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Multicolor Tracking of Molecular Motors at Nanometer Resolution

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Abstract

Molecular motors move processively along cysstoskeletal filaments through stepping of their catalytic head domains. Observation of how the heads step relative to each other reveals the mechanism of motor processivity and various gating mechanisms used by motors to coordinate the cycles of the catalytic heads during processive motility. This chapter will discuss recent developments in simultaneous observation of the stepping motions of the two heads using two-color single particle tracking microscopy.

Keywords

motor proteins; kinesin; dynein; myosin; particle tracking; single-molecule imaging

1. Introduction

Motor proteins of the cytoskeleton use ATP hydrolysis energy to generate motion and force along cytoskeletal filaments. These motors are responsible for a wide variety of cellular functions including contractile and swimming motility of whole cells, intracellular cargo transport, cell division, and organelle positioning. An individual motor's ability to walk processively without dissociating from its track is essential for the efficient transport of intracellular cargos over long distances inside cells. Previously, it was thought that processivity requires strict coordination between the stepping cycles of the two catalytic domains (heads) in order to prevent simultaneous detachment of both heads at a time. However, it had not been possible to directly monitor the stepping movement of both heads of a walking motor in real time, due to the small size (8 – 36 nm) of the steps and lack of microscopy tools to achieve high spatial and temporal resolution.

The motion of fluorescently labeled motor proteins can be tracked at high resolution *in vitro* as they walk along filaments by fitting the fluorescent spot to a two-dimensional Gaussian mask[1]. The accuracy of this technique depends on the number of photons collected from the

Techniques presented: FIONA, multicolor tracking/image registration.

4. Notes

fluorophore and is arbitrarily high[2]. Recent advances in single-molecule imaging enable labeling of the two heads of the motor with different colors of fluorophores for tracking of the stepping movement of the heads simultaneously at nanometer resolution[3]. While two-color single-molecule localization microscopy yields a richer dataset than one-color single-molecule localization microscopy, it is also more technically challenging. Two-color imaging requires proper selection of spectrally separable fluorophores, efficient and specific labeling of motors with these probes, tracking of each fluorophore at nanometer resolution without significant crosstalk between their fluorescence emission, and precise registration of the two fluorescent channels to determine the relative positions of the two probes as the motor walks.

Multicolor tracking has been utilized to study the stepping mechanism of myosin V[3,4], myosin VI[5], and cytoplasmic dynein[6–8]. Organic dyes (Cy3/Cy5[3,8]), quantum dots (QDs, QD565/QD655[4], QD585/QD655[6,7]), and QD and gold nanoparticles[5] are used as fluorophore pairs. These probes are conjugated to the motors using biotin/streptavidin[4,5], calmodulin exchange[3], and HaloTag[6,8] labeling strategies. Usually, the fluorescence signals of two fluorophores are separated spatially using dichroic mirrors[3,5,6]. The locations of the heads of dual-labeled motors are determined by registration of the two fluorescent channels by moving a fiducial marker that emits in both channels in a predictable, known pattern throughout the field of view[3,6]. Alternatively, organic dyes can be excited one at a time and their signal can be collected in a single CCD channel using a time-sharing protocol. This approach prevents splitting and merging of fluorescent channels in image registration, reducing the aberrations in fluorescence detection[4,8]. However, the approach is not readily applicable to QDs because QDs efficiently absorb higher-energy photons than their first exciton peaks, thus cannot be selectively excited.

Two-color tracking of molecular motors revealed direct visualization of hand-over-hand motion in myosin V[3,4], and the presence of both hand-over-hand and inchworm motility in myosin VI[5]. Our lab explored the stepping mechanism of cytoplasmic dynein by labeling dynein's catalytic heads with a QD585/QD655 fluorophore pair and simultaneously monitoring their stepwise movement at nanometer resolution[6]. We found that dynein's two heads move independently of each other, a mechanism fundamentally distinct from the hand-over-hand movement of kinesin and myosin motors. The heads remain widely separated and often walk along different protofilaments of a microtubule, with the leading head preferentially located to the right of the trailing head. Dynein's stepping movement is partially coordinated when the heads are separated from each other, which may lead to the helical motion along microtubules observed during dynein motility[9]. Similar results on yeast dynein stepping have been obtained using organic dye pairs[6,8].

