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Structure and Dynamics of the Kinesin–Microtubule Interaction Revealed by Fluorescence Polarization Microscopy

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Abstract

Fluorescence polarization microscopy (FPM) is the analysis of the polarization of light in a fluorescent microscope in order to determine the angular orientation and rotational mobility of fluorescent molecules. Key advantages of FPM, relative to other structural analysis techniques, are that it allows the detection of conformational changes of fluorescently labeled macromolecules in real time in physiological conditions and at the single-molecule level. In this chapter we describe in detail the FPM experimental set-up and analysis methods we have used to investigate structural intermediates of the motor protein kinesin-1 associated with its walking mechanism along microtubules. We also briefly describe additional FPM methods that have been used to investigate other macromolecular complexes.

I. Introduction

Kinesin is a superfamily of motor proteins that converts the energy from ATP hydrolysis into mechanical work to drive movement along microtubules in a variety of cellular processes such as organelle transport and cell division (Endow, 2003; Goldstein and Philp, 1999). Since the discovery of the founding member of the superfamily, called conventional kinesin or kinesin-1 (from here on simply referred to as kinesin), many experimental approaches in several laboratories have been used to elucidate the mechanism of energy conversion by kinesin motors (Amos, 2008; Valentine and Gilbert, 2007). One approach we have used to address this issue is fluorescence polarization microscopy (FPM), the focus of this chapter.

FPM can be defined as the use of polarized light to determine orientation and rotational dynamics (mobility) of samples imaged in a fluorescent microscope. Like other fluorescence-related techniques, it provides single-molecule sensitivity so that the orientation and mobility of a single fluorescent molecule can be determined.

FPM has been used to study protein conformational changes in several biological macromolecular systems such as membrane proteins (Adachi *et al.*, 2000), DNA (Ha *et al.*, 1999), and the cytoskeletal motor proteins myosin and kinesin (Peterman *et al.*, 2004). FPM is well suited to investigate conformational changes in motor proteins, since they are thought to involve changes in the orientation or angular order of protein domains. In addition, the

single-molecule sensitivity of FPM allows studying conformational changes in motor proteins as they walk, without the need to synchronize them.

The high sensitivity of FPM also allows obtaining structural information on the kinesin–microtubule interaction without saturating the microtubule lattice, a potential source of artifacts (Hackney, 2007; Marx *et al.*, 2006). In a saturated microtubule lattice many kinesin molecules would not have two available adjacent binding sites for their two kinesin heads. This results in an artifactual binding pattern with a subpopulation of molecules with only one motor head interacting with the microtubule. This situation has led to problems of interpretation when analyzing the structure of microtubule-bound two-head kinesin constructs by 3D electron microscopy, a technique that usually requires saturation of the microtubule lattice (Hoenger *et al.*, 2000). By contrast, FPM can provide structural information with much lower kinesin densities and thus avoid saturation and overcrowding artifacts (Asenjo *et al.*, 2003).

One of the first results we obtained using FPM was the unexpected observation that the motor domain of kinesin, in the presence of ADP, can bind to the microtubule with high mobility (Sosa *et al.*, 2001). This result highlights the ability of FPM to identify mobile or disordered states. Such states are difficult to identify and study by other structural techniques such as X-ray crystallography or electron microscopy that usually require ordered samples with many copies of the same molecule in similar configurations. We also found that the neck-linker domain of conventional kinesin (~15 residues located at the C-terminal of each kinesin motor head domain) switches from a highly mobile state to a fixed state, almost parallel to the microtubule axis, upon ATP binding and hydrolysis (Asenjo *et al.*, 2006). This confirmed and extended previous observations providing additional evidence for the role of the neck-linker in biasing the direction of kinesin translocation (Rice *et al.*, 2003, 1999). Also using FPM we obtained evidence that during processive movement at physiological concentrations of ATP both kinesin motor domains spend most of the time in a microtubule-bound configuration (Asenjo *et al.*, 2003). On the other hand, at lower ATP concentrations, in the so called ATP-waiting state, one of the two kinesin motor domains becomes very mobile (Asenjo and Sosa, 2009) (Fig. 1). These FPM results provided important constraints to models for the mechanism of translocation and coordination between the two kinesin motor domains.

