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John F. Beausang
Deborah Y. Shroder
Philip C. Nelson
University of Pennsylvania, nelson@physics.upenn.edu

Yale E. Goldman

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Three-dimensional orientation and dynamic wobble of myosin V by high time resolution single molecule polarized fluorescence

John F. Beausang¹, Deborah Y. Shroder²,³, Philip C. Nelson¹ and Yale E. Goldman²,³

Affiliations

1. Department of Physics, University of Pennsylvania
2. Pennsylvania Muscle Institute
3. Department of Physiology, University of Pennsylvania

Keywords

Myosin V; Single-molecule; polarized fluorescence; single photon counting; change point analysis
Abstract

Myosin V is bio-molecular motor with two actin binding domains (heads) that take multiple steps along actin by a hand-over-hand mechanism. We used high speed polarized total internal reflection fluorescence (polTIRF) microscopy to study the structural dynamics of single myosin V molecules that have been labeled with bifunctional rhodamine linked to one of the calmodulins along the lever arm. Using time-correlated single photon counting technology, the temporal resolution of the polTIRF microscope was improved ~50-fold relative to earlier studies and a maximum likelihood, multi-trace change point algorithm was used to objectively locate times when structural changes occurred. Short-lived substeps displaying an abrupt increase in rotational mobility were detected during stepping, likely corresponding to random thermal fluctuations of the stepping head while searching for its next actin binding site. Thus, myosin V harnesses its fluctuating environment to extend its reach. Additional less frequent angle changes, probably not directly associated with steps, were detected in both leading and trailing heads. The high-speed polTIRF method and change point analysis may be applicable to single molecule studies of other biological systems.
Introduction

Myosin V is a molecular motor that translocates along actin filaments in many cell types, transporting cargos toward the barbed end of actin or tethering them at their destinations (Mehta et al., 1999; Reck-Peterson et al., 2000; Rief et al., 2000; Sakamoto et al., 2000; Veigel et al., 2002; Yildiz et al., 2003; Schroeder et al., 2010; Sweeney & Houdusse 2010; Kodera et al., 2010; Sun et al, 2011). Defects in myosin V are associated with human pigmentation, immunological and neurological disorders (Reck-Peterson et al., 1999). Myosin V consists of two N-terminal globular ATP- and actin-binding domains (‘heads’), a lever arm comprising α-helical light chain binding motifs and six tightly bound calmodulins (CaMs) or calmodulin-like light chains per head, a coiled-coil dimerization domain, and finally a C-terminal cargo-binding tail that is usually absent in studies using recombinant proteins (Sweeney & Houdusse 2010).

In eukaryotic cells, single molecules or very small groups of myosin V molecules can transport cargos by producing ~50 mechanical steps, without dissociating, upon each diffusional encounter with actin, a property termed processivity (Mehta et al., 1999; Sakamoto et al., 2000; Yildiz et al., 2003). Many ensemble and single molecule techniques have been applied toward determining the chemo-mechanical cycle of myosin V (DeLa Cruz et al. 1999; Mehta et al., 1999; Reif et al., 2000; Sakamoto et al., 2000; Veigel et al., 2002; Yildiz et al., 2003; Forkey et al., 2003; Rosenfeld and Sweeney 2004; Kodera et al., 2010), leading to a consensus view of the mechanism in which a state with both heads bound to actin, at a 36 nm separation, occupies much of the ATPase cycle. The biochemical and structural states of the two heads are coordinated, probably via intermolecular strain, which minimizes backwards stepping and
dissociation of the entire molecule (Sweeney & Houdusse 2010). A 36 nm step is taken when the trailing head dissociates, swings 72 nm forward (toward the barbed end) and becomes the leading head. A rapid forwardly directed working stroke rotates the lever arm of the attached head, accounting for most (20-24 nm) of the step (Veigel et al., 2002). The remaining distance is covered by thermal fluctuations of the free head until it binds to an actin subunit ~36 nm ahead of the bound head. Evidence for this postulated thermal search period was provided by tracking gold particles or microtubules bound to the lever arm (Dunn 2007; Shiroguchi 2007) and high speed AFM (Kodera 2010). All of these experiments required attaching a relatively large probe to the lever arm/or slowing down its motions by a nearby adherent surface.

Smaller motions and rotations of the lever arm have been associated with ADP release from the trailing head, possibly accounting for ~5 nm of stepping (Veigel 2002). Additional small steps and lever arm rotations have been detected when the leading-head lever arm strokes before (Capello 2007) or after (Uemora 2004) the trailing head detaches and also apparently independent of productive stepping (Syed 2006; Kodera 2010). Indication that the orientation of the lever arm can change prior to stepping is supported by cryo-electron microscopy (Walker 1999; Oke 2010), whereas combined single molecule fluorescence localization and polarization experiments supported a straight-legged model for stepping (Syed 2006). The roles of these sub-steps, if any, in productive translocation or in coordinating the two heads are not clear.

Single molecule fluorescence techniques (Forkey 2000; Yildiz, 2003; Rosenberg 2005; Joo 2008;) are powerful tools for detecting position and orientation changes of macromolecules using relatively small organic fluorophore or quantum dot reporter probes. Polarized total
internal reflection fluorescence (poTIRF) microscopy measures orientation and rotational motions of dipolar fluorophores by exploiting their preference for absorbing light and emitting fluorescence polarized along their dipole axis. When the fluorophore is bound to the macromolecule in a known position and direction, as is the case with bifunctional rhodamine (BR) linked to a pair of nearby cysteine residues engineered into calmodulin (Irving 1995), molecular rotations can be inferred from the probe angles changes (Forkey 2003; Rosenberg 2005; Beausang 2008a; Lewis 2012).

In addition to the 3-dimensional orientation of the fluorophore and its linked protein domain, rotational mobility can be determined, including microsecond wobbling motions not generally accessible in ensemble fluorescence anisotropy experiments (Forkey 2000; Forkey 2005; Beausang 2008b). Microsecond time-scale wobbling of a protein domain might be functionally important, for instance in detecting the thermal search period during myosin V stepping. Until now, the capability of polTIRF to measure wobble has not been utilized for dynamic measurements, except to verify stable probe attachment. One reason is that the observation time, 40-80 ms, required to cycle through the range of input polarizations needed to measure the 3D orientation and wobble, is longer than the expected lifetime of many structural states, including myosin V’s postulated thermal search.

In this work, we studied recombinant myosin V with BR linked to one of the lever arm CaM subunits to determine, at high time resolution, the events while it was taking translocation steps. The time resolution of the polTIRF microscope was improved ~50-fold by incorporating a single photon counting device and switching the polarization of the fluorescence excitation.
between the requisite directions every 100 µs instead of every 5-10 ms as in most of our previous reports (Forkey 2003; Quinlan 2005; Forkey 2005; Rosenberg 2005; Beausang 2008a; Lewis 2012). Even when the 532 nm laser excitation was made stronger, the signals were noisy due to limited numbers of photons collected during each 100 µs polarization time window. To help interpret the signals, we used a maximum likelihood, multi-trace change point algorithm (Beausang 2011; Methods), that objectively identifies changes in intensity (“change points”), indicating structural changes with single photon precision and no binning of the raw data. We determined the orientation of the BR and the extent of wobble during the intervals between intensity changes and show that the changes often correspond to structural motions of the molecule. Short-lived sub-steps displaying abrupt increases in probe wobble were detected with the pattern expected for hand-over-hand stepping, likely corresponding to the random thermal fluctuations of the detached head during its search for the next actin binding site. In molecules showing these angular fluctuations, they typically occurred when the trailing head stepped, as expected. Additional less frequent angle changes of the lever arm were detected prior to stepping in both leading and trailing positions. This work represents the first use of single molecule polTIRF microscopy to report microsecond time-scale motions relevant to function of a biophysical macromolecule, which might be applied in single molecule studies of other biological systems.
Results

Rotational dynamics using high-speed poITIRF microscopy and change point analysis

The maximum time resolution of standard polarized fluorescence microscopy (5 ms recording gates, 20 – 40 ms cycle time, Forkey 2005; Beausang 2008b) was improved by switching the excitation laser polarizations every 0.1 ms and by cataloging the arrival time of each detected photon (Methods, SIFig 1). In order to obtain 20 – 50 photons within the 0.1 ms gate time, the laser power was increased from our standard 20 mW up to ~50 mW at the sample. [Those sound low to me. Sure they’re not /mm²? I’m pretty sure it should be just “mW” and not “mW/cm²”.

