**Supporting Text**

**Defocused Orientation and Position Imaging (DOPI).** DOPI is a method to visually observe rotational dynamics of many fluorophores that are in the same sample plane. The main advantage of DOPI is the detection of orientations of fluorophores without any angular degeneracy and observing many molecules that are in the same sample plane. If a single fluorescent molecule is moved away from the best focus by ≈500 nm, combinations of lobes and fringes appear on the charge-coupled device (CCD) depending on the orientation of the fluorophore (see Fig. 7). Because the image is taken in a plane where the different light rays have not converged into a single focus, the observed pattern on the CCD becomes larger, and different regions on the CCD correspond to different emission angles of the molecule. The intensity distribution of a single emitter depends on Θ (the axial angle between dipole axis and optical axis), Φ (the azimuthal angle around optical axis), and $\delta z$ is the defocusing amount from the focal plane. The pattern-match analysis technique (1) determines (Θ, Φ) and the lateral position of the fluorophore. The complete theory behind DOPI and the algorithm for pattern matching can be found in refs. 2–4.

**Angular Accuracy of DOPI.** We tested the angular accuracy of DOPI by generating defocused images of BR by using the optical parameters that we use for our experiments (numerical aperture = 1.45; central emission wavelength = 575 nm; pixel size = 100 nm, total number of photons ≈ 10,000; background ≈ 60 per pixel). We also added Poissonian noise to the total number of photons and the background. First, we generated images with fixed in-plane angle values (Φ = 0) and varying Θ values with 3° increments (Θ = 0°, 3°, 90°). Then we fitted these images with our algorithm comparing the noisy images with theoretical images generated at Θ increments of 1°. The fitted images had on average 1° ± 0.70° (means ± SD.) deviations from their actual Θ values. To test axial angle (Φ) accuracy of DOPI, we generated images for fixed theta values of Θ (15°, 30°, 45°, 60°, 75°, and 90°) and varied Φ with 5° increments (Φ = 0°, 5°…355°) and fitted those images using theoretical images calculated at 1° increments in Φ. The deviations of fitted

Φ values from actual Φ values were 5° ± 4° when Θ was equal to 15° and were 1° ± 0.75° when Θ was equal to 90°. These tests demonstrated the power of the DOPI algorithm used and also showed that DOPI is more sensitive for detecting in-plane angle (Φ) when the probe orientation is nearly parallel to the surface, and it gets less sensitive for detecting Φ when probe orientation is nearer the optical axis.

Secondly, we fitted part of our experimental data with different angular increments ranging from 1° to 10° for Φ and 1° to 15° for Θ. Almost all of the extracted angle values for these images overlapped each other with deviations of ±5°. However, a disadvantage of using very small angular increments for Φ and Θ is very long computation time for the analysis (≈5 min per frame on a 3.5 GHz Pentium PC computer). Thus, we used ≈10° increments for detecting the Φ and 15° increments for detecting Θ to reduce the computation time required to ≈30 s per frame. Because of these factors, we mainly chose molecules for detailed analysis that are relatively parallel to the sample plane to obtain better accuracy especially for Φ and less dependence of the accuracy on the specific value of Θ. For myosin V experiments, we used 300 nM ATP to capture ≈5 frames per dwell period between steps using exposure times of 0.5–0.75 s.

**Actin-Based Coordinate System.** As in previous single-molecule fluorescence polarization studies (5-7), the angular position of the probe estimated in the laboratory frame (Θ and Φ; Fig. 1*A*) was transformed into a coordinate frame based on the axis of each actin filament (α and β; Fig. 2*A*) to facilitate interpretation of the motions. Whereas in the previous studies, symmetries across *x*-*y*, *x*-*z*, and *y*-*z* planes reduced the unambiguous angular region detected to 1/8 of the whole spherical space ($0° \le \Theta \le 90°$ and $0° \le \Phi \le 90°$), in the present work this range was increased 4-fold to allow unambiguous determination within a hemisphere (e.g., $0° \le \Theta \le 90°$ and $0° \le \Phi \le 360°$). The remaining ambiguity, between (Θ,Φ) and (180° – Θ, Φ – 180°), cannot be resolved in optical measurements without adding additional information as explained below.