II. Rationale

Fluorescence polarization or anisotropy in bulk solutions is widely used to measure the rotational diffusion of fluorescent molecules. FPM extends this application to very small samples or cells and adds new capabilities for structural investigations (Axelrod, 1989). It provides structural information on macromolecular complexes on timescales and under experimental conditions that are not easily accessible by other structural techniques. In ordered samples, it allows determining the orientation of fluorescently labeled molecules relative to some recognizable structure such as the filament axis of microtubules, actin, or DNA. At the single-molecule-level FPM allows detecting the orientation and position of individual molecules and following conformational changes, without the need to synchronize the activity of the bulk population of molecules (Peterman *et al.*, 2004).

III. Methods

The use of polarized light to determine the orientation and mobility of a fluorophore is based on the vectorial nature of the transition dipole moments responsible for the absorption and emission of light. The probability of the absorption of a photon by a fluorophore is proportional to $\cos^2(\theta)$, where θ is the angle between the absorption dipole moment and the electric vector of the incident light field. The emission of light is also polarized, with intensity proportional to $\cos^2(\xi)$, where ξ is the angle between the emission dipole moment and the electric vector of the emitted light (perpendicular to the direction of propagation) (Lakowicz, 1999). Thus, the orientation of the fluorophore absorption or emission dipole moments can be determined by analyzing the fluorescence intensity corresponding to different polarization directions of the exciting or emitted fluorescence light. Direct information on the orientation of fluorescently labeled proteins or protein domains can be obtained if the direction of the fluorophore dipole relative to the target protein is known.

In the remainder of this chapter, we describe in detail the methods we have used to determine the orientation and mobility of kinesin molecules while interacting with microtubules. We also briefly review other methods used to study other filamentous systems such as the actin–myosin interaction that could equally be applied to the microtubule system.

A. Epifluorescence Linear Dichroism Set-Up

Following is a description of the set-up we have used to study the mechanism of kinesin walking on microtubules (Fig. 2). All the optical components required for fluorescence polarization are placed outside the microscope, allowing straightforward implementation to almost any microscope. Excitation light is provided by a green laser ($\lambda = 532$ nm), with a wavelength close to the excitation maximum of the bifunctional fluorescent probe bis-((N iodoacetyl)piperazinyl)sulfonerhodamine (BSR, see below) used in our studies. Adjustable mirrors are used to direct the beam with fine control into the microscope. The direction of linear polarization of the laser light is alternated between 0, 45, 90, and 135° (0° corresponds to the direction of the microscope left–right axis translation stage, labeled as the *X*-axis in Fig. 2) using an electro-optic modulator (EOM) followed by a $\lambda/4$ wave plate. After setting the beam polarizations, the light is reflected by two identical dichroic mirrors (one outside the microscope and the other in one of the microscope filter cubes) with planes of reflection perpendicular to each other. This arrangement is important to compensate for phase retardations introduced by the dichroics (Peterman *et al.*, 2001), particularly when the polarization direction is not s- or p-polarized (in this set-up this situation would occur with the 45° and 135° polarization directions). A defocusing lens is located between the two dichroics, to provide wide-field illumination. After reflection from the second dichroic, the beam is directed by the microscope objective to the sample. Fluorescence is collected by the same objective and filtered by the emission filter in the microscope filter cube (F1) and an additional filter (F2) to further suppress excitation light. A series of images of the emitted light are captured with a high-sensitivity CCD camera after passing through a 4× relay lens.