Forkey 2005 quotes “~20 mW at the sample”, which seems like a good way to do since the beam is being focused and thus hard to know what the area is. In many recordings the BR probe photo-bleached before a myosin step occurred. The recordings used for analysis represented molecules that photo-bleached after 1-2 seconds of high-intensity illumination, on average, and after 0-6 myosin stepping events (Methods). SIFig 2 shows the photon counts recorded from the 16 combinations of input and detector polarization binned at 0.1 ms intervals. As the molecule walked processively along actin in the presence of 1-40 µM Mg.ATP, tilting of the BR (and myosin lever arm) back and forth between two relatively discrete angles is fairly well-defined angles is clearly evident from abrupt and simultaneous changes in many of the polarized photon count rates (SIFig 2) as reported before (Forkey 2003). All of the channels photo-bleach to the background in a single step, consistent with signals from a single fluorophore.

In order to convert these noisy photon counting series into interpretable intensity and angular time courses, a specialized change point analysis (Watkins & Yang 2004) was developed
for pOTIRF data (Beausang 2011). Instead of binning the photons into intensity traces, the raw time-stamped data were fitted directly by a likelihood function that compares the probability of a change in photon collection rate with that of constant rate. By scanning this likelihood function over all of the photons in an interval from all of the 16 polarization channels, the location and uncertainty of the most likely intensity change was identified (dashed lines in Fig. 1). Iteratively applying this algorithm through the whole trace identifies angle changes, bleaching events and any other large magnitude changes in the individual channels that are clearly seen by eye. Smaller magnitude, but statistically robust, changes are also detected.

The fluorescence intensity for each channel during the interval is the average determined by summing the photons and dividing by the duration between adjacent change points. Each set of 16 intensities is used as input to a maximum likelihood fitting algorithm that estimates the dipole angles ($\theta$, $\phi$), wobble, $\delta$, and brightness, $\kappa$, of the probe during each interval between change points (horizontal lines in Fig. 2B-D). In order to interpret the angles in the biological frame of reference, the orientation relative to the microscope optical axis ($\theta$, $\phi$) is re-expressed in terms of a frame referred to the long axis of the actin filament ($\beta$, $\alpha$) where $\beta$ is the polar angle with respect to the barbed (+) end of the actin filament and $\alpha$ is the azimuthal angle around the actin filament (SIFig 3 and Forkey 2003; Beausang 2008b for details). Due to the dipole character of probe excitation and emission, any one orientation $\Omega = (\beta, \alpha)$ cannot be distinguished from an equivalent orientation pointing in the opposite direction $\Omega' = (180^\circ - \beta, \alpha \pm 180^\circ)$. Instead of imposing a fixed hemisphere for reporting the angles of all molecules as done before (Sun 2007; Beausang 2008a; Reifenberger 2009), an objective method was used to determine a favorable hemisphere for each molecule, based on the initial direction of motility.
and the average direction of the molecule’s probe orientations (i.e., the orientation of the director, see Methods for details). To indicate confidence intervals for the measured angles, four additional estimates of each interval’s intensity were calculated based on the statistical uncertainty of localizing the adjacent change points (Methods, SIFig 4B).

Large changes in $\beta$ and $\alpha$ (Fig. 2B,C) correspond to changes in the polarized count rates and indicate tilting between two stable states with orientation $(\beta, \alpha) \approx (22^\circ, 190^\circ)$ and $(81^\circ, 140^\circ)$ (SIFig 5). In Fig. 2, 4 major stepping events ($\blacktriangle$ and $\blacktriangledown$) are observed at 0.17, 0.37, 0.46 and 0.72 s between $(\beta, \alpha) \approx (25^\circ, 190^\circ)$ and $(85^\circ, 125^\circ)$ as described before (Forkey 2003). The angle changes in $\beta$ and $\alpha$ can be combined into the angle subtended by the probe vector in the two orientations, $\zeta = \cos^{-1}(\mathbf{\Omega}^+ \cdot \mathbf{\Omega}^-) \approx 75^\circ$ (where $- \text{ and } +$ indicate the state before and after stepping, respectively).

Importantly, $\delta$ (microsecond wobble) transiently increased during the recording on alternate steps when $\beta$ changed from low (trailing position) to high (leading position) values, consistent with a labeled trailing head detaching from actin ($\blacktriangle$ in Fig. 2D), undergoing rapid rotational motions and then rebinding to actin as the new lead head with $\beta \approx 90^\circ$ (Fig. 2E). The wobble parameter remains below 50° during the steps when $\beta$ changes from high low values ($\blacktriangledown$). This is most easily interpreted as the non-stepping head undergoing a working stroke, but remaining attached and therefore not wobbling as much. A cartoon showing the sequence of events with the expected alternating periods of high wobble is shown in (Fig. 2E). A gallery (SIFig 6) of angular time courses from additional molecules demonstrates the same stepping pattern with periods of high wobble when the probe switches from low to high $\beta$ angle,
corresponding to the labeled head detaching and wobbling while searching for its next actin binding site.

**Binned Data**

For visualization purposes, the photon arrivals were also binned to produce polarized fluorescence intensities (PFIs) with time resolution traded against photon shot noise (0.8 ms bins; *gray/blue curves* in Fig. 1A,G; 8 ms bins in B-F). Despite the high laser intensities, intensities binned at 8 ms are relatively noisy, resulting in large fluctuations in the individual PFIs and in the estimated orientations. The variability of \((\beta, \alpha)\) during an otherwise constant dwell is larger than might be expected from the variance in the intensity traces, due to the highly non-linear relationship between the magnitude of the PFIs and the corresponding dipole orientation. The wobble parameter \(\delta\) is also noisy (Fig. 2D, but can be seen to increase in magnitude prior to the first and third tilting events (▲) in (Fig. 2), during the transition from low to high \(\beta\). During the final 1/3 of the recording the probe is in a state of prolonged high-wobble \((\delta >75^\circ)\) immediately prior to photo-bleaching, a characteristic that was observed in many other molecules (SIFig 7).

**Dynamics of periods of increased probe wobble during steps**

After manually screening for molecules with robust tilting (i.e., strong anti-correlated changes in the PFIs), 1604 tilting events were detected in 429 molecules, 69 of which contained a total of 84 substeps exhibiting increased wobble (*open symbols* in Fig. 3A) relative to that just before (*filled circles*) and after (*filled triangles*) each step. The fraction of molecules with at least one such high-wobble substep was ~23%, but the fraction of steps showing a high-wobble intermediate was only ~7%. One likely cause of low detection of these sub-steps is the limited number of
photons collected during short duration events. Consistent with this view, molecules recorded at higher laser power contained more high-wobble substeps (~10%) than those recorded at low power (~4%). The substep durations are distributed exponentially with an average duration ~13 ms (filled black dots Fig. 4). A fraction of missing events is expected at or below the detection limit from the exponential distribution (empty black dots). Nevertheless, some molecules with very high signal to background ratio and multiple myosin steps with strong polarization changes did not exhibit detectable high-wobble substeps (SIFig 8).

Another way to test the origin of the sub-steps is to collect the angles preceding and following the high-wobble period (solid red symbol and empty red symbol in Fig. 3B, respectively). Considering all high-wobble substeps (98 events in 73 molecules), there is a strong tendency for low β angles to precede the high wobble state (red points with solid line) and high β angles to follow it. This provides strong support for the sequence of events drawn in Fig. 2, allowing the preceding orientation (low β) to be identified with the trailing lever arm, the state following the step (high β) with the leading lever arm and the high wobble period with the search. The distributions of α before (filled green points in Fig. 3A) and after stepping (empty green points) are uniform and the same before and after stepping.

