Supporting Online Material for

Myosin V Walks by Lever Action and Brownian Motion

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Materials and Methods

Materials. Myosin V was purified from chick brains (*1*). Rabbit skeletal actin was prepared (*2*) and stained (*3*) with phalloidin-tetramethylrhodamine B isothiocyanate conjugate (Fluka). Tubulin purified from porcine brains was labeled with either Alexa Fluor 488 carboxylic acid, succinimidyl ester (Molecular Probes) or biotin-$(AC_5)_2$ sulfo-Osu (Dojindo), and both were co-polymerized with untreated tubulin and stabilized with paclitaxel (Sigma) to make fluorescently labeled and biotinylated microtubules (*4*). Human calmodulin with six histidines at N-terminus was ligated at its C-terminus to human kinesin-1 heavy chain (residues 1-332) with T92N mutation that allows irreversible binding to a microtubule (*5*). The fusion protein was expressed in *E. coli*, purified, and exchanged with intrinsic calmodulin of myosin V essentially as reported (*6*) but at a higher Ca^{2+} concentration and with longer incubation to allow multiple replacements per neck (*7-9*) : calmodulin-kinesin and myosin V at the molar ratio of 10-100 : 1 were incubated for 30 min on ice in 20 mM imidazole-HCl (pH 7.6), 2.5 mM $MgCl_2$, 75 mM KCl, 0.55 mM EGTA, 2 mM DTT, and free Ca^{2+} at 60-400 μM. The reaction was terminated by the addition of 8 mM EGTA followed by >30 min incubation on ice. Myosin V carrying kinesin thus prepared was mixed with the fluorescent microtubules at the tubulin : myosin ratio of 1600–6400 : 1 (one myosin molecule per 1-4 μm of a microtubule) in buffer A (10 mM imidazole-HCl (pH 7.6), 4 mM $MgCl_2$, 100 mM KCl, 1 mM EGTA, 5 mM DTT) with 8 μM paclitaxel, and incubated at room temperature for >5 min to allow binding.

Actin moving assay. A flow chamber was made of two coverslips, bottom one ethanol cleaned, separated by two spacers of 25 μm thickness. All solutions below were in buffer A, and the following infusions (2-3 chamber volumes) were made with 1-2 min incubation in between: 2 mg ml^{-1} biotinylated BSA (Sigma), 5 mg ml^{-1} BSA, 2 mg ml^{-1} streptavidin, buffer A for washing, myosin V-microtubule complex (0.3-2 μM tubulin), and then buffer A with 8 μM paclitaxel. Finally 7.5 or 15 nM actin, pipetted 100-200 times to make filaments short, was infused together with 0.2-0.3 μM ATP, 2.3 μM wild type calmodulin, 8 μM paclitaxel, ATP-regenerating system (1 mM creatine phosphate and 0.1 mg ml^{-1} creatine kinase), oxygen scavenger system (3.2 mg ml^{-1} glucose, 0.2 mg ml^{-1} glucose oxidase, and 0.89 mg ml^{-1} catalase), and 0.5-1% β-mercaptoethanol.

Microtubule bridges. 0.6 pM of 3-μm carboxylated polystyrene beads (Polysciences) were mixed with 0.6 mg ml^{-1} biotinylated BSA in buffer A and incubated for >2 min on ice, followed by addition of 1 mg ml^{-1} BSA and another incubation for >2 min on ice. Immediately before microscopic observation, the beads were washed with buffer A. Infusions into an observation chamber were made as described above under the actin moving assay, except that the first infusion was of the washed beads instead of biotinylated BSA.

Microscopy. We used an Olympus IX70 microscope equipped with a 100× objective (PLAPO100×O LMIR, N.A. 1.4, Olympus), a stable sample stage (KS-O, ChuukoushaSeisakujo, Japan), a dual-view system (*10*) for simultaneous observation of fluorescence at two different colors, with an additional port for bright-field observation (*11*), and a double-beam optical trap system (*12*) that is also based on the dual-view optics. Fluorescence was excited at 475-490 and 545-570 nm, and images of tetramethylrhodamine at 580-625 nm and Alexa 488 at 500-535 nm were captured side by side with an intensified (VS4-1845, Video Scope) CCD camera (CCD-300-RCX,

Dage-MTI). Bright field images (650-730 nm) were recorded with another CCD camera. Observation was made at 23 °C. Centroid of an actin (or microtubule) image was calculated as described (*13*), and the orientation of the filament was determined from the second moments of the intensity distribution ($\tan 2\theta = 2\langle Ixy\rangle/(\langle Iy^2\rangle - \langle Ix^2\rangle)$; *I*, intensity; < >, average). When another filament came nearby, the orientation was judged by eye and centroid was not calculated.