The camera and the EOM input signal are synchronized with two phase-locked waveform generators to alternatively collect images corresponding to the four excitation polarization directions. The intensity of the excitation beam is regulated as desired with a filter wheel

(FW1) containing neutral density filters of varied strength. Another filter (FW2) is used as a beam shutter and to briefly insert a linear polarizer in the beam path at the beginning of each image series data acquisition run. The short segment in the image series, when the linear polarizer is in the light path, is used to determine the phase of the excitation polarization direction periodic changes (i.e., the correspondence between frame number and polarization excitation direction). We use a custom-written program to control the filter wheels and the camera vendor software (Roper Winview or Andor Solis) for image data acquisition.

B. Data Analysis

For efficient fluorescence polarization analysis of data extracted from hundreds of microtubules or single molecules, we have developed a custom graphical user interface program (Frwin) available for Windows PC. The program extracts all the relevant information from the collected image series (currently reads Roper Winview SPE files and Andor Solis raw files) and performs most of the calculations needed in the analysis. The resulting data are exported as text tables for further analysis or plotting with other software.

We measure the fluorescence intensities corresponding to each of the four polarization directions in particular regions (containing a microtubule or a single fluorescence spot) of the images. The fluorescence intensity is estimated as the average pixel intensity in the selected area after subtracting the background. The background is estimated by determining the average pixel intensity in a close-by area with no visible microtubules or fluorescence spots. A correction factor (G) is applied to the fluorescence intensities to account for intensity differences in the excitation and/or absorption between polarization excitation directions. This correction factor is estimated by imaging a sample with isotropic fluorescence absorption (e.g., a concentrated dye solution). In the set-up described here, the differences in fluorescence intensity between excitation polarization directions were less than 3% when an isotropic sample is analyzed.

From the fluorescence intensities, two fluorescence linear dichroism (LD) values are calculated for each microtubule or molecule according to the below equations:

$$LD_{0-90} = \frac{I_{90} - I_0}{I_0 + I_{90}} \quad (1)$$

$$LD_{45-135} = \frac{I_{135} - I_{45}}{I_{135} + I_{45}} \quad (2)$$

where I denotes fluorescence intensity and the subscript denotes the corresponding excitation light polarization directions. From the LD values, we estimate the fluorophore orientation and the extent of fast angular disorder.

We distinguish two types of data, ensemble (Fig. 3) and single-molecule (Fig. 4), depending on the number of molecules contributing simultaneously to the fluorescence intensity in a particular area. Having one case or the other is controlled experimentally by choosing the appropriate amount of fluorescently labeled protein added to the sample. It is also possible to bleach an area with many fluorophores until individual ones can be distinguished.

Whether individual or many fluorophores are contributing to the intensity in an area can be readily determined from the intensity time course. Single molecules typically produce constant fluorescence intensity time traces ending abruptly by photobleaching (Fig. 4C), while the intensity of an ensemble of fluorophores decreases exponentially, due to the gradual photobleaching of the many fluorophores.

First we will discuss how we analyze ensemble data. We estimate the parameter LD^0 , defined as the LD_{0-90} for microtubules with an angle $\omega = 0^\circ$ in the XY microscope stage plane (i.e., parallel to the X -axis in Fig. 2). A cylindrically symmetric arrangement of dipoles around an axis, as is the case for labeled kinesin molecules interacting with a microtubule, can be described by the axial angle β between the fluorescent dipole and the filament long axis. Mobility is incorporated into the model as a fast angular motion of the probe (faster than the experimental data acquisition time) within a cone with axis along β and semi-angle Γ (Fig. 2B). LD^0 is related to β and Γ according to the following equations [rearranged versions of Eq. (3) by Peterman *et al.* (2001)]:

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

$$LD_{0-90} = LD^0 \cos(2\omega) \quad (4)$$

In practice, we estimate the LD^0 values by measuring the LD_{0-90} of many microtubules randomly oriented in the XY plane and performing a nonlinear fit of the data to Eq. (4) (Fig. 3B). We select areas with microtubules in the digitized image and determine their orientation in the XY plane (ω) and their fluorescence intensity to calculate LD_{0-90} and LD_{45-135} according to Eqs. (1) and (2). For each microtubule area, we obtain two independent data points (at $\omega = \theta$ and $\omega = \theta - 45^\circ$) to be fitted with Eq. (4) as LD_{45-135} is equivalent to the LD_{0-90} after a 45° rotation in the XY plane.