Multi-color localization microscopy has enabled a deeper characterization of the stepping mechanism of molecular motors. The method can be further extended to study the conformational dynamics and motility of other complex machineries.

2. Materials

1. Microscope slides

2. No. 1 ½ microscope cover slips, 18×18 mm, Corning
3. Double-sided tape, 3M
4. Dynein lysis buffer (DLB): 80 mM HEPES pH 7.4, 1 mM EGTA, 2 mM MgCl2, 10% glycerol
1. tris(2-carboxyethyl)phosphine (TCEP), 200 mM
2. Sea urchin axonemes[10] or microtubules[11] (see Note 1)
3. Casein (pre-blocking agent), 40 mg/ml dissolved in DLB.
4. Biotinylated GFP-GST-Dyn331kD-DHA, 100 nM (see Note 2)
5. Streptavidin-coated QD-655s and QD-585s (Life Technologies) (see Note 3)
6. ATP, 100 mM dissolved in water, pH adjusted to 7.0 with KOH.
7. Phosphoenolpyruvate and pyruvate kinase (for ATP regeneration) (see Note 4)
8. β-mercaptoethanol, to reduce QD blinking (see Note 5)
9. Hole array coverslip (Miraloma Tech, LLC) mounted on a microscope slide for high accuracy registration of the fluorescent channels[12]
10. Rhodamine Red-X NHS ester (Life Technologies)

3. Methods

Here, we describe a method for dual-color labeling and tracking of yeast cytoplasmic dynein. Similar approaches have been used to study myosin motors. We recommend labeling dynein after it is already attached to filaments (Figure 1) to minimize the attachment of multiple dynein motors to the same QD due to the multivalency of QDs. (see Note 6)

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1. Filaments should be selected based on the purpose of the experiment. For example, microtubules need to be taxol-stabilized and freshly prepared every week for the assays. Axonemes are highly stable and can be used for years when stored at −20 ℃ in the presence of 40% glycerol. Post-translational modifications of tubulin affect the binding rate and processivity of the motors[13]. Therefore, consistency of the results between diferent axoneme and microtubule preparations needs to be verified to validate the results.
2. Tagging and labeling of the motors may affect their velocity and step size. For example, myosin and dynein can tolerate biotin-streptavidin labeling when they are labeled at the head region. In comparison, kinesin is smaller in size (the heads are separated by 8 nm when attached to the microtubule) and cannot tolerate dual labeling of its heads with streptavidin-functionalized QDs (20 nm in diameter). Careful consideration of available bioconjugation strategies should be given that is best for your system. Run length and velocity of labeled motors should be compared to unlabeled motors before further analysis.
3. The peak emission wavelengths of the fluorophore pairs should not overlap significantly. QDs have narrow emission bands (~30 nm full-width at half maximum), and as a result,60 nm spectral separation of the emission maxima of QD 585 and QD 655 does not lead to more than 5% leakage of one fluorescence channel to another. Organic dyes have a wider emission spectrum and hence larger spectral distance between the emission maxima of the two colors may be needed to avoid significant leakage between the channels.
4. Use an ATP regeneration system such as PEP/PK or creatine kinase/creatine phosphate to maintain the ATP concentration and prolong the life of your samples.
5. Antifade or antibleaching reagents enhance the performance of fluorophores. We found that beta-mercaptoethanol enhances the brightness and decreases the blinking of Life Technologies QDots for approximately ten minutes but significantly decreases QD brightness after 10 minutes of exposure to BME. We also saw moderate improvement in QD brightness with the addition of Trolox.
6. Multicolor labeling can be performed on motors in solution or motors already bound to filaments. Motors in solution are more concentrated than those bound to filaments, hence it may lead to a higher percentage of dual-labeled motors using this procedure. However, mixing QDs with motors may lead to crosslinking between different motors or, in the case of a homodimer, within the motor itself. We recommend labeling the motors with QDs when the motors are bound to their track.

