLC), GE Healthcare Life Sciences.
3. Size exclusion column: GE Healthcare Life Sciences, Superdex 200 Increase 10/300 GL.
4. Ultracentrifuge: Beckman Optima TLX with TLA 100 and TLA 100.3 rotors.

3 Methods

3.1 Kinesin Labeling

1. Using a desalting column or size exclusion column, exchange the buffer in which the protein was purified or stored to the labeling buffer.
2. Incubate the protein solution with the BSR probe overnight at 4 °C with a 1:1 BSR:kinesin ratio. Alternatively, a shorter incubation time (40 min) with a higher BSR:kinesin ratio (up to 3.5:1) also produces a good yield of labeled protein. We usually achieve 80–100% labeling efficiency with these protocols. For BR-I₂ or with other proteins different incubation times or different probe:protein ratios may be required (*see Note 6*).
3. Stop the labeling reaction by adding 1 mM DTT for 10 min.
4. To separate the labeled protein from un-reacted BSR pass the reaction through a desalting column equilibrated with purification buffer. The pink color of the labeled kinesin makes it easily traceable while it migrates through the column.
5. Concentrate the kinesin to the required volume for the loading loop of the FPLC apparatus for the next step.
6. To purify the protein from potentially present aggregates or higher molecular weight cross-linked species pass the labeled-kinesin solution through a size exclusion column equilibrated with purification buffer.
7. Check the purity of the preparation with a SDS-PAGE with UV exposition followed by Coomassie staining. Protein labeled with the BSR will emit fluorescence under UV illumination.
8. Check the labeling efficiency Measure the absorption at 549 nm of the purified labeled sample to estimate the BSR concentration and any suitable method to estimate the protein concentration (Bradford assay or measuring the band density in a calibrated Coomassie stained SDS-PAGE gel).
9. To the purified protein add sucrose from the concentrated stock to a final 20% w/v.
10. Aliquot the labeled kinesin (4–10 µl per aliquot), flash freeze and store the aliquots at –80 °C protected from light. For every experiment, new kinesin aliquots are thawed on ice.
11. To check for cross-linking of the target cysteines perform tryptic digestion followed by liquid chromatography mass spectrometry and ensemble FPM measurements (*see Note 7*).
12. Biochemical or motility functional assays should be performed to characterize potential alterations introduced by labeling and the cysteines substitutions introduced. Depending on the type of kinesin used, properties like microtubule activated ATPase activity, microtubule binding, mobility along microtubule, or microtubule depolymerization activity should be tested.

3.2 Flow Chamber Preparation

Samples are imaged in flow-chambers made with two glass coverslips. One of the coverslips is treated with DETA (Subheading 3.2.1) to increase microtubule attachment [21].

3.2.1 Preparation of DETA Treated Coverslips

1. Put 22×50 mm coverslips in a metallic slide rack.
2. Rinse them with ultrapure water.
3. Sonicate the coverslips for 15 min in a solution of 0.5 M KOH.
4. Wash three times for 15 min (3×15) by sonication in ultrapure water.
5. In a glass container, incubate the coverslips with DETA solution. Take into consideration that the DETA solution is corrosive; use gloves.
6. Incubate overnight at room temperature (~ 20 – 25 °C).
7. Wash three times for 15 min (3×15) by sonication in ultrapure water.
8. Place coverslips in an oven at 130 °C until dry.
9. Store the coverslips interleaved with filter paper in a Petri dish in a desiccator. We have stored the slides for over a month without detecting problems with microtubule attachment.

3.2.2 Flow Chambers Assembly—On the DETA treated coverslip apply two double-sided sticky tape bands ~ 5 mm wide along the coverslip leaving a ~ 5 mm space in between. Attach a smaller 18×18 mm untreated coverslip to the tape to form the chamber.