The relationship between LD^0 , the axial angle β , and the mobility cone angle Γ is illustrated in Fig. 5. Values for LD^0 range from -1 to 1 corresponding to dipoles aligned parallel and perpendicular to the microtubule axis, respectively. An LD^0 value of zero corresponds to dipoles with an axial angle $\beta = 54.7^\circ$ or to dipoles that are fully mobile ($\Gamma = 90^\circ$). For LD^0 values closer to 0, there is an increased range of β and Γ angles consistent with a given LD^0 . For example an LD^0 value of -0.7 is consistent with a range $\beta = 0^\circ$ ($\Gamma = 45^\circ$) to $\beta = 31^\circ$ ($\Gamma = 0^\circ$), while an LD^0 value of -0.1 is consistent with a range $\beta = 0^\circ$ ($\Gamma = 83^\circ$) to $\beta = 52^\circ$ ($\Gamma = 0^\circ$). To obtain separate estimates of probe mobility and axial angle, we turn to single-molecule FPM. Single-molecule FPM also allows obtaining orientation information of nonsynchronized transient events such as the ones occurring during kinesin processive walking.

We select individual fluorescence molecules for analysis using kymograph plots (Fig. 4B). These plots represent the pixel intensity along the microtubule over time. Kymographs are a convenient way to quickly review all molecules that interact with a given microtubule and select the ones to be further analyzed. A kymograph reveals in a single image the location

and intensity as a function of time for single-molecule events, allowing one to determine the moment of photobleaching, whether a molecule is stationary or moving, its velocity and direction, and whether several molecules overlap. We manually select the molecule to be analyzed as trajectories in the kymographs using a graphical user interface program (Frwin). The program then refines the XY coordinates of the center of the fluorescence spots in all the image frames in the series by centroid determination or fitting a 2D Gaussian function to the point spread function image (Thompson *et al.*, 2002; Yildiz *et al.*, 2003). The fluorescence intensities of the spot in each image along the time series are then calculated as the average pixel intensity (minus background) of an area around the fluorescent spot center (typically $0.39 \times 0.39 \mu\text{m}^2$). The intensities in the time series are then separated according to the polarization excitation direction used on each image in the series. From such intensity traces, time-averaged values of LD_{0-90} and LD_{45-135} can be calculated for each molecule. Alternatively, for time-resolved records, we calculate running LDs by using the fluorescence intensity of four consecutive images in the series (corresponding to the four polarization excitation directions).

From the two LDs corresponding to a single fluorophore, it is possible to estimate separately the angle of a fluorophore projected in the XY plane (ϕ) and its mobility (Peterman *et al.*, 2001). The projected angle relative to the microtubule axis (α) is calculated by subtracting the angle of the microtubule in the XY plane ($\alpha = \phi - \omega$). From the distributions of projected angles of many randomly placed fluorophores along a microtubule (but preserving cylindrical symmetry around the filament axis), it is possible to estimate the axial angle β . A uniform distribution of dipoles with an axial angle β would have a corresponding distribution of projected angles relative to the microtubule axis with a peak at β and a progressively less populated tail toward 0° (Fig. 6). Therefore, the position of the peak in the distribution of projected angles of many filament-bound fluorophores will indicate the value of the axial angle β .