3.1 Total Internal Reflection Fluorescence microscopy

1. An objective-type TIRF microscope was custom-built with a Nikon Ti-Eclipse microscope body, 100× 1.49 Plan-Apo objective.
2. The fluorescence signal is separated into two channels using an Opto-Split II (Cairn), equipped with 615 nm long pass dichroic beamsplitter, and 585/40 and 655/40 emission filters (Figure 2).
3. Fluorescence images are collected using an electron-multiplied charge-coupled device (EM-CCD) camera (Andor Ixon+, 512×512 pixels, 160 nm effective pixel size).
4. The sample is excited with a 15 – 50 mW 488 nm laser beam (Coherent Sapphire).
5. The microscope is outfitted with a piezoelectric nanopositioner (P527.3CD, Physik Instrumente) for image registration.

3.2 Image Registration

To simultaneously track two fluorophores on a walking motor and measure the distance between them, the relative positions of the fluorophores must be registered to nanometer accuracy. Image registration accounts for positional, rotational, and magnification differences, spherical aberrations, astigmatism, and irregularities in the CCD chip.

1. Illuminate the hole array slide under brightfield. The hole spacing is 2 μm X 2 μm.
2. Scan the entire CCD surface by moving the sample at 20 nm increments in both x and y axes with a full travel area of 2 μm X 2 μm. Acquire data during sample scan.
3. Analyze the recorded movie by localizing the position of the holes in both channels of the CCD chip.
4. Fit coordinates of more than 1,000,000 hole-pairs using non-linear regression with Taylor polynomials to make a subpixel map from these colocalizations.
5. To correct for chromatic aberrations, create fiducial markers made of amine-coated QD-655 and Rhodamine Red-X NHS ester. Rhodamine Red-X is nearly isospectral with the QD585.
6. Move the sample at random intervals and directions across the CCD surface to cover the full chip and obtain more than 100,000 colocalizations.
7. Use nonlinear regression fitting to refine the registration map. This map will be used to measure the distance and position of the two fluorophores.

3.2 Sample preparation, multicolor labeling and data acquisition

1. Dynein motors are expressed in yeast and labeled with biotinylated Halo Tag ligand, as described previously[6].

2. Prepare a flow chamber for the sample using double-sided tape to space out and adhere the coverslip to the slide. Insert all fluids into the chamber from the same side using a micropipettor, creating flow by applying a tissue to the other side to absorb the exiting liquid. The flow chamber holds approximately 10 μl.

3. Prepare dynein lysis buffer with 1 mg/ml casein (DLBC): combine 960 μl DLB, 40 μl of 25 mg/ml casein

4. Flow 10 μl of diluted axonemes in DLB buffer and incubate for 1 minute. Repeat. Remove unbound axonemes by washing with 10 μl DLB and 10 μl DLBC.

5. Flow 10 μl of dynein diluted to 1 nM in DLBC. Incubate for two minutes. Wash three times with 15 μl DLBC.

6. Flow 200 nM Streptavidin-coated QD-655s and QD-585s in DLBC and react with microtubule-bound dynein for 2 minutes. Wash three times with 15 μl DLBC. (see Note 7)

7. Make imaging buffer, containing DLBC, 5–15 μM ATP, 2 mM phosphoenolpyruvate and 0.1 mg/ml pyruvate kinase. Flow 30 μl of imaging buffer.

8. Seal each side of the chamber with clear nail polish and allow to dry for 1 minute. Place the sample in the microscope's sample holder.

3.4. Data Acquisition

1. Adjust laser power to detect on average 10,000 photons from a single QD per frame. At 30 Hz frame rate, we excite the sample with 5 kW/cm^2 488 nm laser beam to detect ~10,000 photons from a single QD per frame.

2. Adjust ATP concentration to lower the stepping rate of the motor to 0.05 steps/frame. This allows detection of the probes position 10–20 times as the motor dwells between subsequent steps. 98% of the steps are detectable under this condition.

