Defocused orientation and position imaging (DOPI) of myosin V

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The centroid of a fluorophore can be determined within ~1.5-nm accuracy from its focused image through fluorescence imaging with one-nanometer accuracy (FIONA). If, instead, the sample is moved away from the focus, the point-spread-function depends on both the position and 3D orientation of the fluorophore, which can be calculated by defocused orientation and position imaging (DOPI). DOPI does not always yield position accurately, but it is possible to switch back and forth between focused and defocused imaging, thereby getting the centroid and the orientation with precision. We have measured the 3D orientation and stepping behavior of single bifunctional rhodamine probes attached to one of the calmodulins of the light-chain domain (LCD) of myosin V as myosin V moves along actin. Concomitant with large and small steps, the LCD rotates and then dwells in the leading and trailing position, respectively. The probe angle relative to the barbed end of the actin (β) averaged 128° while the LCD was in the leading state and 57° in the trailing state. The angular difference of 71° and Paul R. Selvin*‡**...
We switched between an in-focus image, i.e., FIONA, to get the centroid very accurately, and DOPi, to get the angles while myosin V molecules walked along the actin in the presence of ∼300 nM ATP. In total, 32 molecules were observed with alternating focused and defocused imaging. These combined measurements were generally more accurate for measuring the centroid than were pure DOPi measurements. Nevertheless, we also used DOPi exclusively to get the angles and the lateral position of the fluorophore (see later in text and Fig. 4B).

We labeled CaM with a single BR that was attached to two cysteine residues at positions 66 and 73 and exchanged onto the LCD of myosin V (4). We measure the 3D orientation of the dipole axis in a laboratory spherical coordinate frame (θ, φ; Fig. 1A) and subsequently transformed the angles into an actin-based coordinate system (α, β) for the moving myosin V molecules. Here β is the axial probe angle with respect to the actin, and α is the azimuthal angle around actin (see Fig. 2A for Movies 1 and 2 and Figs. 3 and 4, which are published as supporting information on the PNAS web site). Because the myosin V is labeled on one of the light chains, the step sizes alternate between short and long steps (Fig. 2B) (1). For example, if the dye is on the leading lever arm, and the myosin V takes a step, the displacement of the fluorophore is a relatively short 37 ± 2 nm, while x is the in-plane distance of the dye from the midpoint of the lever arm. (That is, x is the distance from the stalk, assuming there is sufficient symmetry.) The following step will show a larger displacement, 37 + 2x nm. To identify the LCD angle obtained after the larger and smaller values corresponding to the leading and trailing states, we combined FIONA and DOPi.

Fig. 3A shows a sample trace obtained from focused and defocused images that show near-44 nm alternating sized steps (see the molecule at the center of the screen in Movie 3, which is published as supporting information on the PNAS web site). The first step in the FIONA trace is near 115 nm (black curve). This step includes two hidden steps that can be tracked from β changes (red curve). During this period the LCD goes through three transitions (trailing → leading → trailing → leading) that will generate two long steps and a short step (2 × 44 nm + 30 nm = 118 nm ≈ 15 nm). After this step, β switches from ∼53° to ∼150° in going from the trailing position to the leading position. The following step sizes in Fig. 3A are alternating short steps (∼30 nm) and long steps (∼44 nm). When going from the trailing LCD to the leading LCD, on average β changes from 60° to 146°. The azimuthal angle, α, changes during the first step shown in Fig. 3A from 43° to 61°.