Samples for microtubule moving assay. Actin filaments were stained with Alexa Fluor 488 phalloidin (Molecular Probes), and microtubules were labeled with 6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (Molecular Probes). Myosin V carrying mutated kinesin (in initial trials of this assay the N-, not C-, terminus of calmodulin was linked to the mutated kinesin) was mixed with the rhodamine-labeled microtubules, which had been pipetted ~100 times to make them short, at the tubulin : myosin molar ratio of 200-800 : 1 in buffer A plus 200 mM KCl (total 300 mM) with 0.5 mg ml^{-1} BSA. The mixture was incubated for >5 min at room temperature. To make an actin bridge (a suspended actin filament), beads coated with α-actinin (Sigma) were prepared. Carboxylated polystyrene beads (3 μm, Polysciences) were mixed with 50 mg ml^{-1} 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (Dojindo) in buffer B (20 mM Pipes-KOH (pH 6.5), 20 mM KCl, 4 mM $MgCl_2$) and gently mixed for 1 h at room temperature. Then the beads were washed three times by centrifugation in buffer C (20 mM Hepes-KOH (pH 8.0), 20 mM KCl, 4 mM $MgCl_2$). 2.0 mg ml^{-1} α-actinin in buffer C was added, and the beads were sonicated for 10 min and gently mixed for 1 h at room temperature. The reaction was terminated by the addition of 50 mM Tris-HCl (pH 6.8). After 10 min of gentle mixing, the beads were washed by centrifugation three times in buffer D (10 mM imidazole-HCl (pH 7.6), 500 mM KCl, 2 mM $MgCl_2$) and twice in buffer A. After sonication on ice for 5 min, 1 mg ml^{-1} BSA was added and sonication was repeated for another 5 min. Finally, the beads were washed three times by centrifugation in buffer A, and kept on ice with the addition of 1 mg ml^{-1} BSA. Free α-actinin remaining in a bead preparation would form actin bundles in the microscope assay; such beads were washed again extensively.

Microtubule moving assay. A flow chamber was made as described in actin moving assay. Solutions in buffer A were infused with 1-2 min incubation in between: 5 mg ml^{-1} BSA, buffer A for washing, myosin-microtubule complex (130 pM tubulin) and 2 nM actin together with 0.1-0.3 μM ATP, 2.3 μM wild type calmodulin, 0.5 mg ml^{-1} BSA, 8 μM paclitaxel, 0.06 pM α-actinin-coated beads, 100 mM 2,3-butanedione oxime (Wako), ATP-regenerating system (see actin moving assay), oxygen scavenger system, and 0.5-1 % β-mercaptoethanol. ATP concentration was corrected for the ATP in the actin preparation estimated by luciferase assay. After the last infusion, the spacers were removed, and the chamber was sealed with silicon oil or nail liquid. We made an actin bridge by moving the microscope stage to let a chosen actin filament attach to two beads held in optical traps. Then, a microtubule of an appropriate length (2~3 μm) was selected and challenged against the actin bridge by stage movement. After binding of the microtubule to the actin bridge, we did not touch the stage.