3. After collecting the movies, perform image registration procedure to obtain the registration map.

3.4. Data analysis

Key steps of data analysis are shown in Figure 3.

1. Split the two fluorescent channels into two separate movies, color them separately and overlay both movies into a single multi-color movie in ImageJ. Inspect the overlay movie visually for colocalized moving spots, which represent dual-labeled dynein motors processively moving along microtubules.

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[7]·The time allowed for labeling should be optimized to maximize the number of targets labeled and minimize the time required to make each sample. The sample preparation procedure described above takes 15 minutes in total.

2. Track each colocalized spot using Gaussian mask fitting. In our lab, we use a custom ImageJ macro that tracks spots designated using the multi-spot pointer tool.
3. Use principal component analysis to decompose the motion of the motor along the length of the filament (on-axis), and along the width of the filament (off-axis).
4. Visually examine traces for the quality of Gaussian tracking. Movement is anticipated to be linear and stepwise in the on-axis plot. Sudden large (>100 nm) changes in probe position or fluctuations between two distant positions likely occur because of the presence of nearby fluorophores. Further inspect these spots in the raw movies and use a smaller fitting to exclude interference of the nearby fluorescent spots in tracking. Select the regions of the traces that fit these criteria and analyze further.
5. Remove false positives for colocalization by measuring the velocities of the traces of each color. If the velocities are significantly different, discard the traces.
6. Remove false positives for one QD whose fluorescence appears in both channels. Compare the intensity of the probes as a function of time using e.g. ImageJ's "Plot Z-axis profile" command. The intensities of the two probes should display unique blinking patterns and should not be correlated. If fluctuations in fluorescence signal are correlated, discard the traces.
7. Fit the remaining dual-color traces for steps using a Schwarz Information Criteron step-fitting algorithm[12]. Set minimum detectable step size to 3 nm.
8. Visually inspect the quality of step fitting. The fit should agree with the data. Plot residuals of the fit to find steps that deviate significantly from data.
9. Overlay the on-axis traces of each color in time (Figure 4).
10. Construct a histogram of step sizes or amount of time spent in one location (dwell time).
11. Analyze the order in which the two heads move relative to each other (i.e. alternating vs. nonalternating steps, see an example trace in Figure 4).
12. Analyze the step size and direction as a function of interhead separation.
13. Analyze the stepping rate as a function of interhead separation.

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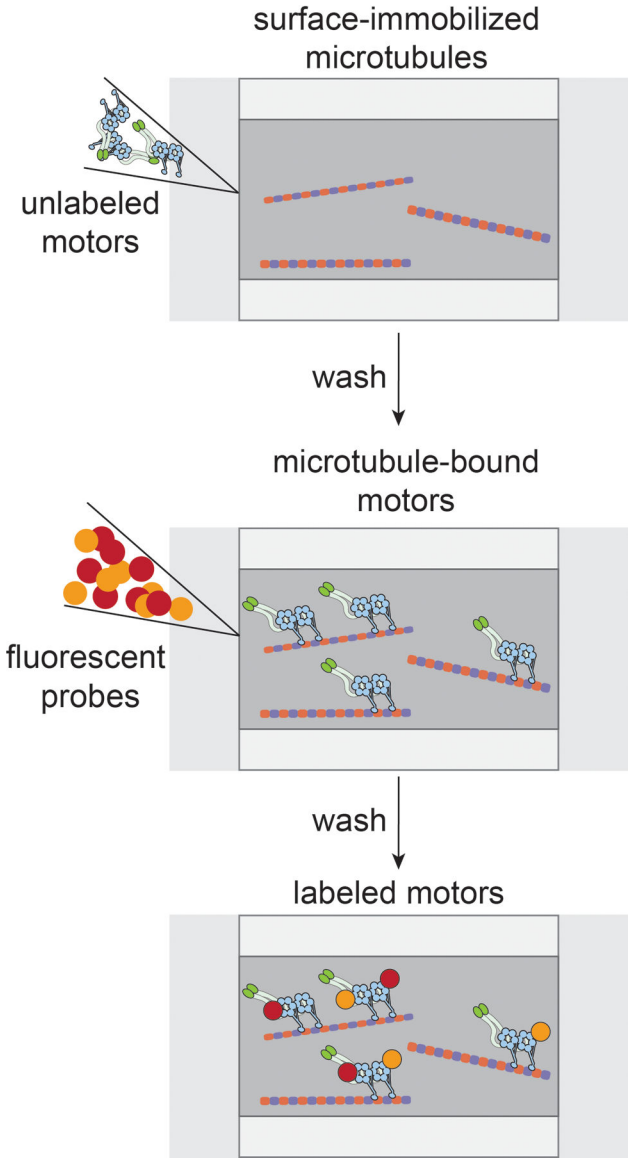