3.3 Microtubules

1. Thaw an aliquot of tubulin on ice.
2. To remove aggregated tubulin, spin the tubulin at $313,000 \times g$ for 10 min at 4 °C in ultracentrifuge and recover the supernatant.
3. Add 1 mM GTP to the supernatant and incubate at 35 °C during 40 min.
4. Add paclitaxel to the GTP microtubules to a final concentration of 20 μ M and further incubate 40 min at 35 °C.
5. Eliminate unpolymerized tubulin by spinning the microtubule solution at $15,000 \times g$ at 25 °C and resuspend the pellet in BRB80 with 20 μ M paclitaxel.
6. Store the polymerized microtubules at 25 °C.
7. Estimate the tubulin concentration of the resuspended microtubules by an appropriate method [22].

3.4 Sample Preparation for an Imaging Session

For each imaging session, a fresh imaging solution is prepared using new aliquots for every component. When starting with a new system, it is advisable to prepare in parallel several

slides having different concentrations of kinesin and microtubules to find the best concentrations for the collection of ensemble or single molecule data.

1. Thaw on ice one aliquot of each of the stock solutions of BSA, PCD, PCA, Trolox, Taxol and the particular nucleotide to be used.
2. Prepare the imaging solution by mixing the appropriate stock solutions. To 165 μ l of BRB12, add at room temperature 20 μ l of BSA, 2 μ l of paclitaxel, 5 μ l of PCA, 2 μ l of PCD, 2 μ l of Trolox, and 4 μ l of the appropriate nucleotide to get 200 μ l total volume. Place 100 μ l of the imaging solution on ice to use for kinesin dilutions.
3. Dilute the microtubules to 40 nM in BRB80 supplemented with 20 μ M paclitaxel (room temperature).
4. Add 5 μ l of microtubule into the flow chamber and incubate for 5 min to let them bind to the coverslip surface.
5. Block the glass surface by adding 15 μ l of imaging buffer at 25 °C. Flow the solution into the chamber by placing the pipette tip at one of the openings ends and the tip of a cut filter paper at the other end. Be careful not to dry the chamber by drawing too much liquid with the filter paper. Incubate for 5 min.
6. Dilute the kinesin in the imaging solution to the desired concentration and flow it into the chamber as done in the previous step (*see Note 8*).
7. To prevent the sample from drying during imaging, seal the two openings of the chamber with rubber cement.

3.5 Laser Illumination Alignment and Beam Polarization Adjustment

Before recording a data set it is important to check and adjust if needed the polarization direction and purity for each of the four polarizations directions used, as well as the general alignment of the microscope laser illumination.

1. If necessary align the laser path moving slightly mirrors M1, M2, and M3 to make sure that the beam comes straight up after reflecting from the dichroic mirror (DM2, Fig. 2) and going through the objective. Then check that the sample illumination center gets imaged near the center of the camera CCD.
2. Check that each of the four voltage settings of the waveform generator used to modulate the EOM produces the correct polarization directions; if not, adjust the EOM amplifier bias and/or waveform amplitude of the waveform generator. To check the polarization directions, place a calibrated polarizer in the beam path on the microscope stage with the beam coming straight up from the dichroic without the objective (wear protective glasses). Set the DC output voltage of the waveform generator to each of the four voltages predetermined to produce each of the four polarization directions to be used. For each voltage rotate the dichroic until minimum intensity is detected. In this situation, the orientation of the polarizer should then be 90° from the desired orientation. If not, adjust the waveform output voltage until this condition is met. After finding the right

voltages for the four polarization directions, create a staircase waveform with these values to be sent to the EOM during movie collection.

3. Check the polarization purity for each of the four polarizations directions. With a calibrated linear polarizer in the path, set the polarization beam in a particular direction η (by adjusting the waveform generator DC output to one of four predetermined voltages), measure the beam intensity with the linear polarizer oriented at η and $\eta + 90^\circ$, and calculate the ratio between the intensities $p_\eta = (I_\eta - I_{\eta+90})/(I_\eta + I_{\eta+90})$. The ratio p measures the polarization purity ranging from zero (non polarized light) to 1 (pure linearly polarized light). Typical p values in our setup for each of the four laser polarization directions used are $p_0 = 0.98, p_{45} = 0.97, p_{90} = 0.99$, and $p_{135} = 0.96$.