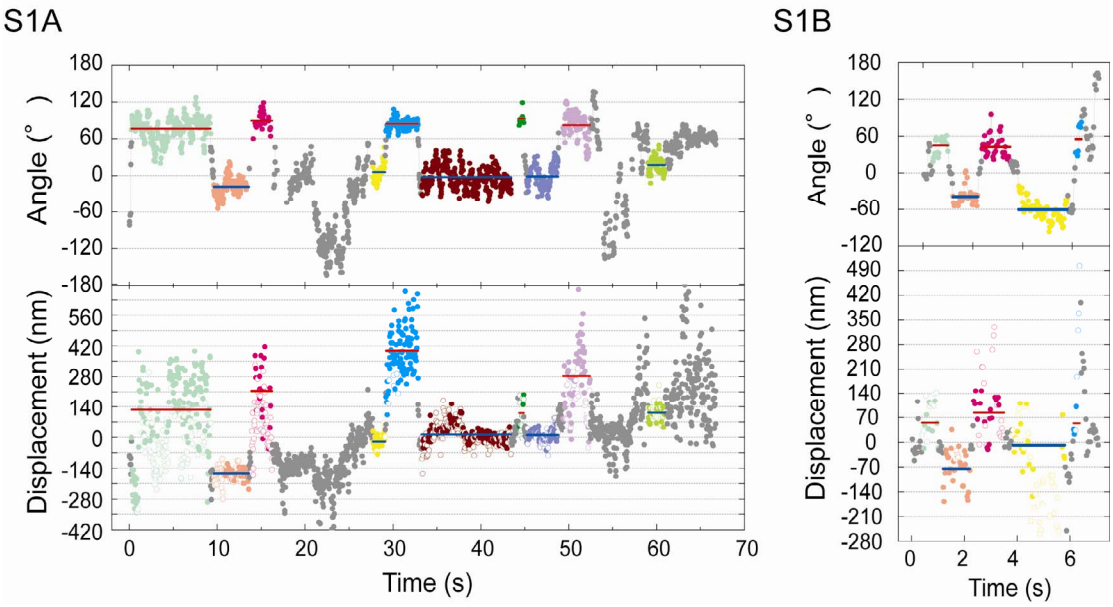
Choice of tubulin : myosin ratio. The binding probability of a floating microtubule to the actin bridge depended significantly on the tubulin-to-myosin ratio during the preparation of the myosin-microtubule complex. At the molar ratio of less than 50 : 1, almost all challenges resulted in binding and the microtubule was often oriented along

the actin filament, suggesting binding by multiple myosin molecules on the microtubule. The binding probability decreased at higher ratios, and was scarce at 1600 : 1. We thus worked at 200-800 : 1, where we could achieve binding a few times in a chamber over a trial period of ~30 min.

Specific binding through the mutated kinesin. To confirm that myosin V bound a microtubule through the mutated kinesin, we performed the following control experiments. In the procedure where intrinsic calmodulin of myosin V was replaced with the calmodulin-kinesin fusion protein, we incubated myosin V with wild-type calmodulin instead of calmodulin-kinesin. The myosin V thus prepared was mixed with microtubules at the molar tubulin : myosin ratio of 50 : 1. No microtubules bound to an actin bridge, compared with the ~100% binding with the kinesin fusion above. In another trial, we mixed, in the normal replacement procedure, tenfold excess of wild-type calmodulin in addition to the calmodulin-kinesin fusion protein. This resulted in a significantly low probability of microtubule binding to an actin bridge. The competition between calmodulin-kinesin and wild-type calmodulin indicates that a microtubule was bound to myosin V at the neck portion through the engineered calmodulin-kinesin that replaced the intrinsic calmodulin.

Supplementary Figures S1 to S6

Figure S1. Analyses of microtubule moving assay. Two more examples of the experiment shown in Fig. 1 in main text, where a microtubule was allowed to move on a suspended actin filament at 0.2 μM (**S1A**) or 0.3 μM (**S1B**) ATP. Colored dots, two stationary angles; dark grey dots, swing phase; red and blue bars, average of colored dots. In the lower panels showing the microtubule centroid, only those instances where the microtubule was within 15° or 13° from the average stationary angle are colored, the remainder being shown in light grey. Note that the centroid of the microtubule image, though calculated within a horizontal band of height 600 nm along the actin filament, moves by the swinging motion alone, and thus displacement should be judged by comparing centroid positions at similar microtubule orientations. That is, only comparison among blue bars, or among red bars, is meaningful. Translocation of the microtubule in **S1A** may be judged by eye in movie S2.



Figures S2 and S3. Durations of unidirectional (**S2**) and fluctuating (**S3**) swings in actin-moving assays. Swings are defined as a transition between two stationary angles which are shown in color in Fig. 2C in main text and fig. S5 below. In each of the eleven swing records containing altogether 77 swings, two swing directions are identified: one accompanying little translocation of actin (altogether 39 swings) and the other accompanying a ~70-nm translocation (38 swings with three exceptions of irregular translocation). The former is presented as anticlockwise swings, or swings from ~0° to a positive angle, in all figures and movies by applying mirror inversion when needed (inverted data: figs. S5A, B, C, D, H, J, and movie S4). Anticlockwise swings defined in this way are all unidirectional without significant reversal, whereas clockwise swings often, but not always, involve fluctuations. Beginning of a swing (frame 1) is the point, judged by eye, when actin significantly deviates from the previous stationary angle; thus, in fluctuating swings, we may have missed the true beginning because a fluctuation could start without appreciable rotation. End is when actin reaches the next stationary angle. The durations in the histograms are the frame number of the end point. White portions in the histograms show swings in which the actin filament was momentarily stuck (black arrows in fig. S5); light grey, swings in which an expected translocational step was missing, possibly due to back stepping in that or the previous swing (green arrows in Figs. 2C and S5). Inset, an expanded histogram for short times. Mean values were calculated from data in dark grey.