Previous work has shown that BDM slows the stepping rate of myosin V by inhibiting the release of ADP (Uemora et al., 2004) and also slows the rebinding rate of the detached head (Dunn & Spudich 2007). Motility experiments were performed in the presence of 50 or 100 mM BDM and approximately two-fold higher MgATP concentration in order to obtain comparable rates of stepping. On those molecules with the most robust measurements of
stepping, 43 substeps were detected in 39 out of 123 molecules containing a total of 380 steps (11% of steps or 32% of molecules contained high-wobble substeps). The detection efficiencies again depended on laser power, indicating detection limited by photon counts: 16% of steps and 34% of molecules contained substeps at high laser powers vs. 6% of steps and 26% of molecules at low laser power. Fitting single exponentials to histograms of the substep durations in the absence (black, Fig. 4) and presence (gray) of BDM leads to estimates of the average sub-step durations of $12.7 \pm 2$ ms and $16.4 \pm 4$ ms respectively (± 95% confidence intervals). Varying ATP concentration from 1 to 20 µM did not systematically affect the duration of the high-wobble substep in the absence (black) or presence (gray, Fig. 5) of BDM. These effects of BDM are smaller than expected from results of gold particle labeled myosin V (Dunn & Spudich, 2007), possibly because our molecules contained only calmodulin in the lever arm, whereas the molecules used by Dunn & Spudich (2007) also included essential light chains, which affected the detached head rebinding rate.

**Minor angle changes**

Occasionally (52 events in 42 molecules), increases in wobble were detected without a corresponding tilting motion of the myosin; instead, the orientation before and after the high-wobble substep is approximately the same (SIFig 7), consistent with the labeled head detaching from actin and rebinding actin without stepping (Syed 2006; Kodera et al., 2010). The average duration of these events was 33 ms ($n = 22$ events in 16 molecules). Nine of these molecules showed this type of non-productive high wobble period in the presumptive trailing head ($\beta < \ldots$)
50°) and 7 in the leading head (β > 50°). Neither ATP concentration nor the presence of BDM appeared to influence the occurrence of these non-step increases in wobble.

Smaller magnitude angle changes also occurred between the major steps. For the molecule in Fig. 2, the lever arm appeared to undergo additional minor tilting at change point #7 on the leading head and at #4, and #9-#12 on the trailing head. The angular displacements (ζ) during these motions were usually less than 10°, but up to 45°. Change points #14-#17 in Fig. 2 during the high wobble period before photobleaching represent statistically significant small changes in intensity, but they lack well-defined, quantifiable orientations.

The number of minor events detected by the change point algorithm preceding and following high wobble sub-steps were similar for the trailing and leading heads (solid and open symbols in Fig. 6A). For both the leading and trailing heads, the majority of steps occurred with no additional angle changes and the overall number of events averaged ~1.1 small change points per major step. Neither the magnitude (Fig. 6B) nor the kinetics (data not shown) of these non-step angle changes were appreciably different between the leading and trailing head states.

Manually reviewing these recordings as well as those for molecules where no high wobble states were detected during stepping (and thus leading and trailing states were less clear), confirmed that few molecules exhibited small, lasting angle changes preceding their steps. Thus, at the ATP concentrations used here (1 – 20 µM), lever orientation is predominantly constant prior to major rotations of the lever arm, consistent with mainly straight-leg stepping (Syed 2006).
Photophysical effects

About 30% of recordings contained an extended period of high probe wobble immediately before photo-bleaching (for example, the last ~0.5 s of Fig. 2D and SIFig 7). Previous studies reported occasional increases in fluorescence intensity or spectral shifting prior to photo-bleaching (Wazawa 2000; Quinlan 2005). We also observed an increase in fluorescence before photo-bleaching in some traces; however, the increase in probe wobble was much more common. Photobleaching times in this population were not different from the ‘normal’ traces and the likelihood of obtaining a period of high wobble before photobleaching did not depend on laser intensity. The phenomenon is probably due to a photophysical artifact of laser light damage to the probe or probe-labeled lever arm, based on several observations. Processes that adversely affect the unlabeled heads would be expected to produce a population of recordings with a prolonged terminal state of constant angle, but molecules without these sustained high wobble episodes tilted back and forth until they photo-bleached. Consequently, we speculate that one of the bifunctional linkers in the probe may break under the high laser illumination, resulting in a singly attached, highly mobile probe that does not discriminate attached actomyosin states from detached ones.

Short bursts of multiple, very rapid polarization change points that were also detected infrequently in a subset of molecules (SIFig 7). The time scale of these motions ~18 ms was much faster than myosin stepping and had no bias toward the leading or trailing head. There were more of these rapid events at higher laser powers suggesting that they may be another photo-physical effect.
Discussion

High speed polTIRF detects periods of brief detachment during myosin V stepping

In previous polTIRF studies of myosin V (Beausang 2008a; Sun 2010; Lewis 2012), the time resolution was limited to 20 ms, and the range of discernible angles was often restricted to 1/8 of a sphere due to symmetries that resulted from confining the input laser polarizations to three orthogonal planes (Forkey 2003; Forkey 2005). Here, laser polarizations aligned in 4 directions (−45°, 0°, 45° and 90°) relative to the plane defined by the input and reflected laser beams (the scattering plane) in both beams decreased the ambiguity four-fold, resulting in a hemisphere of unambiguously distinguishable orientations (SIFig 1 and Beausang 2008a,b; Lewis 2012). This scope is the maximum limit imposed by the intrinsic dipole symmetry of the probe.

Statistically valid change points in the photon collection rates identified times of structural changes, such as tilting and and increased wobble. An important assumption of the change point approach is that the molecule resides in discrete structural states and switches abruptly between these states, resulting in sudden changes in the polarized fluorescence photon count rates. This feature implies that the rates are constant between change points and that the total number of counts divided by the interval between change points is a good estimate of the probe intensity. The sixteen intensities were fitted numerically by a maximum likelihood model of fluorescence dependent on the dipole orientation and wobble, (β, α), and wobble, δ (Forkey 2005; Beausang 2008b; Forkey 2003; Syed 2006; Lewis 2012). Wobble quantifies the depolarization of the fluorescence signal on time scales slower than the fluorescent lifetime (~4 ns) but faster than the measurement time scale (~0.1 ms). The expected microsecond
rotational diffusion time scale of a free myosin V head is thus quantified, for the first time, by this \( \delta \) parameter.

Increases in \( \delta \) during a switch in lever arm orientation were measured in many molecules (SIFig 6). The timing of the structural changes derives purely from the fluorescence intensities, independent of any binning of data or of any model predicting the relative intensities. The brief increases in \( \delta \) are consistent with a highly mobile state of the trailing head after it detaches from actin during the ‘thermal search’ for its next actin binding site. This assignment is supported by several of our results. First, the results of fitting the dipole model to the 8 ms binned data confirm that the high wobble sub-steps occur between steps (Fig. 3A), and are characterized by randomly distributed \( \beta \) and \( \alpha \), consistent with microsecond tumbling of the detached head. Second, in >90% of recordings where more than one high wobble sub-state was detected, they were observed on alternating steps, as expected (for example, Fig. 2E and SIFig 6). Third, the average time for rebinding was independent of ATP concentration (Fig. 5). Lastly, the \(~13\) ms duration of these short intervals (Fig. 4) agrees well with the period of high positional fluctuations of gold nanoparticle labeled myosin V during steps along actin (Dunn & Spudich 2007).

Experimental evidence for the single-head-attached myosin intermediate was obtained with optical trapping (Capello 2007), fluorescence microscopy (Shirgouchi 2007 and Komori 2008) and high speed AFM (Kodera 2010), but measurement of the rebinding rate in these studies was complicated by a bead, a large reporter molecule or surface interactions. The mean waiting time for rebinding of the free head to actin was theoretically estimated to be 0.1 – 1 ms.
from first passage time calculations (Viegel 2002), the processive run length (Smith 2004), and from Brownian dynamic simulations (Craig & Linke 2009). The present work using BR-CaM labeled myosin V (Fig. 4 and Fig. 5) and single particle tracking of 40 nm gold particle-labeled calmodulins (Dunn and Spudich 2007) both measured rebinding times of 10 – 15 ms, even though the 40 nm gold particle probably slowed diffusion of the head. The slower binding compared to the expectation from diffusion-limited models strongly suggests that a structural or biochemical step, such as reversal of the working stroke (Kinosita 2007, Shiroguchi 2010), weak-to-strong actomyosin binding (De La Cruz 1999) or Pi release (Rosenfeld & Sweeney 2004), and not Brownian dynamics, limits rebinding rate.