3.6 Imaging

1. Digital movies are recorded using Andor Solis software to control the camera. Set the desired acquisition parameters in the Solis program (kinetic mode, frame rate externally controlled by the waveform generator, gain, number of frames to be collected etc.).
2. Prepare a sample as described previously in Subheading 3.4. The flow chamber is placed in the microscope with the DETA treated cover slide facing the objective. Adjust the focus and find a region of interest using the minimum possible laser excitation light to avoid bleaching. For single molecule recording we use a kinesin concentration between 0.1 and 4 nM (*see Note 9*).
3. Once that a region of interest is found and in focus, block the beam using the shutter in FW2 and set the beam to the desired illumination intensity using the appropriate ND filter in FW1. Beam intensity and frame rate are set to optimize signal to noise and time resolution. Higher beam intensity is at the expense of a higher bleaching rate and a higher frame rate for better time resolution is at the expense of lower signal to noise ratio. We typically collect data at 10 or 40 Hz (100 or 25 ms/frame) with an illumination intensity between 0.5 and 5 kW/cm².
4. Start movie recording with the Solis software and start the illumination-sequence-protocol. We use a protocol in which a few frames are collected first with the beam blocked, then a few frames collected with the beam open and a tester linear polarizer (0° direction) in the excitation path (FW2 Fig. 2), and then with the beam open and no tester polarizer for the rest of the movie data collection period. Illumination intensity control and the opening beam protocol are performed by rotating the filter wheels (FW1 and FW2, Fig. 2) to the appropriate positions using a custom control program (Wheel_control.exe, *see Note 10*). The frames with the beam blocked are used to determine the background light intensity level of each movie. The frames with the tester polarizer are used to determine the phase of the excitation beam polarization direction (i.e. the correspondence between frame number and excitation polarization direction). After a movie is recorded move to another area and repeat from **step 3** above to collect data from multiple microtubules from each

prepared flow chamber. Individual movies are usually recorded for 3–6 min. We record data for no longer than 1 h per chamber.

3.7 Data Analysis

The raw data collected are digital movies of the fluorescent samples where the excitation light polarization direction changes between four possible values (0° , 45° , 90° , 135°). From these movies, we extract background corrected fluorescent intensity values (I_0 , I_{45} , I_{90} , I_{135}) in space and time for each of the four polarization excitation directions using a custom Windows GUI program (FRVIEW, *see Note 10*). From the intensity values of single and ensemble of molecules we estimate the orientation of the fluorescent dipoles and their mobility (*see Fig. 3* for angle definitions). The program FRVIEW reads the digital movie files (Andor .sif file format) and displays image frames or position vs. time kymographs. The kymographs are used to select single molecule trajectories for analysis (Fig. 4a). Assigning the corresponding excitation polarization direction to each movie frame is done automatically in FRVIEW based on the illumination-sequence-protocol (*see Subheading 3.4*). The program generates tab separated text files with all the pertinent data such as excitation light polarization direction, fluorescent intensity, calculated linear dichroism values (LD), coordinates in the (X , T) plane, time, etc. These files can be conveniently read by other software programs for further analysis.

3.7.1 Analysis of Single Molecule Data

1. Manually mark single molecule trajectories from a position vs. time kymograph display (Fig. 4a, b). Coordinate points between manually selected ones are estimated by linear or polynomial interpolation. The center position of the fluorescent spot at each time frame is then estimated as the center of mass within a region of interest centered at the initially marked coordinates.
2. The fluorescence intensity for each spot frame is determined as the average number counts in an appropriate region of interest (7×7 pixel with our setup) centered at the estimated center of the fluorescence spot minus the background intensity. Estimate the background as the average counts in an area close by without fluorescence trajectories or after the molecule bleaching event. Coordinate and intensity data for each selected trajectory in a movie are saved to tab delimited text files for further analysis.
3. Select trajectories corresponding to single molecules. We restrict the analysis to single molecule trajectories that end by an abrupt drop to near zero intensity due to a photobleaching or unbinding event. Multiple stair case drops in intensity are evidence of more than one molecule present. We exclude molecules that have multiple time and position overlaps with other molecules (evidenced as kymograph trajectories with multiple crossings).
4. Refine the coordinates. A custom python script (evnrawreproc. py, *see Note 10*) reads the files created by FRVIEW and the original movie files to further refine the xy coordinates of the fluorescent spot center positions. The script fit 2D

Gaussian functions to the intensity profile of each selected single molecule trajectory frame.