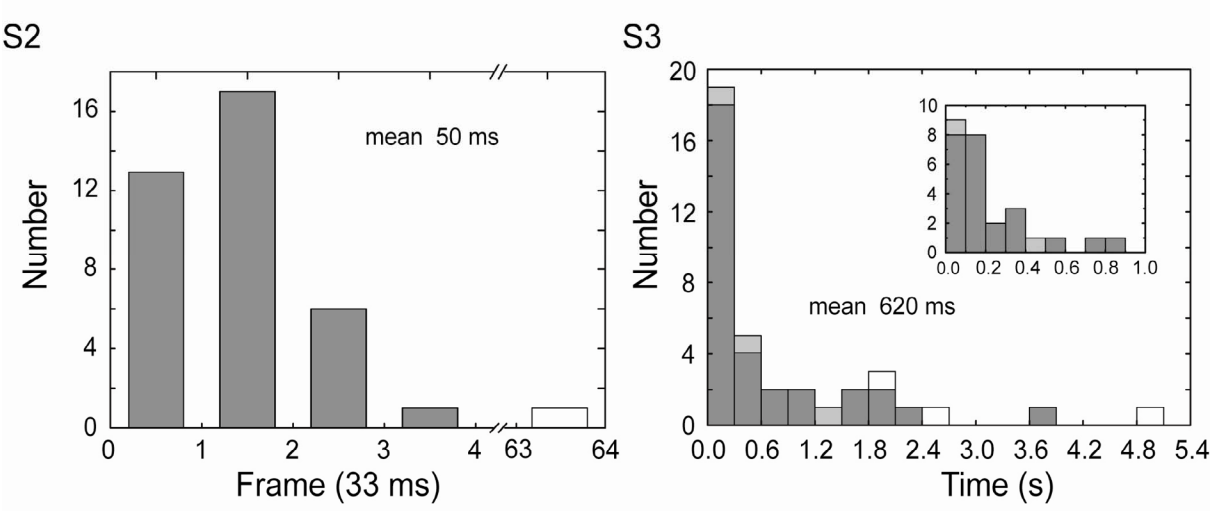


Figure S4. Histograms of dwell times on the two stationary angles. Red, before a clockwise (fluctuating) swing; green, before an anticlockwise (unidirectional) swing. Light colors, swings possibly associated with a back step. See legend to figs. S2 and S3 for details. Mean and s. e. were obtained from data in dark colors by curve fitting with $y=A\cdot\exp(-t/\tau)$.