Because of the dipolar degeneracy, there is another set of possible averaged (α, β) angles, (α = 180°–180°, 180°–β) = (−138°, 120°) for the trailing LCD and (−117°, 32°) for the leading LCD, respectively. The negative α values imply the myosin V molecule occupying the space in the upper hemisphere underneath the actin filament. However, because of the size between the actin and the glass slide by the streptavidin linkage, we expect most of the molecules to walk along the top face of the actin, away from the glass. For most of the molecules analyzed in the present study (94 of 97 molecules), restricting the angular range of the probes to the upper hemisphere, e.g., 0 < β < 180° and 0 < α < 180°, resulted in the probe angle β, relative to the barbed end of actin, being greater for the leading head (after completing a large step) than for the trailing head (after a small step). Because the probe probably lies within ∼40° away from the LCD axis (4), the leading LCD is expected to adopt a larger angle relative to the barbed end of actin than the trailing LCD (17). If we adopt the opposite convention for converting the laboratory coordinate angles into the actin reference frame, then α becomes negative, implying that the myosin V molecules are between the actin and the glass, and the β angle is larger after the smaller LCD step (trailing position) than after the larger step (leading position). The unlikely nature of both of these conditions implies that the bound state after the larger FIONA step and the larger β angle consistently identify the leading LCD. In a few of the molecules that swung very near to the x-y plane,
analyzed by FIONA; black lines, averaged position within each dwelling period; red diamonds, raw of five consecutive defocused images and three focused images. The sample is moved away from the best focus by 500 nm. Black circles, raw position data

V molecule that was imaged by switching between focused and defocused imaging. The exposure time per frame is 0.66 s. We have captured repeated cycles of molecules showed anticorrelated

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a slight azimuthal tilt into the lower angular hemisphere was apparent, as explained in Supporting Text.

Fig. 3B shows a molecule that alternates between ~64- and ~10-nm step sizes (see the molecule at the center of the screen in Movie 4, which is published as supporting information on the PNAS web site). In our earlier work (1), this molecule would have been classified as taking 74- and 0-nm steps because we did not have either sufficient accuracy or the simultaneous angular information that we have here. These results indicate that myosin V does not have a sharp kink structure in the leading lever arm as previously proposed (18). The sharp kink, like the bent knee of a telemark skier (18), is ruled out because (i) an angular transition occurs for every step, as expected for a nonkinked lever arm (Fig. 2B), and (ii) unless the lever arm is kinked, long steps are expected to be followed by short steps (Fig. 2B), but not ~0-nm steps. Notice that if the fluorophore is placed on the MD, e.g., with a green fluorescent protein (GFP), a 74- to 0-nm stepping pattern is expected, as indeed was observed (18). This result is consistent with a more detailed analysis that shows 64–10 nm (19). In Fig. 4, we show a trace of ~53–19 nm stepping pattern (see the molecule at the center of the screen in Movie 5, which is published as supporting information on the PNAS web site).

In Fig. 3B, when going from the trailing LCD to the leading LCD, β changes from ~50° to 148°, a similar β change as in Fig. 3A. Notice that in this case and in Fig. 4, the α and β angles are anticorrelated, meaning they change at the same time but in the opposite direction. Other molecules show correlated changes of α and β (e.g., Figs. 3A and 4B). The amplitude of back-and-forth sideways (α) motions averaged ~27° (see Fig. 9 and Movie 6, which are published as supporting information on the PNAS web site). Approximately 35% of the molecules showed correlated α and β changes, ~50% of molecules showed anticorrelated α and β changes, and in the rest of the molecules, the relationship between α and β changes was not clear. These correlated and anticorrelated α and β changes are likely to be caused by orientation of the fluorescent probe dipole out of the plane of LCD rotation. For instance, taking the probe to be oriented 40° away from the LCD axis (4), and the plane containing the LCD and the probe to be twisted or tilted by 32° relative to the plane containing the LCD and actin, the probe would exhibit the correlated changes of α and β shown in Fig. 3A. The azimuthal angle of the lever arm around the actin filament remains unchanged during this twisting. The estimated actin–LCD geometry for the molecules in Fig. 3 is shown in Fig. 5. If the plane containing the probe and the LCD is twisted or tilted by 51° in the opposite direction, then the probe would exhibit the anticorrelated changes of α and β shown in Fig. 3B. Such azimuthal variation among individual myosin V molecules is expected when their labeled CaM is on different IQ motifs (20). Note that in both of these cases, and in general, when the probe is not located in the plane of LCD tilting, the probe tilts less than the LCD. For instance in Fig. 3A, when the probe is 32° out of the LCD–actin plane, the LCD tilts from β = 122° to 15° when the probe tilts from the observed values of β = 150° to 53°, and αLCD = 17°. Although these calculations (4) show that the observed azimuthal rotations of the probe are not necessarily coming from the azimuthal rotations of the myosin V molecules, there may be other possible effects that might contribute to correlation or anticorrelation between β and α such as leaning of the molecules to the left and right on each step, twisting of LCD around its own axis, or interactions between myosin V and the glass.

