Cryo-electron tomography of cardiac myofibrils reveals a contraction-induced lattice twist in the Z-discs

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Abstract

The Z-disc forms a boundary between sarcomeres, which constitute structural and functional units of striated muscle tissue. Actin filaments from adjacent sarcomeres are cross-bridged by α-actinin in the Z-disc, allowing transmission of tension across the myofibril. Despite decades of studies, the 3D structure of Z-disc has been elusive due to the limited resolution of conventional electron microscopy. Here, we observed porcine cardiac myofibrils using cryo-electron tomography and reconstructed the 3D structures of the actin-actinin cross-bridging complexes within the Z-discs in relaxed and activated states. We found that the α-actinin showed a contraction-induced swing motion along with a global twist in the actin lattice. Our observation suggests that the elasticity and the integrity of the Z-disc during the muscle contraction cycle are maintained by the structural flexibility within the actin-actinin complex.
Introduction

The Z-disc defines the boundary between the two adjacent sarcomeres by crosslinking the two anti-parallel thin myofilaments. The Z-discs transmit tension generated by muscle contraction through the crosslinks mainly composed of $\alpha$-actinin \(^1,2\). The $\alpha$-actinin belongs to the spectrin family and forms a rod-shaped antiparallel homodimer of length $\sim$35 nm.

The $\alpha$-actinin monomer has the N-terminal actin-binding domain, which is composed of two calponin homology (CH) domains; the central rod domain, which is composed of four spectrin-like repeats; and the C-terminal tandem EF-hand domains, which are insensitive to calcium in the muscle-type isoforms \(^3-5\).

The structure of vertebrate Z-disc has been intensively studied using conventional ultra-thin section electron microscopy for decades \(^6-11\). In the transverse sections, the Z-discs show two distinct structural states: the “small square” form and the “basket-weave” form. In the classical studies of fixed muscle tissues, it has been proposed that the small square and the basket-weave forms represent the relaxed and the active contracted states, respectively \(^7,11,12\). According to the recent observations, however, isolated myofibrils immersed in relaxing solution containing EGTA and ATP exhibit the basket-weave form \(^13,14\). Considering the previous observation that non-treated muscle tissues show mixture of the small square and the basket-weave forms within a single Z-disc \(^8\), the variable ionic environment within the cytoplasm of the muscle tissue specimen makes it difficult to obtain a regular lattice
structure of the Z-discs. In this study, therefore, we performed cryo-electron tomography of isolated myofibrils in the presence of either EGTA+ATP or calcium+ATP (Ca+ATP). Under the ion-controlled conditions, we visualized the 3D structural changes within the Z-discs.
Results

Thin-section microscopy of cardiac myofibrils

Before conducting the cryo-electron microscopy, we observed the isolated myofibrils using conventional ultra-thin section electron microscopy because the structure of the Z-disc in the Ca+ATP-activated state has not been reported. Although the Z-discs in the EGTA+ATP (relaxed) state showed a square lattice as expected (Fig. 1A and C), the Z-discs in the Ca+ATP state exhibited a diamond-shaped lattice with inter-axial angles of 80° and 100° (Fig. 1B and D). When we carefully examined the previous studies, however, the reported “small square” lattice is not always square and diamond-shaped “offset-square” lattices have often been reported in mammalian Z-discs. Thus, it is possible that the Z-discs under the Ca+ATP condition are in a fully-activated state, which takes a diamond-shaped lattice rather than a square lattice. We acquired tomograms of ultra-thin sections of the myofibrils and used the averaged subtomograms for the initial references in the subsequent cryo-electron tomography.