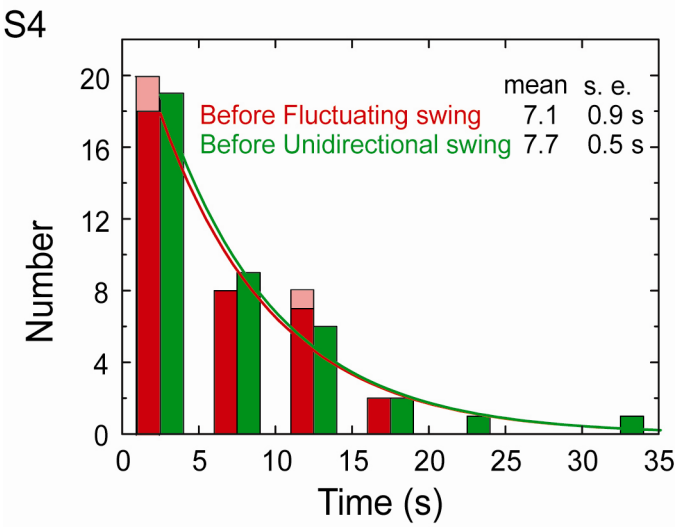
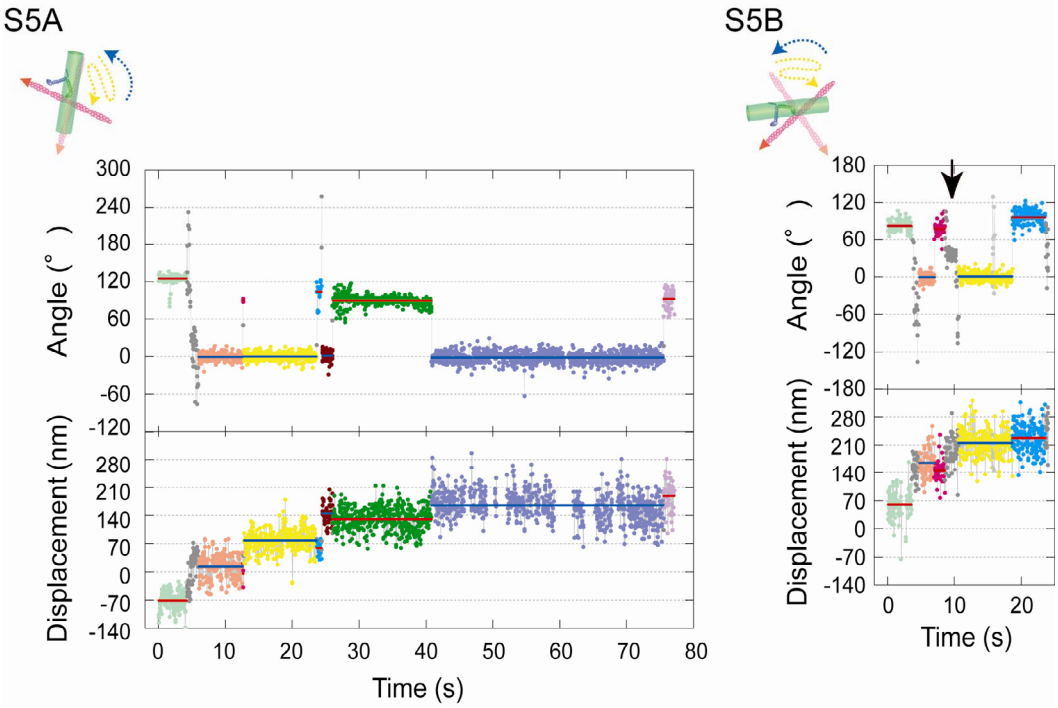
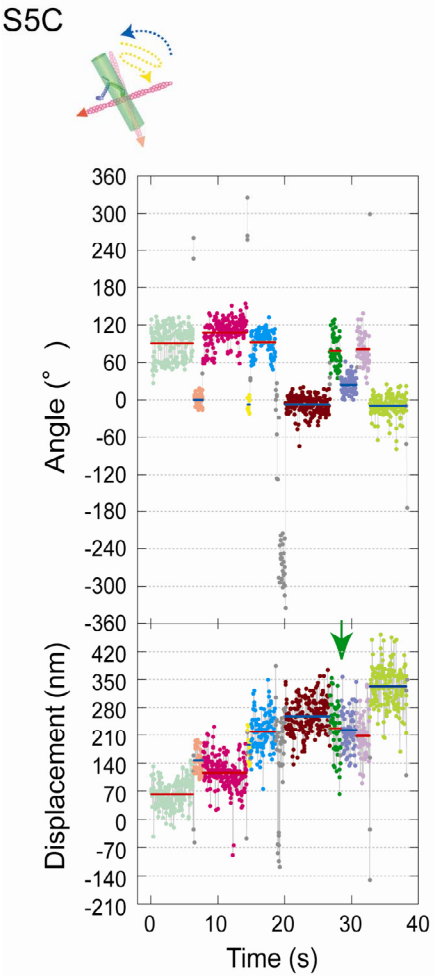