The polarization switching devices (Pockel cells), photon detectors, and single-photon counting circuit are all fast electronic elements with ~microsecond or faster response times. Consequently, the process limiting the time resolution is the number of detected photons from the single probe, here ~50 photons/ms. The periods of increased wobble during stepping of the labeled head last ~10 - 20 ms, leading to ~500 – 1000 total photons detected on average from these intervals. These values are just above the threshold required to detect change points with 95% confidence, which probably accounts for the numbers of such high-wobble sub-steps detected being less (14%) than the expected 50% of all steps. The excitation intensity is not near saturation of the absorption dipole, so higher excitation laser powers would increase the numbers of detected photons per time interval, but at the expense of shorter recording times before photobleaching. Of the ~1,000 total molecules recorded at the laser intensities used, approximately 40% photo-bleached before even one step occurred, thus limiting the practical range of input intensities.
Other factors may result in undercounting the expected number of sub-steps. First, short-lived events that contain enough photons to trigger detection of a change point, but not enough to be reliably fit by the dipole model can be missed because the fitting typically underestimates the wobble of low-photon states. Also, the number of stepping events during a recording might be overestimated because at low ATP and ADP concentrations some large rotational motions are not associated with stepping (Syed 2006; Kodera 2010).

**Distributions of orientation and wobble during myosin V stepping**

The distributions of probe orientations with each step were characterized by two peaks in $\beta$ at $\sim20^\circ$ and $\sim85^\circ$ (Fig. 2A and SIFig 5), which result in lever arm orientations of $60^\circ$ and $120^\circ$ for the leading and trailing configurations when the local orientation of the BR probe on the lever arm is taken into account (Forkey 2003; Lewis 2012). In experiments where both position and orientation were measured (Toprak 2006) the smaller magnitude peak in $\beta_{\text{probe}}$ was determined to be the trailing head, in agreement with the relative orientation between the probe axis and the myosin lever arm (Parker 2010; Lewis 2012). In the high speed polTIRF experiments here, this assignment of angles to the two positions is given additional support by the observation that the high wobble search period is observed when $\beta_{\text{probe}}$ switches from smaller to larger angle (Fig. 2, SIFig 6, Fig. 3A), thereby confirming the trailing to leading positions, respectively, as suggested by the earlier studies.

The fairly uniform $\alpha$ distributions (Fig. 2B and SIFig 5B) are consistent with myosins binding at random azimuthal angles around the actin filament. The magnitude of the power stroke is estimated from angle changes of the stepping head where $(\Delta \beta, \Delta \alpha, \zeta)$ as $(-68^\circ \pm 7^\circ)$, -
2.4° ± 4°, 83° ± 4°). The small magnitude of $\Delta\alpha$, leading to similar magnitudes of $\Delta\beta$ and $\zeta$ confirms that the power stroke is aligned closely along the actin filament axis.

**Minor Angle Changes**

Previous single molecule work on myosin V using optical traps (Viegel 2002; Uemora 2004; Capello 2007), electron micrographs (Walker 1999; Oke 2010), fluorescence (Syed 2006) and AFM (Kodera 2010) have suggested that, in addition to the high-wobble search period during an active step, there may be mechanistically important sub-steps prior to or following a step. The base of the lever arm in the leading head might tilt forward while the remainder of the lever is restrained by its attachment to the rear lever arm, causing a kink to form the so-called telemark configuration (Walker 1999; Syed 2006; Snyder 2004; Oke 2010). In our data, non-step change points were sometimes detected during the intervals between steps, but there was no bias toward leading or trailing heads in either the number of events (Fig. 6A) or their magnitude (B). The occasional larger magnitude angle changes that were detected may be consistent with an alternate reaction pathway (Kad 2008). In our experiments, the most common configuration while the molecule waited for ATP binding was with both heads bound to actin and both lever arms relatively straight. Upon ATP binding, the stepping motion is normally rapid and proceeds without a preceding tilting of either lever arm. These results apply to the specific conditions optimized for detection of the high wobble state: a relatively narrow range of ATP concentrations, no added ADP, and a relatively brief recording interval limited by photo-bleaching. High-speed AFM images of myosin V also showed mainly straight lever arms at micromolar ATP in the absence of ADP. The leading lever arms became kinked more often at higher ADP concentrations (Kodera 2010).
nucleotide concentrations might identify a role for such auxiliary rotations in the myosin molecule.

**Conclusions**

An upgraded polTIRF microscope was used to investigate myosin V motility with high time resolution. Brief ~10 – 15 ms sub-steps, characterized by highly disordered lever arm orientations, were detected during stepping events and likely correspond to the detached head searching by random fluctuations for its next actin binding site. The majority of myosin V molecules contain straight lever arms in both leading and trailing positions (i.e., not bent or kinked) and additional rotations of the lever arm were uncommon on both the leading and trailing heads. Since biological macromolecules generally in an environment characterized by significant thermal fluctuations, other important enzymatic steps and short-lived intermediates might be detected by rotational flexibility that can be measured at the single molecule level using high speed polTIRF microscopy.

**Materials and Methods**

**Biological Samples**

Recombinant chicken myosin V, with its full length lever arm (amino acids 1 - 1099) and a FLAG affinity tag at its C-terminus was co-expressed with calmodulin in SF9 cells (Purcell et al., 2002). Chicken calmodulin (CaM), with residues Pro66 and Ala73 mutated to cysteine, was expressed in E. coli (Putkey et al., 1985), purified, and labeled with bifunctional rhodamine (a generous gift from Dr. J.E.T. Corrie; Corrie et al., 1998; Beausang et al., 2008b). Myosin V was
labeled by exchanging endogenous wild-type chicken calmodulin (WT-CaM) with exogenous BR-CaM at low stoichiometry (Forkey et al., 2003; Beausang 2008b). G-actin was obtained from rabbit skeletal muscle (Pardee and Spudich, 1982). Alexa 647-labeled F-actin was prepared from G-actin, Alexa 647 actin (Molecular Probes, Carlsbad, CA), in a 5:1 ratio at 1 µM total actin subunit concentration and stabilized with 1.1 µM phalloidin (Molecular Probes, Carlsbad, CA). In some experiments 0.05 µM biotin-actin (Cytoskeleton, Denver, CO) was also incorporated during polymerization.

PolTIRF experiments were performed as described previously (Beausang 2008b; Beausang 2010). Briefly, all reagents are made in myosin buffer (M5; pH = 7.6) containing 25 mM KCl, 20 mM HEPES, 5 mM MgCl₂, and 1 mM EGTA except the motility buffer, M5⁺, for single-molecule myosin V motility assays, which is M5 buffer with 1 - 40 µM ATP, 100 mM DTT, 100 µg/ml wild-type CaM (WT-CaM), and ~100 pM of BR-CaM labeled myosin V. For some experiments 2,3-butanedione monoxime (BDM, Sigma) prepared fresh from powder is included in the final motility buffer (final concentration 50 - 100 mM) and the pH is re-adjusted as needed with KOH. Sometimes, M5⁺ buffer included 10 mM phosphocreatine (Sigma P-7936) and 0.3 mg/ml creatine phosphokinase (prepared daily from powder, Sigma C3755), but no influence on myosin velocity or stepping kinetics was detected. 100 mM DTT was used as a reducing agent with no deoxygenating system to avoid BR blinking (Rosenberg et al., 2005).

Myosin V processivity experiments were performed in a flow cell consisting of a plasma cleaned quartz slide (Quartz Scientific, HPFS grade quartz, 212000-001), spin coated with 2 mg/ml PMMA (Aldrich Chemical, secondary standard grade 37 003-7), and glass cover slip (Fisher, No
1.) held together by pieces of double–sided tape (Scotch, Cat. No. 665). In most experiments, 60 nM NEM (N-ethylmaleimide) treated myosin II (Veigel C, Barsoo ML, White DC, Sparrow JC, Molloy JE (1998) Biophys J 75(3):1424–1438.) was flowed into the lane, incubated for 5 minutes and followed by a rapid flow of a 200 nM Alexa 647-actin filaments. The surface is passivated by a 5 min incubation with 1 mg/ml unlabeled BSA (Sigma A0281). Finally the motility buffer containing the myosin and ATP is flowed into the chamber. Slight variations in the protocol were also implemented, including substituting the NEM myosin and Alexa 647 labeled actin with 1 mg/ml biotinylated BSA (Sigma, A8549), an incubation with 0.5 mg/ml streptavidin (Sigma, S-4762) and biotin-Alexa 647-actin filaments, but no significant differences were detected.