Cryo-electron tomography of native cardiac myofibrils

Cryo-electron tomography of myofibrils has been challenging due their large sizes (diameter of ~2 μm). However, we happened to notice that cardiac myofibrils are often branched into thin (300~500 nm) sub-fibrils when intervened by mitochondria or thick collagen bundles. We purified these “thin” myofibrils by gentle homogenization and
differential centrifugation and conducted cryo-electron tomography to reconstruct the repeating unit within the Z-discs (Fig. 2A-D). We compared the sarcomere lengths in the EGTA+ATP and the Ca+ATP states and confirmed that the Ca+ATP treatment properly activated the myofibrils (Fig. S1A). We extracted subtomograms based on the lattice points appeared on the cross-sections of the Z-discs (Fig. 2E), and the resulting averaged subtomograms (Fig. 3A-B) showed a central F-actin (gray), four opposite-polarity F-actins (yellow), and eight α-actinins (orange). Due to the structural heterogeneity within the actin lattice, however, other neighboring F-actins were blurred out. Therefore, we shifted the center of the subtomograms from the central F-actin to the neighboring F-actins and conducted additional alignments to generate shifted maps. By combining six shifted maps with the original map (Fig. 3C-D), we could see the actin lattice in the basket-weave form (EGTA+ATP) and the diamond-shaped form (Ca+ATP) (Fig. 3E-F). When we aligned the shifted maps with the original map, we noticed that densities of the α-actinin dimers were not fully visualized in the original map (Fig. 3A-B, orange), and the shifted maps showed the remaining lower halves of the dimers (Fig. 3E-F, green). These observations suggest that there is a structural heterogeneity within the α-actinin dimer and the 3D refinement was biased toward one of the α-actinin molecules that binds to the F-actin at the center of the subtomogram.

We attempted to improve the resolutions of the actin-actinin complexes by focused refinement, but we could not align the F-actin and the α-actinin densities simultaneously,
suggesting that the interaction between the F-actin and the actin-binding domain of the \( \alpha \)-actinin is highly flexible \(^{18,19}\). Thus, we performed multibody refinements \(^{20}\), which aligned the central F-actin (gray), the opposite-polarity F-actin (yellow), and two monomers of the \( \alpha \)-actinin dimer (orange and green), separately (i.e. four-body refinement). We repeated this multibody refinement for all the four anti-parallel F-actin pairs, and reconstructed the whole repeat unit of the Z-disc with significantly improved resolutions (Fig. S1B, Fig. 4A, and Movies S1-2). The resolutions of the \( \alpha \)-actinin densities were high enough to fit the crystal structure \(^3\) (Fig. 4A, red and green models).

In both EGTA+ATP and Ca+ATP states, nearly 40% of the variance was explained by the first and the second eigenvectors (Fig. S1C and 4B). In both states, the first eigenvector represents a tilting motion of the central F-actin about its center. This motion can be related to the presence of the flexible linker between the N-terminal actin-binding domain and the rod domain of the \( \alpha \)-actinin \(^3\). The second eigenvector in both states represents a rotation of the opposite-polarity F-actin about the central F-actin. This eigenvector probably reflects the irregularity in the lattice angles \(^9\).

Contraction-induced lattice conversion in the Z-disc

The assembled refined bodies showed large structural changes between the EGTA+ATP and the Ca+ATP states (Fig. 5A-C). The \( \alpha \)-actinin dimers showed a \(~21^\circ\) swinging motion about the central F-actin, and the opposite-polarity F-actin was displaced by \(~80\ \text{Å}\) along
with a ∼13° turn about its long axis. The conformation of the α-actinin in the EGTA+ATP state was similar to the proposed model of the basket-weave form \(^8,^{14,18}\). Although it is difficult to compare the previously reported 3D structure of the “small square” Z-disc \(^{21}\) with our Ca+ATP map due to the limited resolution of the previous thin-section tomogram, the straightened conformation of the α-actinin in the top view is consistently observed in both observations. These motions of the α-actinin caused sliding of the actin-binding domain along the surface of the F-actin (Fig. 5C, right). Together with the first eigenvector motions (Fig. 4B), it is likely that the interface between the F-actin and the actin-binding domain of the α-actinin is not strictly defined, which is similar to the F-actin-tropomyosin association \(^{22-24}\).
Discussion

Comparison with the previous reports of the Z-disc structure

In this study, we reported the first native 3D structure of the mammalian Z-disc. The 3D structure of the native invertebrate Z-disc has previously been reported 25, but the invertebrate Z-discs are separated from the actomyosin system under a harsh condition (extraction using 0.6 M potassium iodide). Moreover, the reconstructed map severely suffers from the missing wedge of information because the isolated invertebrate Z-discs are uniformly oriented perpendicular to the beam axis. Thus, our observations were physiologically more relevant for analyzing the conformational changes of the Z-disc in the context of active myofibrils.