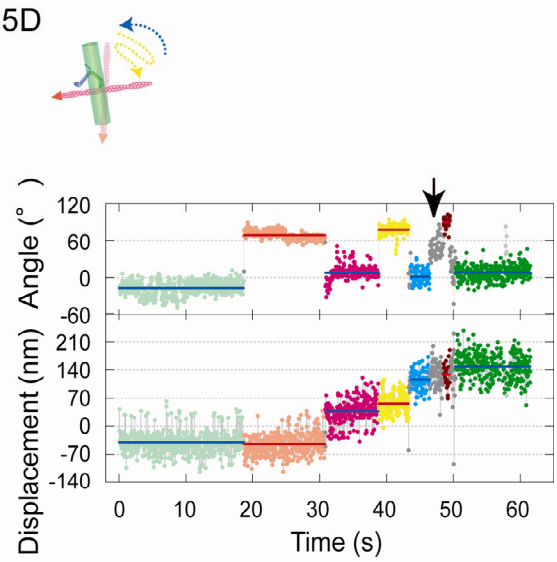
Figure S5. Time courses of angle and position of actin filaments in actin-moving assays on a surface. Experiments as in Fig. 2 in main text, at 0.2 μM ATP. Cartoons at the top of each figure show relative orientations among myosin, actin, and microtubule deduced from the observed motion. Angles in the graphs are defined such that swings accompanying little translocation are presented as anticlockwise swings starting from ~0°. For this, mirror inversion has been applied to some of the data, as indicated by microtubule-on-top-of-myosin cartoons; these microtubules were presumably attached to the 'right-hand' side of a neck whereas others were on the 'left-hand' side. See legend to figs. S2 and S3 for details. The cartoon in **S5A** shows the configuration in movie S4. Orientation of microtubule was not determined in **S5H** and **S5J** because of the presence of another nearby microtubule. Coloured dots in time courses show periods in which actin stays in one of the two stationary angles, dark grey being swing phases in between. Light grey dots show momentary fluctuation in a stationary phase; because the actin resumed the same angle after the fluctuation, we interpret these events as transient unbinding of a head followed by rebinding to the same or nearby site. Red and blue horizontal bars, average in a stationary angle before a clockwise (fluctuating) or anticlockwise (unidirectional) swing, respectively. Green arrows, swings that did not accompany an expected translocation, possibly due to a back step at the arrow or in the previous swing. Black arrows, momentary sticking to a surface. Breaks in horizontal axes indicate focus adjustment, which resulted in the apparent shift in the displacement record. In **S5B**, the stage drifted continuously, and the displacement record was corrected by referencing to a fixed bright object in the image. One swing in **S5F** (grey arrow) could not be analyzed because of a nearby actin. In **S5E**, the actin was not lying on the image plane in one of the stationary angles, and thus displacements can be compared only among red or among blue bars.