5. The fluorescence intensity for each of the four polarization directions (I_0 , I_{45} , I_{90} , I_{135}) in a given time period is calculated as the average of all the values corresponding to each polarization direction during such a period (with the shortest possible period corresponding to four frames, one for each polarization excitation direction).
6. From the four intensity values (I_0 , I_{45} , I_{90} , I_{135}) calculate the two linear dichroism ratios LD_1 and LD_2 ($LD_1 = LD_{0,90}$ and $LD_2 = LD_{45,135}$) as:

$$LD_{\eta, \eta+90^\circ} = \frac{I_{\eta+90} - I_\eta}{I_{\eta+90} + I_\eta} \quad (1)$$

7. The mean projected angle Φ of the fluorophore in the XY plane and the order r factor is calculated from LD_1 and LD_2 as (Eqs. 5, 6, 7 from [20]):

$$\begin{cases} r = \sqrt{LD_1^2 + LD_2^2} \\ \cos^2(\Phi) = \frac{1}{2} \left(1 - \frac{LD_1}{r} \right) \\ \cos^2(\Phi - 45) = \frac{1}{2} \left(1 - \frac{LD_2}{r} \right) \end{cases} \quad (2)$$

The order r factor depends on Γ and θ according to the following expression derived from [19]:

$$r(\Gamma, \theta) = \frac{3\sin^2(\theta)}{1 + \frac{4}{\cos(\Gamma) + \cos^2(\Gamma)} - 3\cos^2(\theta)} \quad (3)$$

The r factor strongly depends on Γ , the mobility cone semi-angle (Fig. 3d) and therefore gives an indication of the probe mobility going from zero for fully mobile probes to 1 for perfectly localized ones.

The projected angle relative to the microtubule axis α is calculated as:

$$\alpha = \Phi - \omega \quad (4)$$

From the distribution of α values of many kinesin molecules the axial angle β of the probes with the microtubule axis can be inferred (Fig. 3c).

8. The resulting simultaneous recording of fluorophore orientation, mobility and position obtained by smFPM can then be used to obtain structural and functional insights into the molecule of interest. For example using this method we were

able to determine that the two motor domains of kinesin-1 alternate between a disordered and a well oriented configuration as the molecule walks processively along microtubules at limiting ATP concentrations [12]. We also observed a correlation between the angular orientation of the kinesin-13 motor domain and its ability to diffuse along the microtubule lattice [13].

3.7.2 Analysis of Fluorescence Polarization of Microtubule Covered by an Ensemble of Kinesins

1. We usually collect ensemble FPM data before smFPM experiments as they: (a) can serve to check if the labeling strategy worked (*see Note 7*) and (b) provide data complimentary to the smFPM data (*see Note 11*). Ensemble FPM data is collected in a similar way that the smFPM data but the kinesin concentration used in the experiments is higher (typically around 100–200 nM) and the larger fluorescence intensity produced by the ensemble allows the use of lower laser excitation (typically 0.02–0.2 kW/cm²).
2. Collect digital movies of microtubules decorated with fluorescently labeled proteins randomly oriented in the (X, Z) plane.
3. From the digital movies measure the average intensity of the microtubule area (MTa) and an adjacent area (BKGa) to estimate the fluorescence intensity (I) of the microtubule over the background ($I = I_{MTa} - I_{BKGa}$) for each of the four excitation polarization excitations used. From the four fluorescent intensities calculate the LDs values as done with the smFPM data (This is all done automatically in FRVIEW by selecting a region of interest on the microtubule, another in an adjacent area and given the time period to average intensities).
4. Estimate the LD⁰ value. LD⁰ is equal to the LD₁ of microtubules oriented with their axes oriented parallel to the X axis ($\omega = 0^\circ$) and is estimated from the LD₁ values of many randomly oriented microtubules by non-linear fitting the data to the equation [19]:

$$LD_1 = LD^0 \cos(2\omega) \quad (5)$$

For a distribution of fluorophores with cylindrical symmetry (such as the one produced by microtubules decorated with many labeled kinesin molecules) the LD⁰ is related to axial angle of the fluorophores with the microtubule axis β and their mobility semi cone angle Γ by the following equation [19]:

$$LD^0 = \frac{-3}{1 + 8/[3\cos^2(\beta) - 1](\cos(\Gamma) + \cos^2(\Gamma))} \quad (6)$$