Figure 1. On-slide labeling of motor proteins to minimize crosslinking between QDs.

Biotinylated dynein motors are flowed into a microscope chamber containing filaments adsorbed onto the surface of a cover slip. Functionalized QDs are then added to the chamber for labeling. Unbound motors and QDs are washed away after each step.

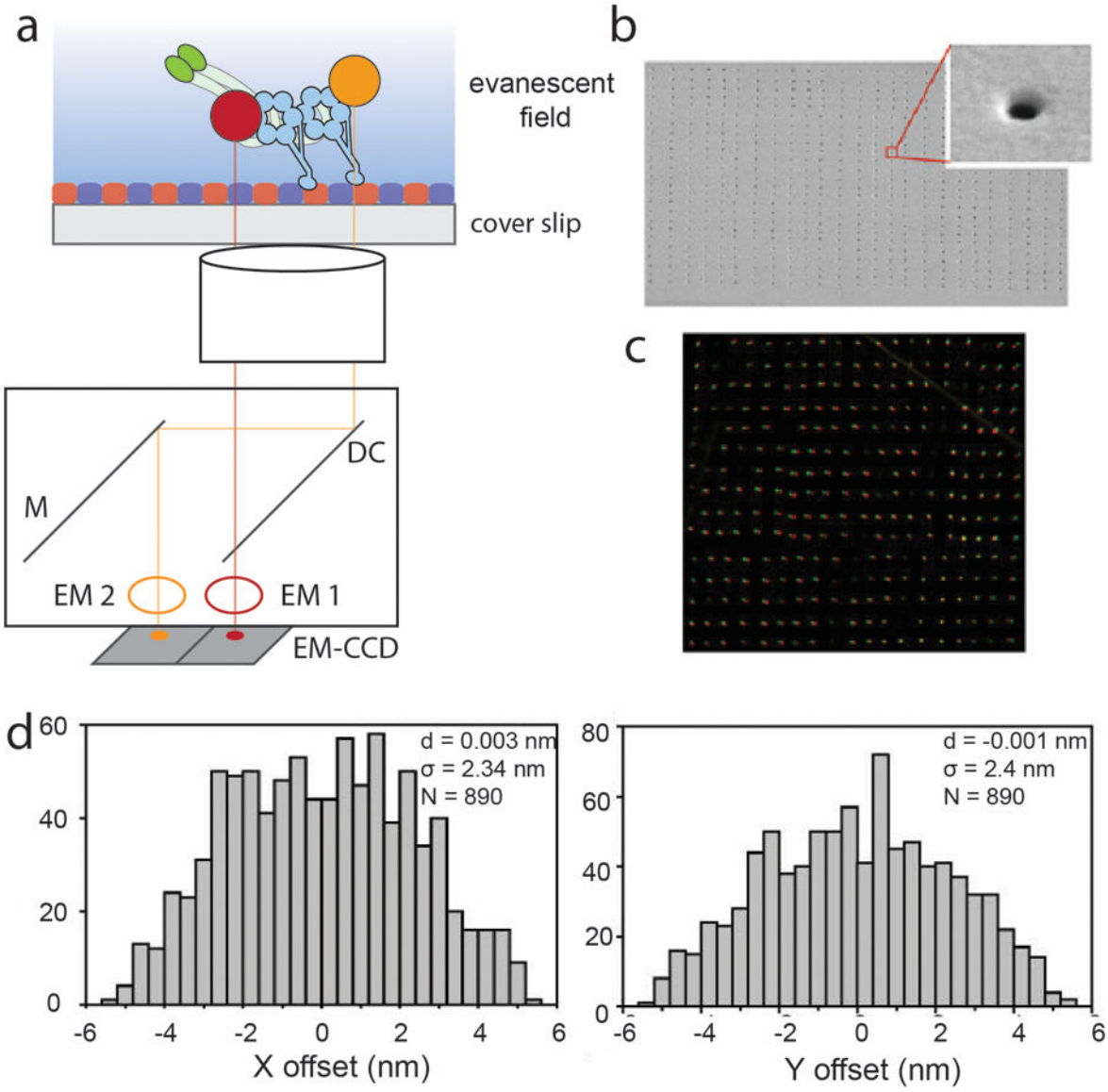

Figure 2. Two-color optical imaging setup and construction of fiducial map.

a. Total internal fluorescence microscope equipped for two-color single-particle tracking. Processive motility of labeled monitors is monitored by exciting the fluorescent probes with an evanescent field at the glass-water interface and splitting the fluorescence signal into two channels on a CCD detector. DC = dichroic; M = mirror; EM = emitter. b. Transmission electron microscopy image of nanohole array used for channel registration. c. Map generated using brightfield images from nanohole map. d. Histogram of calculated distance between fluorescence from QD655-Rhodamine Red X probes appearing in respective channels.

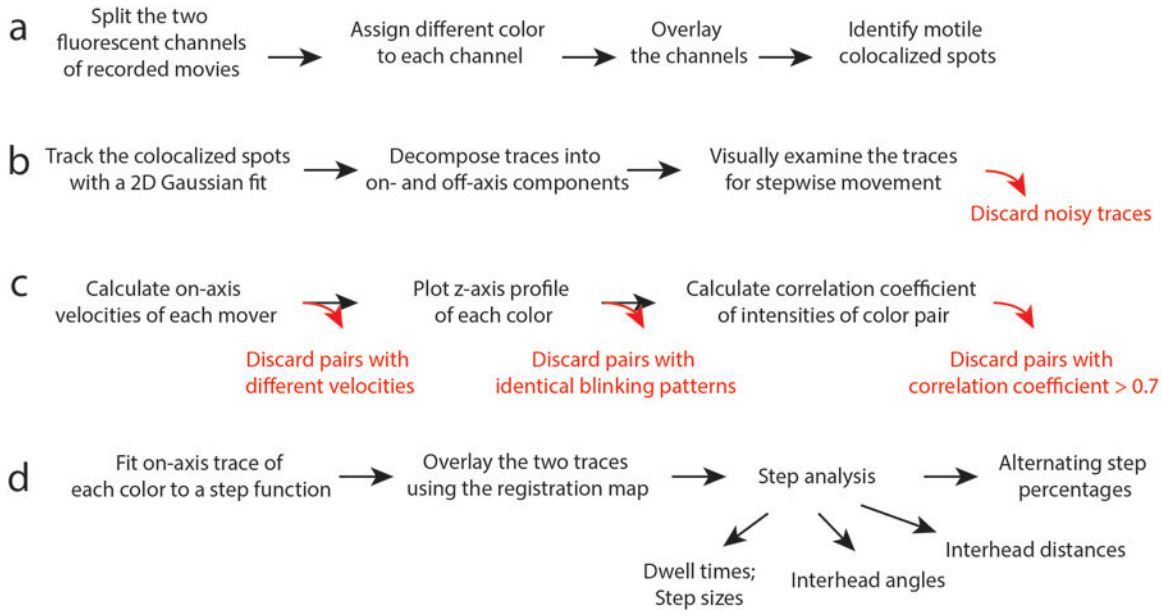

Figure 3. Data analysis.

a. Visual inspection of two-color movies for colocalizing QDs. b. Single-particle tracking analysis and decomposition of motion into the on-axis and off-axis components relative to microtubule orientation. c. Verification that traces from purported colocalizers originate from QD probes on the same motor and are not cross-talk between channels from the same QD probe. d. Quantitation of data using step analysis.