The amount of mobility in the probe is captured in the order parameter r defined as

$$r^2 = \text{LD}_{0-90}^2 + \text{LD}_{45-135}^2 \quad (5)$$

For nonmobile dipoles $r = 1$, increased mobility reduces r and for a fully mobile probe ($\Gamma = 90^\circ$) $r = 0$. The projected angle ϕ of the dipole in the XY plane is calculated according to the following equations:

$$\cos^2(\phi) = \frac{(1 - (\text{LD}_{0-90}/r))}{2} \quad (6)$$

$$\cos^2(\phi - 45^\circ) = \frac{(1 - (\text{LD}_{45-135}/r))}{2} \quad (7)$$

Equations (5), (6), and (7) are derived from Eq. (4) in the work of Peterman *et al.* (2001). Note that two angles (ϕ and $180^\circ - \phi$) satisfy Eq. (6) or (7), but only one satisfies both at the same time. This is one reason why we collect fluorescence intensity data for four

polarization excitation axes: this allows us to resolve the ϕ or $180^\circ - \phi$ ambiguity. In addition, it makes it possible to obtain separate estimates of probe orientation and mobility (see below).

A convenient way to visualize the relationship between the LDs, mobility, and projected angles is to plot LD_{45-135} versus LD_{0-90} (Fig. 7). In this plot mobility affects the radial position while the projected angle determines the angular location. Nonmobile dipoles locate around a circle of radius 1 ($r = 1$) and dipoles with increased mobility locate at smaller radius.

C. Other Methods

1. Modulation of Excitation Polarization in Conjunction with Measurement of Emission Polarization—Goldman, Irving, Corrie, and coworkers have developed methods to determine the orientation of actin-bound proteins in muscle fibers (Dale *et al.*, 1999; Corrie *et al.*, 1999b; Hopkins *et al.*, 1998; Irving, 1996). The core of their approach was to vary the excitation polarization and, in addition, to measure the fluorescence in two perpendicular polarization channels (Hopkins *et al.*, 1998). The additional knowledge of the emission polarization allows determination of the orientational mobility (“wobble”) of the probe with respect to the protein complex in ensemble-type experiments. With this knowledge, average axial orientations of the dipole moments can be determined in the presence of wobble (Dale *et al.*, 1999; Irving, 1996).

For single-molecule analysis, Goldman and coworkers have altered their fluorescence excitation scheme to use prism-type total internal reflection (TIR) illumination (Forkey *et al.*, 2000, 2005, 2003). TIR excitation has the benefit that only a thin slice of the sample, up to about 100 nm, from a glass–water interface is illuminated, reducing background fluorescence. Control over the polarization of the resulting evanescent wave is more complex than in epi-illumination and involves, in addition to an EOM, physical switching of the propagation direction of the excitation beam (Forkey *et al.*, 2005). An additional advantage of this set-up is that the evanescent wave contains a strong polarization component in the z -direction, which allows determination of the 3D orientation of the dipoles. This approach has been applied to determine the rotation of the myosin V lever arm during processive stepping along actin (Forkey *et al.*, 2003).

2. Confocal Microscopy—One of the limitations of the approaches described above is their relatively low time resolution. Changes in dipole orientation that occur faster than the rate of polarization switching will be averaged out and cannot be detected. This limitation is due to the use of cameras in wide-field microscopy, which require integration times of several tens of milliseconds in order to obtain single-molecule images with high enough signal-to-noise ratio. The most straightforward way around is to use confocal fluorescence microscopy. In that case a point detector, most frequently an avalanche photodiode is used, which can time tag detected photons with sub-microsecond accuracy. This set-up could be combined with a polarizing beam splitter and two detectors in the emission light path to obtain angular information (Moerner and Fromm, 2003), as has been done to study conformational changes in nonprocessive myosin (Warshaw *et al.*, 1998).

Recently, we have developed a novel, confocal assay to measure fluorescence intensity fluctuations on walking kinesin (Verbrugge *et al.*, 2007). The crux of this approach is to use autocorrelation analysis of time traces, in order to access the submillisecond time regime. Förster Resonance Energy Transfer can be readily detected by determining the cross-correlation signal between a donor and acceptor channel (Verbrugge *et al.*, 2007). One could envision using this confocal assay in conjunction with circularly polarized excitation light and two perpendicular polarization channels to measure angular changes with a time resolution of less than 1 ms.