Fig. 4B shows simultaneous angular and displacement trajectories for a moving myosin V measured by pure DOPI analysis (see the molecule at the center of the screen in Movie 7, which is published as supporting information on the PNAS web site). The raw displacement data from DOPI by itself is usually not precise
enough to detect alternating short and long steps because of the limited positional accuracy. However, the angular information (\(\phi_1\) and \(\phi_2\)) gives clear indications that steps occurred. The averaged step sizes are \(44\) nm for long steps and \(32\) nm for short steps for that particular molecule. Average \(\phi_2\) value for the trailing LCD is \(68^\circ\) and \(131^\circ\) for the leading LCD. The \(\phi_1\) value at the beginning of the plot is \(18^\circ\), which indicates that the probe is very close to the glass surface. Although the molecule initially has small \(\phi_1\) changes, correlated with \(\phi_2\) changes, \(\phi_1\) increases by \(20^\circ\) after five steps. This \(\phi_1\) increase indicates that the molecule walked with a slight right-handed pitch moving from side to the top of the actin.

Fig. 6 shows the histogram of \(\phi_2\) for 97 myosin V molecules undergoing 1,151 tilting events, for all of the data from focused and defocused experiments and for the purely defocused data as well.

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**Fig. 4.** Displacement and 3D orientation trajectories of two different myosin V molecules, showing \(\approx53–19\) nm and \(\approx44–32\) nm stepping. (A) A sample trace of a myosin V molecule that was imaged by switching between focused and defocused imaging. The exposure time is \(0.75\) s, and we have captured cycles of consecutive four defocused images and two focused images. For the step at \(t=19.5\) s, the image of BR is focused; therefore, the position is available, but not the orientation. The position information at \(t=9.75\) s is not shown because the image was shifting due to defocused-to-focused imaging. The sample is moved away from the focus by 500 nm. \(\phi_{\text{actin}} = 0^\circ\). (B) A sample trace of a myosin V molecule that was imaged by pure defocused imaging (DOPI). The exposure time is \(0.75\) s, and the sample is moved away from focus by 500 nm. \(\phi_{\text{actin}} = 24^\circ\).

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**Fig. 5.** Cartoon showing the estimated geometries of LCD–actin for the molecules in Fig. 3 A (correlated) and B (anticorrelated). The actin is represented by a cylinder, and the dipoles are shown in blue. The angle between the lever-arm axis and the dipole axis is \(\approx40^\circ\), and the azimuthal angle of the dipole axis around the lever-arm axis is variable for different CaM positions. Labeled light chains are shown in orange.

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**Fig. 6.** Histogram of the dwell-averaged \(\phi_2\) values for moving myosin V molecules in the presence of \(\approx300\) nM ATP. A total of 1,151 tilting events are observed for 97 myosin V molecules, and the resulting histogram is fit into a two-peaked Gaussian function \((r^2 = 0.945)\). The peak with the lower value \((\phi_1 = 57^\circ)\) corresponds to the trailing state, and the peak with the higher value \((\phi_2 = 128^\circ)\) corresponds to the leading state. The standard deviation is \(22^\circ\) for \(\phi_1\) and \(17^\circ\) for \(\phi_2\).
well. All moving myosin Vs (n = 97) had lever arm tilts (see Movies 1 and 2), in contrast to the anomalous results previously seen (4). In the absence of ATP, none of the myosin V molecules (n = 30 molecules) showed rotations. For those subsets where we looked at myosin V by means of alternating defocused and focused imaging, all of the stepping events (n = 183 steps) and tilting events were found to be coincident with each other. The averaged β values for all moving molecules switch between β1 = 57 ± 22° and β2 = 128 ± 17° (means ± standard deviations). The standard deviations indicate that the β distributions for both of trailing and leading states of the LCD are quite broad, possibly because of differences in the CaM position on the LCD. The difference between the two β angles is 71°, which can be compared with a β difference of 45° in the previous measurement (4) considering the fourfold angular degeneracy of SMFP in this study. It should be mentioned that the degeneracy problem of SMFP can be removed by making additional polarizations in the four samples of the LCD. The 71° rotation of the 24-nm LCD alone gives 28-nm translation ([=24 nm × (cos 57° − cos 128°)]) along the actin per stroke. This 28-nm translation is an average calculation assuming that both of the leading lever arm or twisting of the labeled CaM in focus. The axial probe angle difference of 45° in the previous measurements of fluorescence intensity. Images were captured by using a back-thinned charge-coupled device camera (512 × 512 pixels, 16-bit digital output pixel size; Andor, South Windsor, CT). A piezoelectric z-axis stage sample (Nano-Z100; Mad City Labs, Madison, WI) was used to control the distance between the sample and objective. The image acquisition and defocusing were synchronized by using a custom C++ program.