For the conversion from the laboratory coordinate frame to the actin-based frame, the orientation of actin and the direction myosin V movement need to be known. We use the position data from fluorescence imaging with one-nanometer accuracy (FIONA) or DOPI to calculate the actin direction by fitting a line to the displacement trajectory. Let $\Phi_{actin}$ be the angle between myosin V's walking direction and +*x* axis and $\Phi_r$ be the azimuthal angle of the probe dipole around the microscope optical axis relative to the direction of myosin V motion. Then $\Phi_r = \Phi - \Phi_{actin}$. The dipole axis $\vec{D}$ is given by

$$\vec{D} = \langle \sin\Theta.\cos\Phi_r, \sin\Theta.\sin\Phi_r, \cos\Theta \rangle \text{ , } \quad \textbf{[1]}$$

in the laboratory coordinate frame, and as

$$\vec{D} = \langle \cos\beta, \sin\beta.\cos\alpha, \sin\beta.\sin\alpha \rangle \text{ , } \quad \textbf{[2]}$$

in the actin-based frame. α and β are then calculated equating these two expressions:

$$\langle \sin\Theta.\cos\Phi_r, \sin\Theta.\sin\Phi_r, \cos\Theta \rangle = \langle \cos\beta, \sin\beta.\cos\alpha, \sin\beta.\sin\alpha \rangle \text{ . } \quad \textbf{[3]}$$

Because of multiple values of the arctangent function, if $\Phi_r < 0$ or if $\Phi_r > 180°$, then α + 180° replaces α.

**Dipolar Degeneracy.** Although DOPI has no degeneracy in measuring the orientation of the dipole axis within any preselected hemisphere, the bidirectional symmetry of the optical dipoles still presents an ambiguity between (Θ, Φ) and (180° – Θ, Φ + 180°). For most of the molecules analyzed in the present study (94 of 97 molecules), restricting the angular range to the upper hemisphere in the sample chamber, e.g., $0 < \Theta < 90°$, which implies $0 < \alpha < 180°$, resulted in the probe angle β, relative to the barbed end of actin, being greater for the leading head (after a large step) than for the trailing head (after a small step), as expected from EM (8) data and the cartoon representations in Figs. 1 and

2. Thus, the myosin V molecules appear to walk mainly on the top surface of the actin, away from the glass.

For dipoles that are almost parallel to the sample plane ($\Theta \approx 90°$), however, small out-of-plane angular fluctuations or noise might cause $\Theta$ to become transiently >90°. Restricting the molecule to the upper hemisphere in this case would have the effect of artifactually causing sudden, nearly ≈180° rotations of $\alpha$. Only 3 molecules (out of 97 myosin V molecules analyzed) showed this behavior. Fig 8 shows an example of this effect. The time base for the molecule of Fig. 4*B* has been extended to show data at earlier and later times. The $\alpha$ trace in Fig. 8 *Bottom* shows nearly 180° jumps near the beginning of the trace and at ≈70 s (shown with green open triangles). Fig. 8 *Bottom* shows the effect of replacing $\alpha$ with $\alpha - 180°$, when $\alpha > 90°$ in this molecule (filled green triangles). The large jumps are eliminated and slightly negative $\alpha$ (lower angular hemisphere) results. The filled diamond symbols in Fig. 8 *Middle* show $180° - \beta$ for these same time points, and open triangle symbols show the original $\beta$ data. The few molecules displaying this type of sudden 180° $\alpha$ jumps that are eliminated by choosing the opposite end of their dipole show that the molecules are not restricted absolutely to the upper hemisphere. The large majority of molecules, however, walk along the top of actin as expected.

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