For a distribution of probes close to perpendicular or parallel to the microtubule axis (β close to 90° or 0° respectively) LD⁰ is strongly dependent on the mobility

cone semi-angle Γ , going from zero for fully mobile probes ($\Gamma = 90^\circ$) to 1 or -1 respectively for fully localized ones ($\Gamma = 0^\circ$) (Fig. 3e).

4 Notes

1. We use PIPES from Sigma (PIPES dipotassium salt, P7643), which produces less autofluorescence than other PIPES we have used.
2. To increase kinesin-microtubule binding we usually use a lower ionic strength buffer (BRB12 vs. BRB80). Experiments performed with BRB12 also have less background fluorescence (autofluorescence).
3. A higher pH than the regular BRB80 (7.4 vs. 6.8) buffer improves the labeling yield.
4. To avoid autofluorescence we use high purity BSA (Sigma #A0281, fatty acid and globulin free). In addition we spin the BSA stock solution ($220,000 \times g$, 15 min) to precipitate and eliminate impurities. After centrifugation, the supernatant is taken and the small black precipitate that usually forms is discarded.
5. The nucleotide to be included in the imaging solution depends on the experiment to be performed. Other nucleotide analogues conditions we have used are presence of ADP + AlF_4^- (made from 100 mM ADP, 198 mM AlCl_3 and 1 M KF) and absence of nucleotides (by including the enzyme apyrase to hydrolyze any ATP and ADP present in the solution).
6. For experiments with the BR-I₂ probe a step-wise addition of the fluorophore at room temperature (addition of four equal amounts of probe every 30 min to a final 2:1 probe:protein ratio) has been used [17].
7. If possible, the location of the target cysteines should be chosen such that the direction of the probe dipole will be near parallel or perpendicular to the microtubule axis in some standard condition (e.g. when a labeled motor domain is strongly bound to the microtubule). In these conditions, successful labeling and cross-linking with the probe can be verified by the expected signed anisotropy values obtained in ensemble FPM measurements (see Subheading 3.7.2). If the labeling location on the kinesin protein is such that the probe would be near perpendicular ($\beta = 90^\circ$) or parallel ($\beta = 0^\circ$) to the microtubule axis then verification that they produce the expected LD^0 in the given conditions is strong confirmation that the probe is cross linked to the protein as expected (i.e. LD^0 values near 1 or -1 respectively for perpendicular or parallel oriented probes). Lack of anisotropy ($\text{LD}^0 \sim 0$) in these conditions suggests highly mobile probes, either because the protein does not bind to the microtubules as expected or the bifunctional probe is not properly attached to the protein (single rather than double attachment).
8. An alternative way to prepare the sample (**steps 2–6**) is to pre-incubate microtubules and kinesin together in the imaging solution and flow the mix into the flow chamber.

9. Focusing and finding microtubules to image can be difficult at low kinesin concentrations given that we usually use unlabeled microtubules. To avoid this problem, it is recommended to have the focus position of the microscope pre-determined before placing the experimental chamber in the microscope stage. This can be done by focusing a cover slip with the surface marked by lines made with a red sharpie magic marker (they conveniently fluoresce when illuminated with the laser light). If kinesin decorated microtubules are still not found, this may indicate a problem with the microtubules (e.g. low concentration of polymer) or the kinesin preparation. This can be quickly ruled out by doing an experiment with a labeled prep known to bind well to microtubules and using a higher kinesin concentration. It is also advisable to start any imaging session with a high kinesin concentration (appropriate to ensemble imaging) and then lower the concentration as needed to resolve single molecules.
10. All custom software programs referenced in the manuscript are available for academic use upon request to the authors.
11. The ensemble polarization data provides anisotropy LD^0 values that depend on the axial angle (β) of the probes with the microtubule axis and the probe mobility (expressed as the mobility cone angle Γ). The ensemble LD^0 values must be consistent with the angles and mobility values derived from the distribution of many smFPM measurements obtained in similar conditions.