In conclusion, by using DOPI-FIONA, we have ruled out the possibility of nonstepping myosin V molecules (4), as well as 74- to 0-nm steps (1). We observed myosin V primarily undergoing 28-nm translation, for those subsets where we performed following the protocol described by Sakamoto et al. (24).

**Materials and Methods**

**Quantum Dots.** Q-dots (QS655) were purchased from Quantum Dot (Hayward, CA).

**Proteins.** Myosin V was prepared from brains of newly hatched chickens, and labeled on one of its CaM light chains with BR, according to published procedures (4). Actin was purified from rabbit muscle according to a published protocol (22) modified by Murray et al. (23). Actin polymerization and biotinylation were performed following the protocol described by Sakamoto et al. (24).

**Actin Immobilization.** M5BufBH (20 mM Hepes/2 mM MgCl₂/25 mM KC1/1 mM EGTA, pH 7.6) was prepared weekly for all incubations and dilutions. Observation chambers were prepared by using a glass microscope slide and a coverslip (0.17-mm thickness) separated by double-sided adhesive tape to obtain a volume of ~20 μl. Actin immobilization was done in three steps, with each step followed by washing with 100 μl of M5BufBH. Steps were as follows: (i) incubation with 50 μl of 0.67 mg/ml BSA—biotin (A-8549; Sigma) for 5 min; (ii) incubation with 50 μl of 0.5 mg/ml streptavidin (S-888; Molecular Probes) for 5 min; and (iii) incubation with 30 μl of 100 nM biotinylated F-actin (1 biotin/20 actin monomers) for 5 min.

**In Vitro Motility Assay.** Thirty microliters of ~2 nM BR-labeled myosin V molecules in M5BufBH++ (10 mM DTT and 0.1 mg/ml CaM in M5BufBH) was added to the immobilized actin in the absence of ATP. After the actin decorated with myosin V molecules was visualized, 30 μl of imaging buffer containing ~300 nM ATP was added (imaging buffer is prepared freshly during actin immobilization process). One hundred microliters of imaging buffer contained 2 μl of 20% glucose solution in distilled water, 1 μl of 2-mercaptoethanol, 1 μl of glyoxy, 10 μl of 10 mg/ml casein, 6 μl of 5 M ATP, and 80 μl M5BufBH++ (pH 7.6). Gloxy was prepared weekly with 1,665 units of glucose oxidase (G-7016; Sigma) and 26,000 units of catalase (106810; Roche) in 0.2 ml of M5BufBH buffer, passed twice through 0.2-μm syringe filters, and centrifuged at 13,000 × g for 5 min.

**Imaging.** Fluorophores were excited with a 532-nm diode-pumped Nd:YAG laser (CrystalLaser, Reno, NV) by using an objective type total internal reflection (TIR) microscope setup that included an IX-71 inverted microscope (Olympus, Melville, NY) with 1.6× magnification unit and an infinity corrected 100× objective (Olympus Planap 100×/1.45 OIL). (1) DOPI is not sensitive to the polarization of the excitation illumination, but circularly polarized excitation light was used to reduce orientation-dependent fluctuations of fluorescence intensity. Images were captured by using a back-thinned charge-coupled device camera (512 × 512 pixels, 16 × 16-μm pixel size; Andor, South Windsor, CT). A piezoelectric z-axis stage sample (Nano-Z100; Mad City Labs, Madison, WI) was used to control the distance between the sample and objective. The image acquisition and defocusing were synchronized by using a custom C++ program.

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