**High-speed polarized TIRF setup**

The time resolution of a polarized total internal reflection fluorescence (polTIRF) microscope (Forkey 2005; Beausang 2008b; Beausang 2010) was improved by decreasing the time each laser polarization illuminated the sample from earlier 5 ms periods to 0.1 ms and by storing the arrival time and the polarization state of the illumination for each detected photon. Two alternating beams from a 532 nm Nd:YAG laser (SIFig 1) were projected through a coupling prism at a glancing incident angle producing an evanescent field at the quartz slide/aqueous interface. Switching between the two beams, termed path 1 and path 2, was achieved by computer-controlled voltages applied to a Pockels cell (PC0) and a polarizing beam splitting (PBS0) cube. Similarly, the polarization of the laser in each beam was switched between four different linear polarizations (s, 90°, p, 0°, L, +45° and R, -45° relative to the plane defined by the incident and reflected beams, SIFig 1) with an additional Pockels cell and a Berek compensator in each beam (PC1/BC1 and PC2/BC2). Each polarization illuminates the sample
for 0.1 ms in the sequence $s1, p1, p2, s2, R1, L1, L2,$ and $R2$ (letter indicates incident polarization, number indicates path).

Fluorescence emission was collected by a Leica $100\times1.2$ NA water immersion lens, passed through a long pass blocking filter, and either imaged onto an intensified charge-coupled device (CCD) camera (Cascade II, Photometrics) or directed through a polarizing beam splitter (PBS1) that resolves its $x$ and $y$ components between two avalanche photodiodes (APDx and APDy). A time correlated single photon counting PC adaptor board (TCSPC, SPC-130, Becker and Hickl), operating in FIFO mode, and a pulse router (HRT-82, Becker and Hickl) were modified to record the excitation polarization state, according to a suggestion by Dr. Wolfgang Becker, details available upon request. The photon pulses during first 3 µs of each polarization interval, while the Pockel cell voltage was settling, were ignored. A 10 MHz pulse train from a digital delay generator (DG645, Stanford Research Systems) triggered the timing of the TCSPC circuit and a 10 kHz pulse train, generated by same DG645 unit, triggered digital counters that sequenced the high voltage amplifiers driving the three Pockels cells.

Immediately before each polarization recording, two images of the field of candidate fluorophores were recorded with the CCD camera and superimposed on an image of the Alexa 647 labeled actin filaments and displayed on a monitor. These images were used to estimate the position and direction of the BR-CaM labeled myosin V. A moving molecule was selected for polarization analysis and identified by a mouse click. A custom-built LabView program centered the molecule above the objective via a piezoelectric stage, rotated in a removable mirror (RM) that directed the fluorescence emission onto the APDs, and sent a trigger signal that reset the
Pockel cell drivers to the initial polarization setting and started the TCSPC. Photon counts were recorded for 5s, corresponding to 6250 cycles of the 16 polarization channels. During recording of polarized fluorescence, spatial information from the fluorophore was not available.

**Multiple Channel Change Point (MCCP) algorithm**

A change point algorithm tailored to single photon polTIRF experiments was developed (Beausang 2011) where a likelihood function compares the ratio two hypotheses: H) that there is a single, abrupt intensity change at the \(i^{th}\) photon in an interval containing \(N\) photons in time \(T\) and the null hypothesis \(H_0\) that there is no intensity change at the \(i^{th}\) photon. Assuming that the fluorescence emission is random, then the only user-defined parameter is the probability of a false positive change point, here chosen to be 5%, which defines a threshold that the likelihood function must exceed for significance. The likelihood function, \(L_i\), is calculated for each photon

\[
L_i = \left[ \sum_{j=1}^{16} \left[ n_{ij} \ln \left( \frac{n_{ij}}{N_j} \right) + \left( N_j - n_{ij} \right) \ln \left( 1 - \frac{n_{ij}}{N_j} \right) \right] + \left( \sum_{j=1}^{16} n_{ij} \right) \ln \left( 1 - \frac{1}{16} \right) \right]
\]

Where \(n_{ij}\) is an \(N \times 16\) dimensional matrix of the accumulated number of photons \(i\) for each of the \(j\) PIFs (SIFig 9), \(t_i\) indicates the arrival time of the \(i^{th}\) photon and \(N_j\) is the total number of photons in the \(j^{th}\) PFI. A non-uniform distribution of false positives across the interval is mitigated by applying correction terms to the likelihood function and also including a small exclusion region that prevents change points from being detected in the first or last 2.5% of the photons in the interval (Beausang 2011 and Watkins and Yang 2004).
The algorithm is applied iteratively to the data until no additional change points are detected. The likelihood surfaces for the change points at 0.46 s and 0.49 s (\#5 and \#6 corresponding to the magenta and red lines in SIFig 4C) exceed the threshold (black line) for 95% confidence by over 10 log units and correspond to abrupt changes in the PFIs (four of 16 are shown in A) and the kink in the accumulated photon trace (SIFig 4B). The likelihood surface of a 3rd change point at ~0.4 s (\#4, blue line) is broader and less pronounced with only a small change in the PFIs, but it still exceeds the threshold for significance by ~ 8 log units. The peaks of the likelihood functions determine the most likely location of the change points (vertical black dashed lines), and the relative sharpness of the likelihood peaks determines the 95% confidence interval (gray shaded regions), which for change points \#4, \#5 and \#6 are 1000, 226, and 248 photons or 15, 3.6 and 4.5 ms, respectively.

**Ambiguities in the Dipole Model for Orientation and Wobble**

The 16 combinations of 8 time-multiplexed input polarizations and 2 simultaneously measured emission polarizations used here, including ±45° linear polarized excitations enables unambiguous determination of probe angle within a hemisphere of orientation (Beausang, 2008a; 2008b; Sun, 2007; Lewis, 2012). In additional to allowing a greater range of angles to be discerned, including the ±45° laser polarizations also resolves a more subtle ambiguity associated with δ. When the probe is completely free to rotate on the microsecond time scale, δ should be 90° (θ and ϕ are not defined at high δ); however, when only s and p (i.e., 0° and 90°) input laser polarizations are used (e.g., Forkey 2003) and the probe has high wobble, an orientation in the laboratory frame of reference of (θ, ϕ) = (54.7°, 45°) and δ ≈ 50° is usually reported by the algorithm that fits the dipole model because the corresponding 8 PFIs are the
same as the case when $\delta = 90^\circ$. In previous work, this artifact was not noticed because periods of large probe wobble were not explicitly investigated.

**Choice of Hemisphere**

For any given measurement of dipole orientation, either of two vectors may apply $(\theta, \phi)$ or $(\pi - \theta, \phi + \pi)$, due to the two-fold symmetry of the probe dipole. The probe bound to the protein is not symmetrical, however, due to its specific attachments to the two different Cys residues of the protein, so it is meaningful to consider which of the dipole ends is being expressed by the angle and, particularly, to report the same end of the probe in successive measurements for a given molecule. In order to maintain the same end in successive measurements, information is required beyond the individual polarization measurements. If the true rotational motions of the molecule are smaller than $180^\circ$, then all of the orientations for one end of the dipole will fall into one hemisphere of orientations. We can find a hemisphere that fulfills this requirement if we assume that all of the measured angles in a processive run are within $90^\circ$ of the average angle. This assumption is reasonable because the recordings usually contain similar numbers of leading and trailing positions and the total angle change for lever arm of myosin V when it steps is substantially less than $180^\circ$ as indicated by cryo electronmicroscopy (Walker et al., 1999) and AFM (Kodera et al., 2010).

