Supporting Information

The Antiparallel Coiled-coil Domain Allows the Multiple Forward Step Sizes of Myosin X

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1. Materials and methods

Constructs and purification

Full-length myosin X (amino acids 1-2058) was created starting from the amino acids DNFFTEGTRV and ending at the amino acids RSASSQGSSR. Full-length myosin X was constructed with a FLAG-tag (peptide sequence: DYKDDDDK) at the N-terminus to purify myosin X using an anti-FLAG antibody affinity column. mApple (a red fluorescence protein) and a spacer of three amino-acids were added to the FLAG-tag. mApple that has an excitation peak of 568 nm and an emission peak of 592 nm was used to track the motility of single myosin X molecules.

Chimera with the antiparallel coiled-coil domain consisted of the motor domain and the first IQ motif from myosin V (amino acids 1-790 of myosin V, starting from the amino acids AASELYTKFA and ending at the amino acids LRKKYLRMRK) followed by the second and third IQ motif and the rest of myosin X HMM (173 amino acids, starting from the amino acids LYCVVIIQKN and ending at the amino acids RLEEEACRAA - the end of myosin X HMM).

Chimera with the parallel coiled-coil domain was constructed from the motor domain, 3 IQ motifs and the single alpha helical (SAH) domain from myosin X (amino acids 1-881 of myosin X, starting from amino acids DNFFTEGTRV and ending at the amino acids AELTRELEKQ) and the coiled-coil domain of myosin V (119 amino acids, starting from KIEARSVERY and ending at QETEQLVSNL). The coiled-coil domain of myosin V was followed by a leucine zipper (KQLEDKVEELLSKNYHLENEVARLKKLVGE) to allow the chimera to form a dimer.

Mutant myosin X was constructed from amino acids 1-938 of myosin X (starting from the amino acids DNFFTEGTRV and ending at the amino acids RLEEEACRAA). This construct contained 4 mutation points including I890A, L893Q, I897A, K904A in the

antiparallel coiled-coil domain. The antiparallel coiled-coil domain was followed by a linker (SEGGSGGSGGSGGSAASAA) with a leucine zipper and an Avi-tag (peptide sequence: GLNDIFEAQKIEWHE).

The chimeras and mutant myosin X contained mApple and a spacer of 3 amino-acids in the N-terminus, and a FLAG-tag in the C-terminus. All the proteins were expressed in baculovirus expression system and were purified from SF9 cells.[1] Since all the constructs contained a FLAG-tag, these proteins were purified using anti-FLAG antibody affinity columns.

Actin filaments and actin bundles

Globular actins (G-actins) were obtained from the rabbit skeletal muscle as previously described.[2] Fascin expressed in *E. Coli* was purified as described in the previous report.[3] Actin filaments and actin bundles were prepared as described in the previous report.[4] Actin filaments were polymerized in the polymerization buffer containing 10 µM G-actin, 100 µM MgATP and 5 µM phalloidin. F-actin was incubated at 4°C for 12 hours before use. In order to prepare actin bundles, 8 µM F-actin was mixed with 3 µM fascin in buffer and incubated on ice and stored at 4°C for two days before performing experiments.

Single-molecule motility assays

Sample chambers used in experiments were prepared as described in the previous report.[5] Firstly, we sonicated the microscope slides and coverslips using Milli-Q water for 30 minutes and rinsed with milliQ water 3 times. Next, we sonicated the microscope slides and coverslips with acetone for 30 minutes and rinsed with Milli-Q water 3 times. After that, we sonicated the microscope slides and coverslips with 1 M KOH for 30 minutes and rinsed with

Milli-Q water 3 times. Then, we sonicated the microscope slides and coverslips with Milli-Q water for 30 minutes and rinsed with Milli-Q water 3 times. After finishing the sonication, we dried the microscope slides and coverslips, coated the coverslips using 15 μL nitrocellulose, and incubated it for 1 hour to let the coverslips dry. Finally, we assembled the chamber with coated coverslips and a microscope slide using double-sided tape and sealed with epoxy glue. Sample chambers were stored in a dehumidifier.

Motility buffer for single-molecule motility assay contained 20 mM HEPES, 2 mM $MgCl_2$, 25 mM KCl, 1 mM EGTA, 10 mM DTT and 5.5 μM Calmodulin. N-ethylmaleimide-modified myosin (NEM myosin) was diluted in motility buffer to reach the final concentration of 0.25 mg/mL and then was loaded into the sample chamber for actin immobilization. After 7-minute incubation at room temperature and washing, actin filaments or actin bundles were introduced into the sample chamber. After another 7-minute incubation, unbound filaments or actin bundles were gently washed out with buffer, and myosin was added into the chamber. The concentration of myosin varied for each construct. To block the non-specific binding, myosin was diluted in buffer with casein (7.6 mg/mL). After incubation to let myosin bind to actin filaments or actin bundle, we observed the fluorescence signals of myosin using a fluorescence microscope to choose the regions where actin filaments or actin bundle were stable and performed fluorescent imaging experiments in those regions. Actin filaments or actin bundles could be observed by illuminating mApple-fused motor domains of myosin with a 561 nm laser. Finally, ATP was added into the sample chamber to observe the motility of myosin. In order to measure step sizes, 2 μM ATP was used. 2 mM ATP was used to measure run-length and velocity of myosin.