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Fig. 1.

Structure of two rhodamine-derived bifunctional probes used for single molecules fluorescence polarization studies. The rhodamine part is indicated with *lighter lines* (red in color figure). The direction of the dipole is indicated by the *double arrow*. (a) BSR (bis-((N-iodoacetyl)piperazinyl)sulfonerhodamine). (b) BR (*N,N'*-Bis[2-(iodoacetamido)ethyl]-*N,N'*-dimethylrhodamine)

Fig. 2.
Main components of the fluorescence polarization setup used. M1, M2, M3, and M4: mirrors. DM1 and DM2 dichroic mirrors. FW1 and FW2: filter wheels. EOM: electro optic modulator. 1/4: quarter wave plate. BE: beam expander. DL: defocusing lens. Obj: Objective lens

Fig. 3.

Probe orientation and angles definitions. (a) Model of a kinesin microtubule complex in the typical tight bound configuration with the BSR probe attached in one of the target regions used in smFPM studies. The Kinesin motor domain is represented with ribbons (*yellow* in color figure), the microtubule as a gray surface, and the BSR probe as atom and sticks (*magenta* in color figure). The *double arrow* indicates the direction of the BSR fluorescence transition dipole moment. (b) Definition of the angles used in the eqs. (X, Y) is the plane of the microscope stage with the X axis oriented as the left-right translation direction of the microscope stage (see also Fig. 2). The direction of the fluorescence transition dipole moment is represented as a *double arrow* and noted D . Its projection on the (X, Y) plane is noted $P_{XY}(D)$. β is the mean axial angle (referred simply as the axial angle) with the filament axis of a probe that pivots rapidly within a cone of semi-angle Γ (mobility cone angle). α is the probe dipole angle projected in the XY plane minus the filament axis angle in the plane (ω). (c) and (e) shows the relationship between β and Γ for a cylindrically symmetric distribution of probes (such as the one expected for a kinesin decorated microtubule) with the values α , r , and LD^0 obtained from single molecule and ensemble FPM measurements. (c) Simulated frequency distribution of single molecule α values for a distribution of probes uniformly bound around a cylindrical axis with axial angle β without

adding noise (*bars*) or adding Gaussian noise (*lines*) to the simulated intensity values. The $|\alpha|$ distribution has a peak near the β angle and a tail towards 0° . **(d)** Contour plot showing the relationship between the order r factor (contour values and *grey level*), the mobility cone angle Γ (Y -axis), and the angle θ (X -axis) of the probe from the Z axis (from Eq. 3). **(e)** Contour plot showing the relationship between the ensemble LD^0 (contour values and *grey level*), the mobility cone angle Γ (Y -axis), and the axial angle β (X -axis) according to Eq. 6

Fig. 4.

Example of time resolved single molecule data with estimation of the dipole direction projected angle (α) and order factor (r) along the trajectory. The kinesin used is a construct of the *Drosophila melanogaster* kinesin-13 KLP10A labeled with BSR in the location indicated in Fig. 2a. In this example a kinesin molecule is bound to the microtubule with high rotational mobility (low order r factor) while undergoing one-dimensional diffusion along the microtubule and then become stationary (at time 50 s in **b**) with reduced angular mobility (higher order r value). (**a**) Image of the microtubule analyzed. To produce this image several frames involving several kinesin binding events were averaged. (**b**) Position vs. time kymograph taken from the microtubule shown in **a**. The kymograph shows a single kinesin labeled molecule undergoing one-dimensional-diffusion. (**c**) Fluorescence intensities corresponding to each of the four excitation polarization directions for the trajectory molecule shown in (**b**). (**d**) Calculated projected angle α of the molecule (three frames-set running average). The median α value during each of the two movement phases is indicated by the *magenta dashed lines*. (**e**) Calculated order r factor (three frames-set running average). The median r value during each of the two movement phases is indicated by the *dashed lines*. The traces show changes in angular mobility (rapid changes in orientation of the

kinesin motor domain to which the BSR probe is attached) that correlate to whether the molecule is undergoing one-dimensional-diffusion or being stationary

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