The polar axis of this molecule-specific hemisphere is inferred from the set of dipole orientations for that molecule. The axis is defined as the director of the nematic liquid crystal order parameter $\Xi$:
\[
\Xi_{i,j} = \frac{1}{n_D} \sum_{k} \left( \theta_{k,i,j} \hat{\delta}_{k,i,j} - \frac{1}{3} \hat{\delta}_{i,j} \right)
\]

where \(n_D\) is the total number of dwell periods in the recording, \(\hat{\theta}_k\) is the \(k^{th}\) orientation of either end of the dipole represented as a unit vector in Cartesian coordinates

\[
\langle \sin(\theta_k) \cos(\phi_k), \sin(\theta_k) \sin(\phi_k), \cos(\theta_k) \rangle, i,j = 1,2,3 \text{ representing the } x, y \text{ and } z \text{ directions, and}
\]

\(\hat{\delta}_{i,j}\) is the Kronecker delta function equal to 1 when \(i = j\) and zero otherwise. Note that for each angle, regardless of whether \(\{\hat{\theta}_{z,x}, \hat{\theta}_{y,z}, \hat{\theta}_{x,y}\}\) or its dipole symmetry related vector

\[
\{\hat{\theta}_{z,x}, \hat{\theta}_{y,z}, \hat{\theta}_{x,y}\}
\]

is used, \(\Xi\) is unchanged. Because \(\Xi\) is a \(3 \times 3\) matrix there are 3 orthogonal eigenvectors and the polar axis of the hemisphere is chosen to align with the dominant eigenvector, that is, the eigenvector with the largest eigenvalue.

This vector is the average orientation during the processive run of a myosin V. The director can point in any direction and a hemisphere aligned along it will contain only one end of the probe dipole if the molecule remained within 90° of that orientation. Which end of the director axis to align with the analysis hemisphere is still to be chosen, but for relative motions during a run, it is irrelevant. The opposite hemisphere also corresponds to the molecule walking on the opposite side of the actin (Lewis, 2012). Which side is traversed is not known for any individual molecule. The end closest to the initial direction of motion of the molecule, as measured from the initial CCD images recorded prior to the polarization analysis, is arbitrarily chosen as the hemisphere director. When \((\theta, \phi)\) is rotated into the actin frame of reference \((\beta, \alpha)\) the hemisphere does not change.
References


Acknowledgments

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Figure Legends

High-time resolution polarized fluorescence intensities (PFIs). (A) 16 PFIs are summed into one total intensity with photons binned according to each 0.8 ms cycle (gray) and averaged every 10 cycles (black) is relatively constant before photo-bleaching to background counts at ~1.5 s. The total intensity (B) and the 16 PFIs (C-F) comprising the interval prior to photo-bleaching (blue) are shown expanded in the following panels but with intensities are averaged over 10 cycles. The measured intensities depend on the relative orientation between the fluorophore dipole axis and the direction of the input laser polarizations $p$ (blue), $s$ (red), $L$ (green) and $R$ (magenta) and the polarized fluorescence detected on APDx (C an E) and APDy (D and F) for beams 1 and 2, respectively (see SIFig 1 for details on the experimental setup). (G) To illustrate the high time resolution of each PFI, the photons collected during each 0.8 ms cycle of the laser polarizations are shown for $\iota_2 I_x$ (blue) and $\iota_2 I_y$ (red) binned at both 0.8 ms (lighter jagged lines) and averaged over 10 cycles (darker jagged lines). The results of the MCCP algorithm prior to the photo-bleaching event indicate the location intensity change points (vertical black dashed lines) along with a 95% confidence interval (gray area). The average intensity between neighboring change points as well as four estimates of the intensity corresponding to the confidence intervals (5 dark colored horizontal lines) are also shown (B-G). Similar figures (8 ms bins SIFig 10 and 0.8 ms
bins SIFig 2) but without the change point results (*vertical dashed lines and horizontal colored lines*) are included in the supplement.

**Fig. 2#Angles#**

Results of the dipole model for the 8.0 ms binned intensities (*lighter jagged lines*) and the change point intensities (*5 horizontal lines*, are often very close to each other because the uncertainty in localizing the change points is small). (A) The intensity scale factor $\kappa$ (*gray*) is similar to the total intensity (Fig. 1B) and is relatively constant despite large changes in the underlying PFIs, the hallmark of an orientation change. (B) The polar angle $\beta$ (*red*) shows 4 large angle changes (denoted by ▼) that are most likely due to myosin stepping events at change points #1, #3, #5 and #8 where $\beta$ alternates between a low value of 25° and a high value of 85°. The magnitude of the total angular displacement $\zeta$ (see text) between change point intervals is represented by a small cross (×). (C) The azimuthal angle $\alpha$ (*green*) also changes with each step, alternating between 200° and 125°. (D) The extent of rapid probe motion during the measurement (wobble) is represented by a cone with half-angle $\delta$ (*blue*) and shows relatively low values during a dwell and increases during steps from the low $\beta$ state (between change points #1-2 and #5-6), but not after steps from the high to low $\beta$ states (i.e., change points #3-4 and #7-8). Wobble at the end of the trace is also elevated, possibly indicating a laser-induced breakage of one of the bifunctional bonds on the probe, resulting in a monofunctional, and thus highly mobile, attachment preferentially observed at the end of many traces. (E) A cartoon depicting the BR-CaM labeled myosin lever arm (*double-headed red arrow*) translocating along an actin filament (*blue helix*) that is attached to the quartz slide (*light gray*) by NEM myosin II (*dark gray*). Increases in probe wobble between steps due to the detached head rapidly diffusing...
before rebinding actin (red disk) occurs during every other step of the steps, consistent with the hand over hand mechanism where the labeled head detaches from the trailing position but not when the labeled head is attached to actin undergoing a power stroke in the leading head position. A similar figure but without the change point results (vertical dashed lines and horizontal colored lines) is included in the supplement (SIFig 11).

Fig. 3#PrePostDists+Wob#

Distributions of probe angle and wobble before and after steps when an increase in probe wobble was detected for 98 events in 73 molecules. (A) The distributions of $\beta$ (red) and $\alpha$ (green) during the intervals immediately preceding (solid with filled symbols) and following (dotted with open symbols) those steps where a high wobble state was detected. Steps with a high wobble state allow trailing and leading lever arm states to be identified unambiguously as the intervals preceding and following the step, respectively. The distribution of $\alpha$ before and after the step is approximately uniform with no discernible difference between leading and trailing states. The distributions of $\beta$ before and after the step, however, illustrate the tendency for trailing states to have lower values of $\beta$ whereas leading states have larger values of $\beta$. (B) The distributions of $\delta$ (blue) during the intervals immediately preceding (filled circle with solid line) and following (filled triangle with solid line) those steps where a detected high wobble state was detected is peaked at lower values ($\sim$30°) compared with the distribution of $\delta$ during the high wobble state (open squares with solid line) which is peaked at large values ($\sim$70-80°).

Fig. 4#Histo#
Histogram of the duration of the high wobble interval between large orientation that likely correspond to detached heads rebinding to actin. Single exponential fits, excluding the first bin (open symbol) in each distribution result in average lifetimes of $13 \pm 2$ ms and $16 \pm 4$ ms without and with 50-100 mM BDM present in solution.

Fig. 5#BDM+ATP#

The duration of the high wobble state for molecules in the absence (black) and presence (gray) of BDM over a range of Mg.ATP concentrations agrees within uncertainty (S.E.) with the mean duration (horizontal dashed line) indicating that the kinetics of this state are independent of Mg.ATP concentration. Experiments with BDM in solution were performed at a higher ATP concentration in order to compensate for the reduction in velocity (Uemora et al., 2004). The outlier at 2 µM Mg.ATP is likely the result of insufficient statistics as there were only 6 events detected at this concentration.