3. Defocused Orientation Imaging—There is an additional way of obtaining the orientation of a single emitting dipole moment without requiring the use of polarizers in the excitation or emission light paths. Slightly defocused images (500–1000 nm) of a single fluorophore taken in a conventional wide-field epi or TIRF configuration have characteristic shapes that depend on the orientation of the dipole (Bartko and Dickson, 1999; Bohmer and Enderlein, 2003; Patra *et al.*, 2004). The defocused image of a tilted emitting dipole, close to an interface, is not circular but shows additional lobes and fringes. Such distorted images, for known dipole orientation, distance to surface, and defocusing, can be predicted using exact wave-optical calculations (Bohmer and Enderlein, 2003). By comparing the theoretical images with experimentally images, using a pattern matching algorithm, the orientation of the dipoles can be determined (Patra *et al.*, 2004). Using this approach, angle accuracies of 10–15° have been obtained from images taken in 0.6 s (Enderlein *et al.*, 2006). The advantage of this approach is that no complex polarization equipment is required; only good focus control is needed. The disadvantage is that complex and time-consuming image analysis is required. Furthermore, this is a strict single-fluorophore approach; for overlapping fluorophores, the image effects will be masked. In addition, movement of the orientation of the dipole within the image integration time complicates the analysis. This defocusing and image analysis approach has been used to determine the changes in orientation and position of myosin V motor proteins walking along actin filaments (Enderlein *et al.*, 2006).

IV. Fluorescent Labeling for FPM

An important requirement for FPM studies is that the fluorophore does not rotate too much with respect to the protein of interest. Otherwise, the measurements will reflect this mobility and not the mobility of the protein. It is also desirable to know *a priori* the relative orientation between labeled protein and the transition dipole moments of the label to be able to directly relate the measured polarizations to the orientation of the protein under study. The development of bifunctional fluorescent probes (Corrie *et al.*, 1998) has fulfilled both requirements. The two attachment points reduce probe mobility (Peterman *et al.*, 2001) and allow predicting the orientation of the dipole relative to the target protein if its atomic structure is available (Corrie *et al.*, 1999a). Proper attachment of the probe by its two functional groups can be assessed by enzymatic proteolysis followed by mass spectrometry (Corrie *et al.*, 1999a; Peterman *et al.*, 2001).

We have used in all our studies the bifunctional fluorescent probe BSR (B10621, Molecular Probes Invitrogen, Carlsbad, CA), which has excitation and emission maxima at 549 and

575 nm, respectively. This probe has two sulfhydryl reactive groups so that it can be attached to cysteine residues in a protein. Location-specific labeling is achieved by making cys-light mutant versions of the protein under study and introducing unique pairs of solvent exposed cysteines properly spaced from each other (distance between β -carbons ~ 1.5 nm for BSR labeling). The transition dipoles of the BSR probe are parallel to their sulfhydryl-reactive functional groups (Corrie *et al.*, 1999a; Penzkofer and Wiedmann, 1980). Consequently, when the structure of the protein of interest is known, the orientation of the transition dipole moments of the fluorescent probes can be readily determined within the protein's frame of reference.

We have labeled kinesin constructs with BSR in several locations without lack of function (microtubule binding, ATPase activity, or ability to move processively), and other groups have successfully labeled other cytoskeletal proteins with BSR or BR, another rhodamine based bifunctional probe (Corrie *et al.*, 1999a; Julien *et al.*, 2007; Knowles *et al.*, 2008). Needless to say, any new labeled protein or location has to be tested for possible deleterious effect introduced by mutagenesis or probe attachment.

V. Discussion and Future Directions

As indicated in the previous sections, FPM has distinctive advantages that make it uniquely suitable to solve structural questions. It allows studying dynamic processes in real time and at the single-molecule level. In comparison with fluorescence resonance energy transfer (FRET), another fluorescence-based technique that requires a pair of labels (donor and acceptor), at a limited distance range, FPM requires a single label which considerably simplifies the labeling strategy.