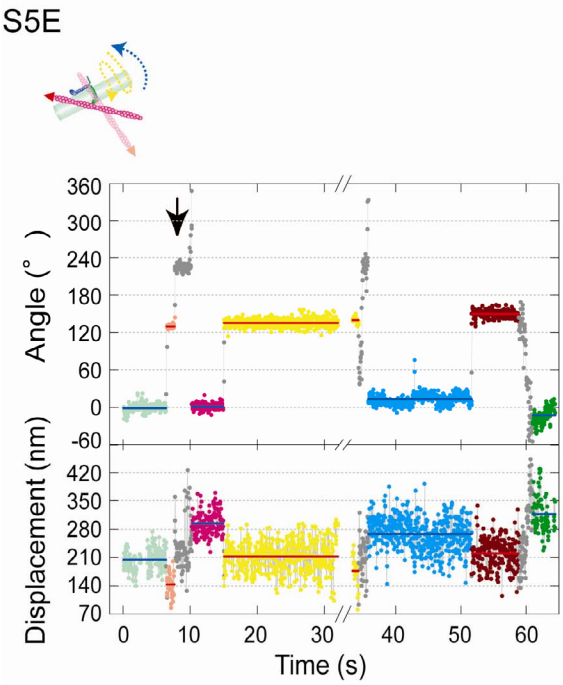
S5C



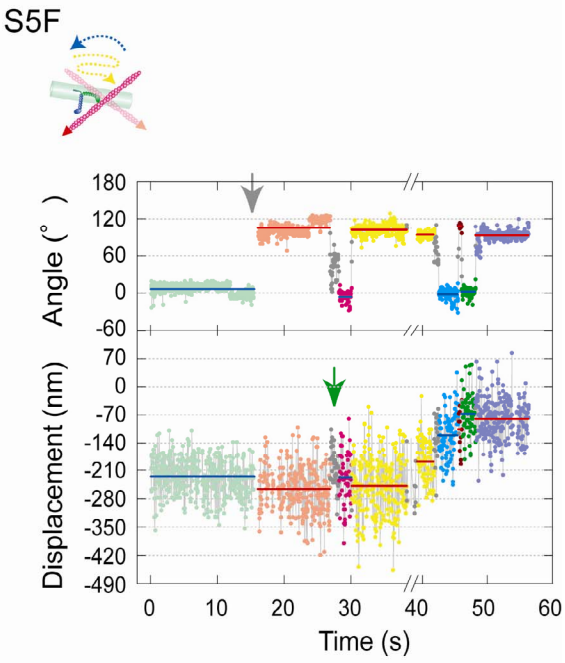
S5D

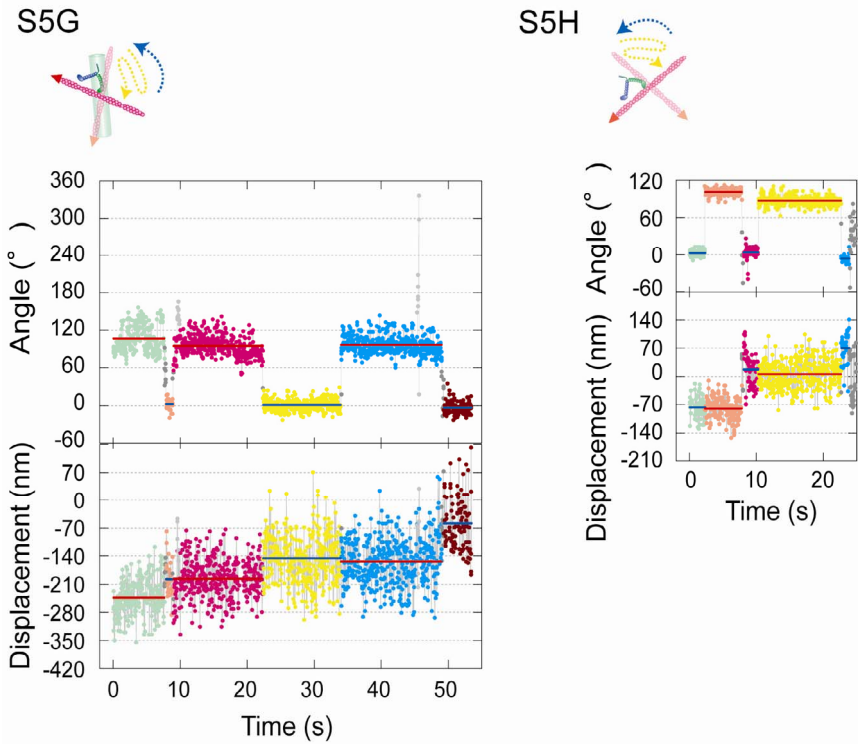


S5E



S5F




S5G
S5H
Angle (°)
Displacement (nm)
Time (s)