Fig. 6#Tlmrk#

Distributions of non-step angle changes ($n=73$) using only steps during which a high wobble state was detected so that the trailing and leading head could be unambiguously assigned to the dwell before and after the step, respectively. (A) The distribution of the number of additional angle changes on the trailing ($n = 59$, solid line) and leading heads ($n = 69$, dashed line) is similar, possibly due to the coupling between leading and trailing lever arms. (B) Histograms of the change in $\beta$ angle during the dwell prior to (filled symbols) and after (open symbols) large wobble events were detected corresponding to changes in the lever arm orientation during the trailing- and leading-head dwells. Similar histograms for $\Delta \alpha$ are included in SIFig 12.
Supplementary Figure captions

SIFig 1/Setup/
SIFig 2/PFI1x/
SIFig 3/RefFrame/
SIFig 4/Substep/
SIFig 5/AngDist/
SIFig 6/GalDS/
SIFig 7/GalOthers/
SIFig 8/NoSubstep/
SIFig 9/kink/
SIFig 10/PFIonoCP/
SIFig 11/AnglesNoCP/
SIFig 12/TmrkAlpha/
To Do:
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Fig. 1
(PFIs)
To do:
1. Squeeze in actin ref. frame pic?
2. Add CP labels to x-axis

Fig. 2
(Angles)
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Fig. 3
(PrePostDists+Wob)
To Do:
1. Combine both figs into one panel
2. Make histogram into bars

Fig. 4
(Histo)

Fig. 5
(BDM+ATP)
To Do:
1. Make histogram into bars

Fig. 6
(Tlmrk)
Supplemental Information
The optics of the polTIRF setup have previously been reported (Forkey 2005) and are unchanged. Briefly, a 532 nm laser is passed through an Pockels cell (PC0) and split by a polarizing beam splitting cube (PBS0) into two paths, each of which pass through a 2\textsuperscript{nd} Pockels cell (PC1 and PC2) and a birect compensator (BC1 and BC2), and the directed by mirrors into a coupling prism at a glancing angle for TIRF illumination. Rapidly cycling the high voltage on the Pockels cells results in four linear input laser polarizations per beam oriented at 0°, ±45°, and 90° for \( p \), \( L/R \), and \( s \) polarizations (insert). Fluorescence emission from single BR-CaM labeled myosins is collected by the objective, passed through a filter and directed onto a CCD camera. A molecule translocating along actin (Alexa 647 labeled and imaged by a separate 532 nm laser, not shown) is selected in custom LabView software for polarization analysis. The candidate molecule is centered in the image plane by a piezoelectric stage (not shown) and removable mirror (RM) rotated into position and the polarized fluorescence emission passed through a beam splitting cube (PBS1) and finally collected onto two single photon counting avalanche photodiodes (APDs). Electrical pulses representing each single photon are passed through a filter to reject photons collected during the 3 \( \mu \text{s} \) interval required for the Pockels cells to change and passed through a router (Becker and Hickl HRT-82) that has been modified to tag each photon with the polarization state of the input laser. The arrival time of these pulses relative to an initial trigger pulse are then precisely measured using a time correlated single photon counting device (TCSPC, Becker and Hickl, SPC-130). In order to maintain a synchronous time base the 10 MHz oscillator from the delay generator, which is used to control the timing of the pockels cell Driver is converted to a square wave with a custom circuit and used as the SYNC signal in the TCSPC device. More details can be found in Beausang 2010.
To do:
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All 16 the 0.8 ms binned intensities from Fig_PFI instead of just the two shown in Fig_PFI_G.
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SI Fig. 3
(RefFrame )
To do:
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Add PFI and angle labels to each graph
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Time on x axis
Re order plots

SIFig. 4 (Substep)
Likelihood function for three change points. (A) The likelihood (jagged colored lines) of an intensity change point is calculated for each photon in an interval, and if the peak exceeds the threshold for significance (black horizontal line at zero) then its location is chosen as the most likely location for the change (vertical dashed lines). 95% confidence intervals (gray area behind vertical dashed line) is determined by the region where the likelihood curve exceeds the peak value minus two Ln units (see text for details). (B) 4 of the 16 PFIs binned at 0.8 ms (light jagged line), averaged over 10 cycles (dark jagged line), and averaged between the change points (horizontal lines) demonstrate the intermediate intensities between CP#5-6 that were detected by the algorithm using the single photon data (see SIFig_kink). (C) the total intensity (gray) is constant despite the changes in the PFIs. (D) Polar angle $\beta$ and (E) wobble $\delta$ were the result of the dipole model during this interval for the PFIs binned at 8.0 ms (jagged lines) and averaged between change points (horizontal lines) and demonstrate the increase in probe wobble between CP#5-6 when $\beta$ changes from $\sim 25^\circ$ to $\sim 90^\circ$. 

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SI Fig. 5
(AngleDist)
Distributions of the probe angles (A) The distribution of the polar angle $\beta$ has two peaks at $21^\circ \pm 2^\circ$ and $84^\circ \pm 4^\circ$ (mode $\pm$ S.E. by bootstrapping), due predominately to the trailing and leading configurations of the lever arm, respectively. (B) The distribution of $\alpha$ is approximately uniform with a slight prevalence for the probe to be aligned parallel to the sample plane ($\alpha = 0^\circ$ or $180^\circ$). This is consistent with the azimuthal freedom due to the molecule landing at any of helical actin binding sites. (C) The distribution of wobble is centered at $39^\circ \pm 0.4^\circ$, which is characteristic of myosin during a two-head bound dwell state, with a small tail extending to $90^\circ$, which includes both the high wobble states detected when the detached head rebinds to actin and the high wobble state that occurs in many molecules immediately prior to photo-bleaching. (D) Combining $\Delta \beta$ and $\Delta \alpha$ for each step into a single total angle change ($\zeta$), results in a distribution that is peaked $\sim 80^\circ$. 

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SI Fig. 6
(galDS)

β and δ for 6 molecules showing short-lived increases in delta (orange boxes) prior to stepping (i.e., large changes in beta). Periods of high wobble prior to photo-bleaching (blue) sometimes occur with similar magnitude delta.
SIFig. 7
(galOthers)

(A) Example of molecule with increased wobble prior to photobleaching (blue box).
(B) Intervals of rapid polarization changes likely represent a photophysical effect (red boxes).
(C) Some molecules with robust tilting did not show increases in wobble.
(D) Increases in wobble typically occur during a change in beta (orange box) but similar states were sometimes observed without changes in beta (green box) and may represent non-step angles changes (Syed 2006 and Kodera et al., 2010).
Some molecules with clear anticorrelated changes in the PFIs and high signal to background ratio showed clearly changes in the beta angle, but nevertheless, substeps during steps at CP #1, #2 or #3 were not detected. See Fig_PFI and Fig_Angles for a description of the traces.
SI Fig. 9
(kink)

Single photon traces for all 16 PFIs for the interval shown in Fig_substep. Instead of binning the single photon data, plotting the accumulated number of photons in each PFI against each photons arrival time results in photon traces where constant intensity is represented by a constant slope and a change in intensity is seen as a kink or sudden change in slope.

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SIFig. 10
(PFInoCP)
Plot of the binned intensities from Fig_PFI without the change point intervals. See Fig_PFI for details.
To do:
- Format similar to Fig_Angles

SI Fig. 11
(AnglesNoCP)

Plot of the dipole model results from only the binned intensity data. See Fig_Angles for details.
Distributions of non-step angle changes ($n=73$) using only steps during which a high wobble state was detected so that the trailing and leading head could be unambiguously assigned to the dwell before and after the step, respectively. The distribution of $\Delta \alpha$ changes on the trailing head (solid line) is more narrowly distributed around zero than the relatively broad distribution on the leading head (dashed line). $\Delta \beta$ is included in Fig_Tlmrk_B.
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SIFig_SPC