Total internal reflection fluorescence microscope

To acquire fluorescence signals, an inverted microscope (IX73, Olympus) with a 100X objective with a numerical aperture (NA) of 1.49 (UAPON, Olympus) was used. mApple-fused myosin was excited by a 561 nm laser (Coherent Inc.). The laser beam was expanded through a 15X beam expander (Edmond Optics) and was focused on the back focal plane of the objective through a lens. The lens was used to adjust the incident angle to achieve a critical angle for total internal reflection. Fluorescence signals were acquired with an EMCCD camera (Ixon Ultra, Andor) with an exposure time of 100 msec or 150 msec via a frame-transfer mode through a dichroic mirror (ZET488/561rpc, Chroma) and an emission filter (ZET488/561m, Chroma) in a microscope. An additional emission filter (ET595/50m, Chroma) was used in front of a camera.

Data analysis

The localization of single mApple-fused myosin was made by fitting the point spread function (PSF) into a two-dimensional Gaussian using the following equation[6]

$$I(x,y) = A_0 + A_1 \exp\left[-\frac{1}{2}\left(\frac{x-x_0}{\sigma_x}\right)^2 - \frac{1}{2}\left(\frac{y-y_0}{\sigma_y}\right)^2\right]$$

with A_0 is the background, A_1 is the peak intensity of the PSF, x_0 and y_0 are the centroids in the x and y direction, σ_x and σ_y are the corresponding standard deviations in the x and y direction.

Custom-made IDL programs were used to localize the centroids of mApple-fuse motor domains of myosin. The trajectories were generated by plotting the centroids of PSFs in every imaging frame. The steps in trajectories were detected using a MATLAB program that was created in Ahmet Yildiz's lab at the University of California, Berkeley. Using the

function normalMixEM of the mixtool package in R language and cross-validation technique, the histograms of step sizes were fitted with mixture of multiple-peak Gaussian functions.[7]