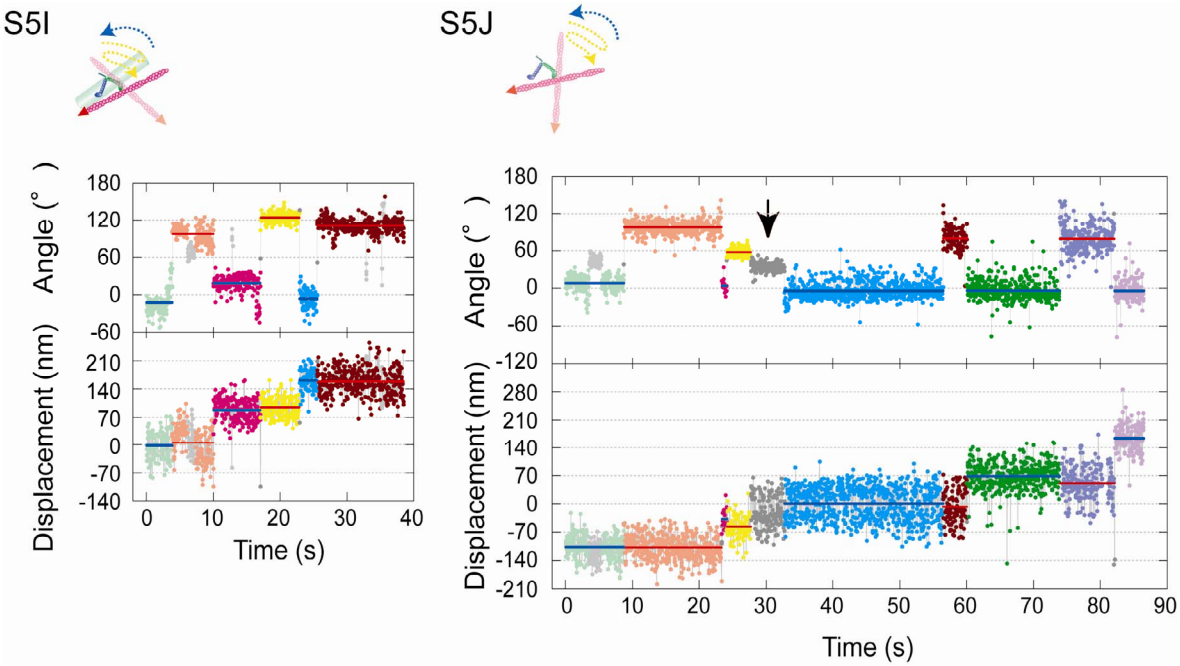
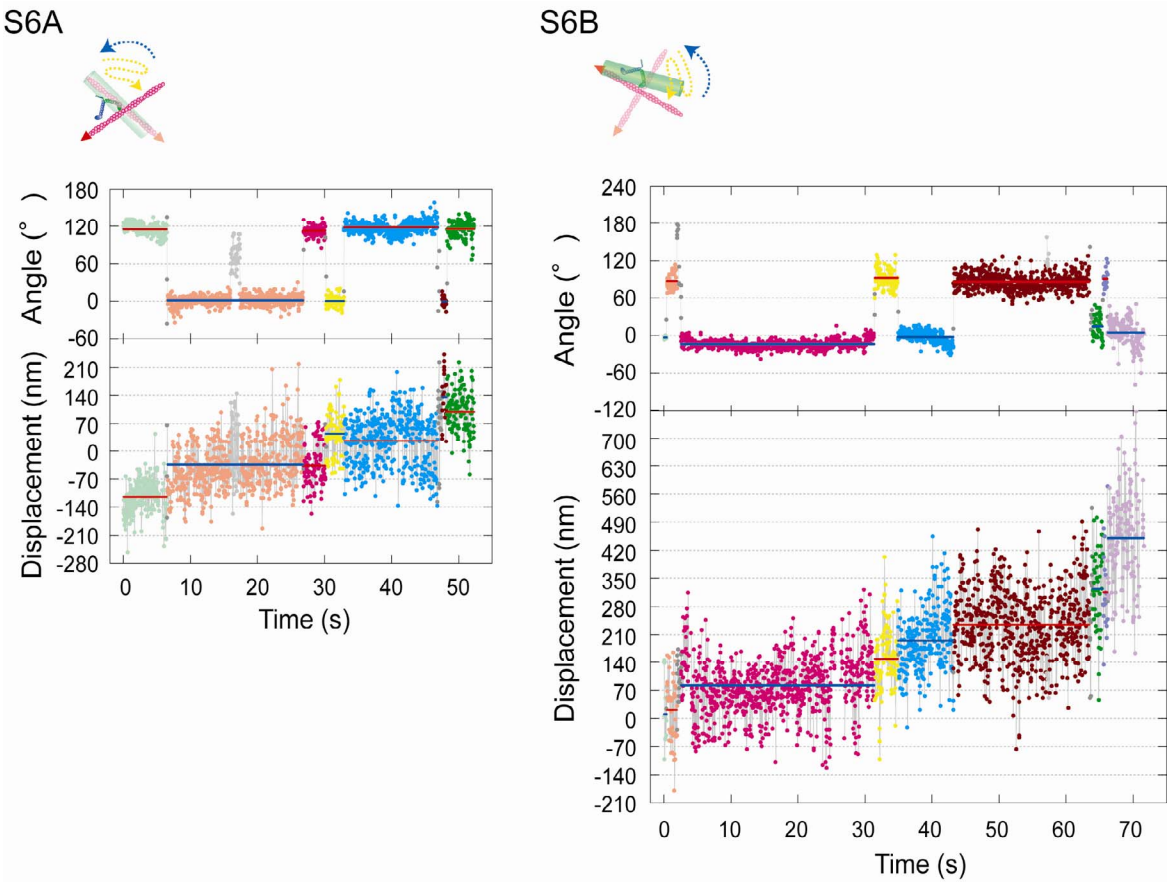

S5I
S5J
Angle (°)
Displacement (nm)
Time (s)

Figure S6. Time courses of angle and position of actin filaments in actin-moving assays on a microtubule bridge. Experiments as in Fig. 4 in main text, at 0.2 μM ATP. Symbols are as in fig. S5. The cartoon in **S6A** shows the configuration in movie S6. Data in **S6B** have been mirror-inverted.



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