When applied to single molecules, FPM can also provide fluorophore position information with nanometer accuracy depending on the signal/noise level of the images (Thompson *et al.*, 2002; Yildiz *et al.*, 2003). Combining orientational and positional information will help determining the structure of multiple subunit macromolecular complexes by providing spatial constraints to the possible ways that the subunits arrange in a complex. This information will complement methods to produce atomic models of multisubunit macromolecular complexes that combine medium-resolution 3D electron microscopy data with atomic resolution structures of the individual subunits obtained by X-ray crystallography or nuclear magnetic resonance (NMR) (Fabiola and Chapman, 2005). Advantages of FPM over these approaches is that it can be applied under more native-like conditions (room temperature, low concentrations, no crystals) and allows direct observation of dynamics on the millisecond timescale.

Important areas for further development of FPM are improving the time resolution and the development of new suitable fluorophores. The current single-molecule FPM methods used have a time resolution of over 20 ms, which limits its use to follow relatively slow dynamic events. Using FPM in combination with higher time resolution techniques, like the recently developed FRET confocal-correlation analysis (Verbrugge *et al.*, 2007), will allow one to follow changes in orientation/mobility in the sub-milliseconds time range.

Development of more bifunctional fluorescent probes with different functional groups and spectral characteristics will allow the orientation of different proteins or protein domains to be determined simultaneously by multicolor FPM. One group of currently available probes with very good potential but which has not yet been used extensively in FPM studies is the bifunctional fluorescent arsenical derivatives known as FIAsH and ReAsH (Adams *et al.*, 2002). These probes bind specifically and with low mobility about the attachment point to a genetically encoded sequence (Machleidt *et al.*, 2007). Use of these probes will reduce the number of mutations necessary to achieve specific labeling as only the required target sequence would be needed. It will also facilitate extending FPM studies to live cells.

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Fig. 1.

High- and low-mobility transitions of a kinesin motor domain walking processively along a microtubule at low [ATP]. (A) Order parameter r (low r values correspond to high angular mobility) plotted as a function of time for a kinesin molecule fluorescently labeled in one head. The solid symbols connected by the thin lines correspond to the r -values determined from the ratios between fluorescence intensities (LDs) corresponding to different polarization excitation directions. The solid thick line (red) corresponds to the idealized r -factor transition events detected using a hidden Markov algorithm (Qin, 2004). (B) Interpretation of the observed mobility transients. At sub-saturating [ATP], a kinesin molecule pauses between steps in the ATP-waiting state. The fluorescently labeled head alternates between microtubule-bound (low mobility, high r) and tethered configurations (high mobility, low r) as the two heads alternate positions during processive walking. For further details, see text and the work of Asenjo and Sosa (2009). (See Plate no. 31 in the Color Plate Section.)

Fig. 2.

Fluorescence polarization microscopy (FPM) set-up and angle definitions. (A) Experimental set-up. The FPM set-up used by the authors is built around an inverted microscope system (Eclipse TE300, Nikon, Melville, NY) according to the figure. Laser (Model 142H-532-200, Lightwave Electronics, Mountain View, CA); BE: 3× Beam expander (Melles Griot, Albuquerque, NM); M1, M2: Mirrors; EOM: Electro-optic modulator (M350-80, Conoptics, Danbury, CT); WP: $\frac{1}{4}\lambda$ wave plate (Tower Optical, Delray Beach, FL); FW1, FW2: Motorized filter wheels (Prior Scientific, Rockland, MA); D1, D2: Dichroic mirrors (DM545LP, Chroma, Rockingham, VT); DL: Defocusing lenses; OBJ: Objective (Nikon 100× Plan Apo TIRF, NA: 1.45); F1, F2: Emission filters (XR3002 538AELP Omega, Brattleboro, VT and D570/40, Chroma, Bellows Falls, VT); MS: Microscope XY translation stage; 4XRL: 4× Video relay lens (Nikon, Melville, NY); CCD: Charged-coupled device camera (I-Pentamax, Roper Photometrics, Tucson, AZ or iXon^{EM} 897, Andor Technology, South Windsor, CT); WFG1, WFG2: Arbitrary waveform generators (33120A, Agilent Technologies, Santa Clara, CA). (B) Angle definitions. Microtubule filaments lay flat in the XY-plane. ω is the angle of the filament long axis with the X axis. β is the average axial angle between the fluorophore absorption dipole and the filament axis, and ε is the azimuthal angle around the filament axis. Different fluorophores bound to equivalent sites on the filament with cylindrical symmetry (as would be the case for kinesin-labeled molecules bound to a microtubule) will have variable ε angles and the same β angle. Γ is the semi-angle of the cone in which the dipole is able to pivot very rapidly (mobility cone angle). (See Plate no. 32 in the Color Plate Section.)