2. Supporting Figure

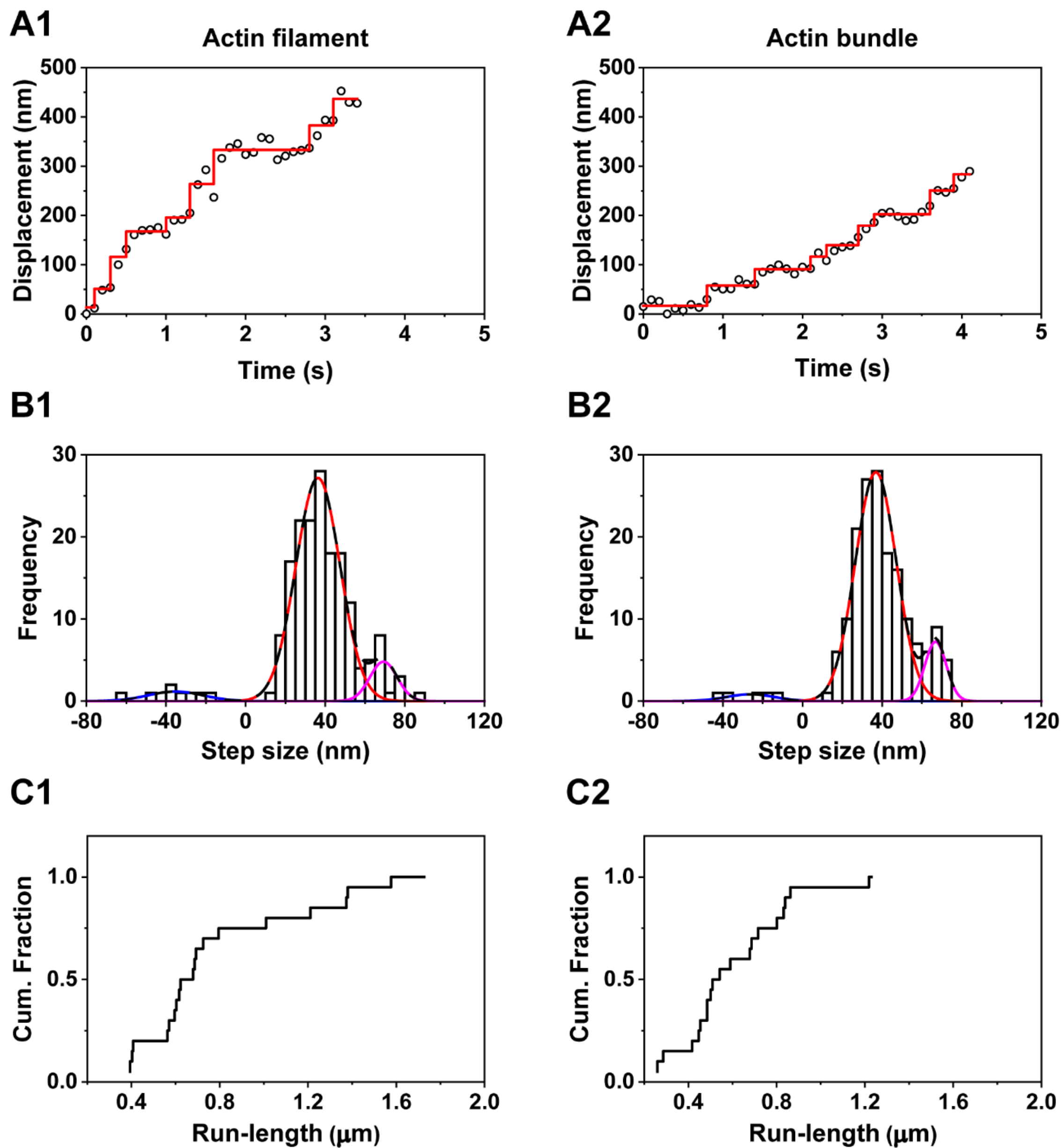

Figure S1. The motility of myosin V. (A) Representative traces of myosin V moving on actin filaments (A1) and actin bundles (A2). (B) Step size histograms of myosin V moving on actin filaments (B1) and actin bundles (B2) at 2 µM ATP. The step size histogram of myosin V moving on actin filaments was fitted with the sum of 3 Gaussian components centred at -35.3 ± 13.7 (± standard deviation (SD)) nm, 36.3 ± 11.1 nm, 69.3 ± 6.8 nm. (n = 176 steps). The step size histogram of myosin V moving on actin bundles was fitted with the sum of 3 Gaussian components centred at -26.2 ± 12.1 nm, 36.9 ± 10.3 nm, 66.9 ± 5.4 nm. (n = 169 steps). (C) Run-length of myosin V on actin filaments (C1) and actin bundle (C2) at 2 mM ATP. Average run-length of myosin V on actin filaments was 0.83 ± 0.40 (± SD) µm (N = 20 molecules). Average run-length of myosin V on actin bundles was 0.64 ± 0.26 µm (N = 20).

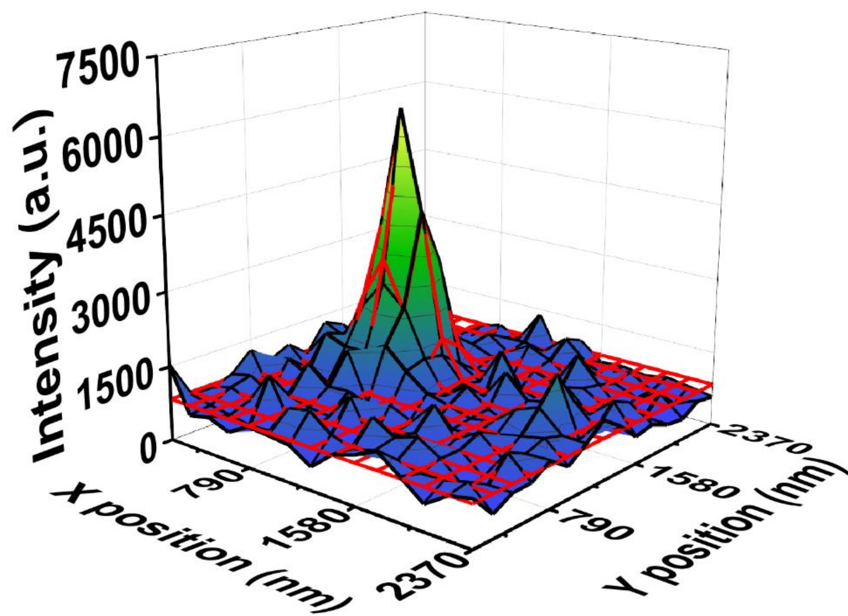

Figure S2. Point spread function. The red mesh is the 2D Gaussian fitting of the point spread function (PSF). Using the obtained centroid from 2D Gaussian fitting, we localized the centroids of single mApple molecules.

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