Single photons collected by either APDx or APDy during one complete cycle of 8 laser polarizations arrive randomly during each laser polarization illumination interval (colored dots and labels), referred to here as a gate (black dashed vertical lines). $t_g = 0.1 \text{ ms}$ so that 1 cycle $= 8 \times t_g = 0.8 \text{ ms}$. Binning the single photon data only requires counting the number of photons in each gate; however, the MCCP change point algorithm uses the raw arrival times directly.
Change points with large magnitude likelihood values are consistent with myosin stepping. Calculating the average dwell between change points with likelihood values greater than 30 log-units results and then sorting the molecules according to their average velocity results in excellent agreement between the measured dwell time and the expected velocity (gray line). The expected velocity is a plot of the equation: \( \text{dwell} = \frac{36 \text{ nm}}{\text{velocity}} \) were 36 nm is the step size of myosin and velocity is the average velocity for \(~10\) molecules on the slide measured from multiple images recorded on the CCD camera either before or after polarization recordings.
This seems like a reasonable bin size.
This bin is OK, but look at more molecules to confirm difference in stdev.
In this molecule steps were detected at CP#1, #2 and #7. No substep was detected when the wobble changed from the high to low $\beta$ at CP#1, presumably since this is the power stroke on the leading head. During the step at CP#2 and high wobble substep was detected during the transition from low to high $\beta$ consistent with the duration of the rebinding of detached head. Between CP#5-6 high wobble was again detected however there was no change in $\beta$ angle, possibly due to a so called foot-stomp event where the leading head detaches and rebinds to the same actin monomer (Syed; Kodera 2010). A third step occurs at CP#7 with no increase in probe wobble, again consistent with a power stroke on the leading head. Lastly, the probe remains in a high wobble state, likely due to a photo0physical effect on the dye prior to photo-bleaching after $\sim$1.3 s.
Cumulative distribution of non-step $\beta$ (A, C) and $\alpha$ (B, D) angle changes ($n=73$) using only steps during which a high wobble state was detected so that the trailing (solid curves in A and B) and leading head (dashed curves in A and B) could be unambiguously assigned to the entire dwell before and after the step, respectively. For the trailing and leading heads, the angle during the interval immediately preceding (red for $\beta$ in A and C) and following (green for $\alpha$ in B and D) the step was compared to the remaining angles during the respective dwells (magenta for $\beta$ in A and C; brown for $\alpha$ in B and D). Additional angle changes during the dwell were indistinguishable from the angles associated with stepping for $\beta$ in the leading head (C) and $\alpha$ in either the leading (B) or trailing heads (D). Conversely, for the $\beta$ angle on the trailing head, stepping was more likely to occur from smaller $\beta$ values. See Fig_Tlmrk for additional details.
The distribution of $\delta$ during intervals at the beginning of a recording (blue) are peaked at moderate $\delta$ of $\sim40^\circ$ whereas wobble during intervals immediately prior to photo-bleaching (purple) are noticeably shifted to larger values with a peak near $\sim80^\circ$. These distributions are insensitive to high (solid) or low (dashed) laser power, but a larger fraction of the recording prior to photo-bleaching contains high wobble states occupy at high laser power, suggesting some type of photophysical effect.
In this molecule a clear step as detected at CP#1 from a low to high $\beta$ angle, suggesting a trailing head detachment and rebinding, however no increase in wobble was detected. A second step likely occurs between between CP #2 and #10 but its location is obscured by the intervals of rapid polarization changes that occurs again for a second period between CP#11 and CP#17. These periods correspond to random highly variable $\beta$ and $\alpha$ angles, but usually not higher wobble $\delta$ (see SI Fig. Rapid for distributions). Clearly myosin stepping on this rapid ~20 ms time scale is not feasible at rate-limiting ATP concentrations. Presumably a photophysical effect of the dye is the cause. It is also interesting to note that the increased time resolution can distinguish an apparent dual photo-bleach in the binned intensities at 0.7 sec that is actually a brief blinking event followed by a photo bleach.
Occasionally, periods of rapidly changing PFIs were detected by the MCCP algorithm during an otherwise typical recording (see also SIFig_rapidOsc). (A) The distribution of $\beta$ (red) and $\alpha$ (green) during these intervals is consistent with a random distribution of orientations; specifically, a uniform distribution for $\alpha$ (green line) and a sinusoidal distribution for $\beta$ (red line). For sufficient statistics $\beta$ was restricted between $0^\circ$ and $90^\circ$ (black vertical dashed line). The distribution of $\delta$ (blue) is peaked at $40^\circ$ and is comparable to other distributions of probe wobble when both heads of myosin are bound to actin (see SIFig_AngDistsDurWob_A). (B) The histogram of the intervals (gray bars) between these changes resulted in a distribution that was fit (omitting the under-populated first bin) by a single exponential with decay constant of $18 \pm 2$ ms (solid). These events are relatively rare but typically occur in a burst of several changes, thus making them relatively easy to identify but hard to subscribe to a biological function. For example, the abundance of these events is in excess of what would be expected for stepping at the Mg.ATP concentrations used (dashed line).
The histogram of observed high wobble rebinding events during myosin stepping (green dots) or their cumulative distribution (gray dots) suggests most of the events with less than ~250 photons are not detected. Either of these distribution can be predicted from the known sensitivity of the MCCP algorithm (blue curve), which is a fit to the detection efficiency determined by using the algorithm to find the high wobble state in a series of simulations over a range of durations and signal to background ratios, and the expected distribution if 100% of the events were detected (red curve), which is determined by fitting an exponential (magenta line) to the tail of the observed distribution (green dots). Treating the signal to background ratio as a fit parameter results in excellent agreement between the predicted distribution (solid green lines). This analysis combines all high wobble events detected during stepping (i.e., with and without BDM present in solution), but does not include those events that are detected by the algorithm but not identified as high wobble due to insufficient photons used in probe model.

To do:
- Decide if we want to use this curve
- If so, clean it up and use better colors.
- Maybe include the fit using the observed signal to background ratio (i.e., zero fit parameters!)

SIFig_DetecFrac

Just mention this in words
The efficiency of the probe model to accurately identify a high wobble state can be assessed by simulating $\delta = 90^\circ$ over a range of photons and signal to background ratios. The range of possible $\delta$ values returned by the model can then be used to determine if the experimentally observed wobble is consistent with high wobble under similar conditions. Here the lower bound of the 95% confidence interval for $\delta$ (y axis) is reported for a range of photons (x axis) at various signal to background ratios where $\infty$ indicates the ideal case of zero background photons. The gray curve represents an exponential distribution of rebinding events with a mean of 600 photons estimated from our experimental results. For example, if a recording has a signal to background ratio of 3 and an interval with 500 photons is detected during a stepping event, then probe wobble estimated by the dipole algorithm can be as low as $55^\circ$ and still be consistent with a high wobble event. Physically, as the number of photons decreases the relative size of these fluctuations can be large, which has the effect of making the probe appear as if it is strongly polarized in the direction of the PFI associated with the fluctuation and thus lower the observed value of $\delta$.

Try to Use this to back calculate a consistent wobble?
Just mention this in words
Relegate to Supp.
Remove dashed line

Promote to supp.

Omit, but say in words
Omit, but say in words
Include these 6
Gallery of angle changes

110504D_055: 4.9, (5.5 ms) short

110504E_026: 4.0, weird bleach

110504C_113: 5.0, (12 ms) rapid CPs, 2D scan
Gallery of angle changes

110504D_055: 4.9, (5.5 ms) short

110504C_113: 5.0, (12 ms) rapid CPs, 2D scan

110504E_026: 4.0, weird bleach

More of these
110519G_069... 5 changes, but too noisy?

More of these
Gallery of Post-Wobble angle changes

100420B_010...4 changes

110519G_069... 5 changes, but too noisy?

More of these
Pre and post

110519G_069...good bleach, sort of noisy, not HoH?

More of these
Pre and post
110503F_068

More of these

3/23/2012
101207F_031: 5.2, 3 steps, lo wob

110504C_073: 4.7, good

110503C_095: 4.6, good

110519G_025

Only this one
Gallery of foot stomps

110504E_014: 4.9, foot stomp, rapid CPs

110518M_018: 4.7, not great, large wob

Only this one
Gallery of high wobble at end

Either of these

omit

Rapid too

omit
### Summary of change points and 95% confidence interval

<table>
<thead>
<tr>
<th>Index</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>6</th>
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<td>[3, 6]</td>
<td>[4, 5]</td>
<td>[5, 6]</td>
<td>[6, 7]</td>
<td>[7, 8]</td>
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<td>7.66967</td>
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### Summary of change points and 95% confidence interval

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### Index

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<th>psi I</th>
<th>phi-actin(deg)</th>
<th>dist (px)</th>
<th>dist (nm)</th>
<th>delta-t (sec)</th>
<th>vel (pix/sec)</th>
<th>vel (nm/sec)</th>
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Avg.Cnt.Rt.(bleach, bak, ratio): {61.5525, 15.0256, 4.09651} cnts/ms (no cal)

APD: (X,Y,X,Y,c1,c2): {23.3853, 24.2697, 0.982965, 0.981828, 1.13815} cnts/ms(y cal)
bak: {bm1,bm2,bm3,bm4,bm5,x12est} = {6.52561, 9.30299, 1.42561, 1.41743} cnts/ms (yes cal)
director eigenvalues: {0.697534, 0.26815, 0.0263314}
direction: (β, α) = (40.866, 156.633)^a, (θ, φ) = (74.9608, -34.4581)^a.

1/23/2012