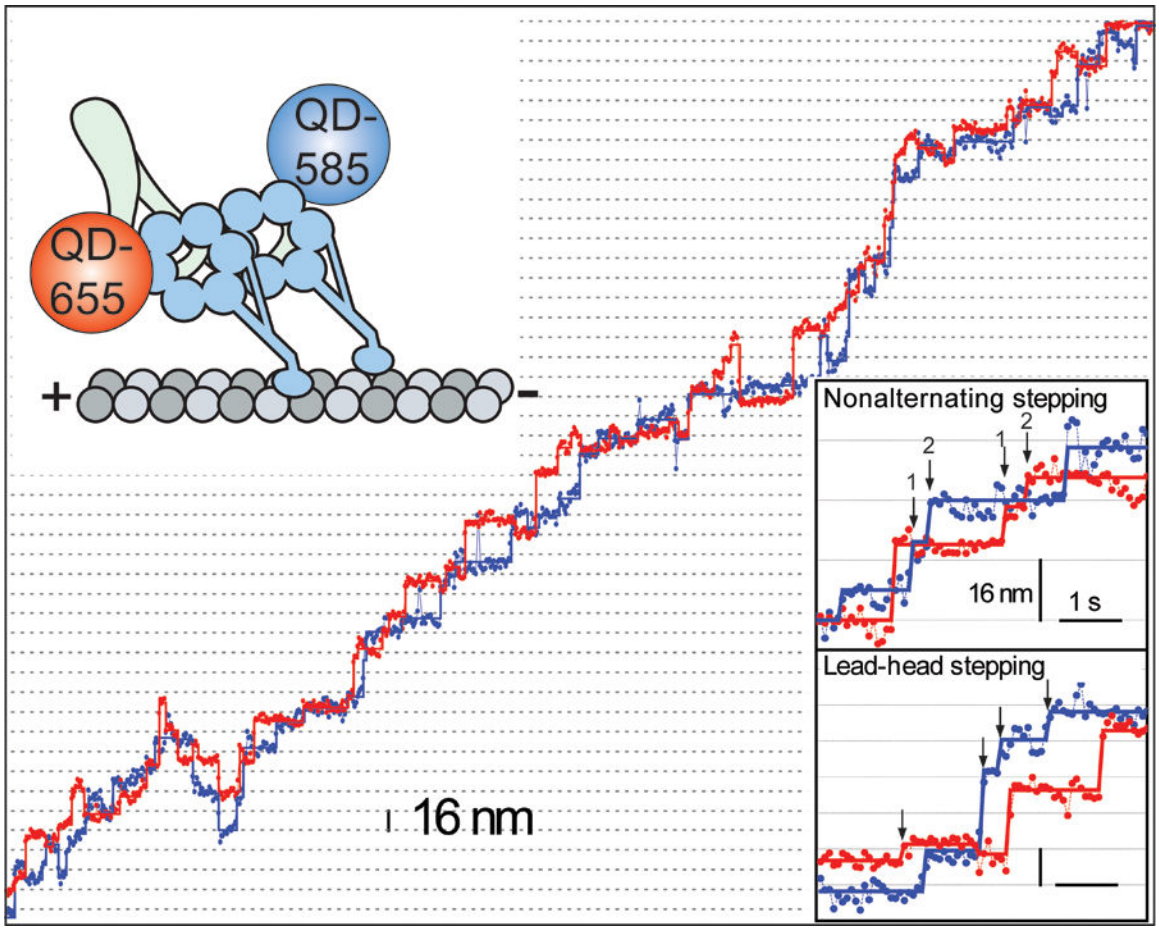

Figure 4. Multicolor tracking of dynein motility.

(Top insert) Dynein is labeled on both heads with QD-585 and QD-655. (Middle) Stepping trace of a dual-labeled dynein motor. Horizontal lines represent the fit of the trace to a stepwise function. (Bottom insert) Details of the trace show uncoordinated stepping of the two heads during processive motility. Figure is modified from reference 6.