Fig. 3.

Ensemble fluorescence polarization microscopy (FPM) data. (A) Sequence of fluorescence images corresponding to four polarization excitation directions (indicated by the numbers in each panel). Note the clear fluorescence anisotropy of this sample: the fluorescence intensity depends strongly on the angle between the microtubule and the polarization excitation axes. The anisotropy is due to the alignment of the fluorophore dipoles relative to the microtubule axis. (B) LD_{0-90} versus microtubule angle in the XY plane (ω). Each data point corresponds to an LD_{0-90} measurement of a microtubule segment decorated with many fluorescently labeled kinesins. The line corresponds to a nonlinear fit of the data to Eq. (4). LD^0 is the LD_{0-90} for microtubules parallel to the X -axis ($\omega = 0^\circ$). In this example the LD^0 estimated from the fitted curve is -0.73 , indicating that the fluorophore dipoles are nearly parallel to the microtubule axis.

Fig. 4. Single-molecule fluorescence polarization microscopy (FPM) data. (A) Fluorescent image of a microtubule with individual fluorophores bound. (B) Kymograph made from part of the microtubule shown in A. (C) Fluorescence intensity trace of a single molecule. Each line corresponds to a different polarization excitation direction as indicated in the figure (90° , 135° , 0° , 45°). The clear anisotropy of the molecule in this example indicates low mobility. The relatively constant intensities over time together with the sudden photobleaching (occurring here at ~ 14 s) is the typical behavior of single-fluorophore records.

Fig. 5.

Contour plot of LD^0 as a function of axial angle β and mobility cone angle Γ . The plot represents LD^0 as a function of the angles β and Γ according to Eq. (3). The darkest gray level corresponds to $LD^0 = -1$ and the lightest to $LD^0 = 1$. Iso-contour levels are shown at each 0.1 LD^0 units. Dashed and continuous contour lines correspond to negative and positive LD^0 , respectively.

Fig. 6.

Distribution of simulated projected angles relative to the microtubule axis (α). The inset on the right illustrates the definition of projected angle. The figure shows the distributions of absolute projected angles $|\alpha|$ (binned in 1° intervals) for dipoles uniformly distributed around the filament with axial angles β of 20° (light bars and dashed line) and 70° (dark bars and solid line). The range of possible values of projected angles α is -90 to 90° , but they are grouped according to their absolute value $|\alpha|$ because the distributions are centro-symmetric about 0° ($P(\alpha) = P(-\alpha)$). The modeled distributions were calculated from 10,000 vectors uniformly distributed around the microtubule axis ($\varepsilon = 0-360^\circ$ or $\varepsilon = 0-180^\circ$) with the indicated axial angles α without adding noise (bars) or adding Gaussian noise (lines). As shown the peak of the projected angle distributions coincide with the axial angle β and the effect of adding experimental noise is to broaden the distributions.

Fig. 7.

LD₄₅₋₁₃₅ versus LD₀₋₉₀ plot. The two LDs obtained from a single fluorophore have a characteristic relationship, depending on the angular mobility and projected angle ϕ . In this plot the order r -factor is equal to the radial position. Thus, fluorophores with increased mobility will locate at smaller radius. Circles with r values of 1 (no mobility) and 0.31 (mobility) are shown. Probes with similar ϕ values (-90° to 90° possible values) will locate along similar radial lines as indicated.