Although it has been accepted that the Z-disc takes a square lattice conformation irrespective of whether it is in the small square or in the basket-weave form, diamond-shaped lattices were observed in unstimulated rat skeletal muscle (inter-axial angles of 80°/100°) 26, in dog cardiac muscles (inter-axial angles of 82°/98°) 8, and in the nemaline rods of human myopathy patients (inter-axial angles of 75°/105°) 9. As the thin and thick myofilaments in the A-band constitute a hexagonal lattice 7, it is possible that tension applied by the actomyosin contraction deforms the square lattice of the Z-discs into the diamond-shaped lattice.

It has been demonstrated that tetanized muscle tissue induced by high-frequency electrical stimulation shows the Z-discs in the basket-weave form 6, 15. This observation contradicts
our results and other reports that the basket-weave form is observed in the presence of EGTA and ATP. The physical state of the Z-disc in the tetanized muscle may be different from that under the normal contracting condition.

Mechanism of the conformational change

We observed a swinging motion of the α-actinin about the central F-actin along with a twist in the F-actin lattice (Fig. 5A-B). This swinging motion of the α-actinin can be attributed to the presence of its flexible linker between the N-terminal actin-binding domain and the central rod domain, which mediates the actin-actinin interaction in various orientations. Although the interface between the F-actin and the actin-binding domain of the α-actinin in the EGTA+ATP state was similar to the previously reported CH-domain-decorated F-actin structures, the Ca+ATP treatment displaced the α-actinin along the surface of the F-actin (Fig. 5C, right). As in the case of the interaction between the F-actin and tropomyosin/troponin, the actin-binding domain of the α-actinin could bind to a wide area of the F-actin surface. This hypothesis is related to the previous observation that the *in vitro* reconstituted actin-actinin bundles quickly disintegrate upon removal of free α-actinin molecules by washing with buffer. The interaction between the F-actin and α-actinin is likely to be highly dynamic, and the other molecules such as titin or nebulin/nebulette may reinforce the cross-bridges within the Z-discs.
Symmetry mismatch between the square lattice and the F-actin helix

The one-start helix of F-actin has a helical parameter of 27.5 Å-rise and 166.6°-rotation, giving an approximately 90°-rotation per seven actin monomers. Although this innate 90°-rotation within the helical symmetry of the F-actin matches the square lattice model (Fig. 6, asterisks), there are large angular discrepancies at other positions. We suppose that these discrepancies between the helical symmetry and the lattice angles underlies the existence of the two lattice forms in the Z-disc. The one-start helix of F-actin can be considered as a two-start helix with 55 Å-rise and -26.8°-rotation. If we focus on one strand of this two-start long-pitch helix, two α-actinin dimers bind to the F-actin every seven actin monomers (Fig. 6, red/blue). As seven is an odd number, the distance between the two longitudinally-neighboring α-actinin dimers was not constant and there are two patterns; 165 Å-rise and -80.4°-rotation, and 220 Å-rise and 107.2°-rotation (Fig. 6, stars). We believe this is another innate feature of the helical symmetry of F-actin, which gives rise to the diamond-shaped lattice with 80°/100° rotation angles in the Ca+ATP state.

The discrepancy between the helical symmetry of the F-actin and the lattice symmetry cannot be fully resolved in neither of the basket-weave and the diamond-shaped forms. The observed high flexibility between the F-actin and the actin-binding domain of the α-actinin (Fig. 5C, right) could compensate the angular mismatches between the helical and the lattice angles. Although it is counter-intuitive that the stout structure of the Z-disc is maintained by such flexible interactions, the structural adjustability of the α-actinin is
thought to be essential for the muscle tissue to withstand the high mechanical stress during the contraction cycles.
Methods

Isolation of myofibrils

Fresh porcine hearts were obtained from a local slaughter house. Cardiac myofibrils were dissected from the left ventricular papillary muscles and homogenized in a hypotonic buffer (Hepes 30 mM, pH 7.2) supplemented with protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan) using a kitchen juice mixer. The homogenized tissue was resuspended in a hypotonic HK buffer (Hepes 30mM, pH 7.2, 60 mM KCl, 2 mM MgCl₂, protease inhibitor cocktail) and washed extensively by centrifugation at 750 ×g for 15 min at 4 °C until the upper half of the pellet turned white. The brown-colored lower half of the pellet containing collagen fibers were carefully removed and the upper white part was incubated in HK buffer plus 1% Triton X-100 for 15 min at 4°C. The demembranated myofibrils were washed three times with HK buffer and were gently homogenized with a Dounce glass homogenizer to release thin myofibrils. Large fibrils were removed by centrifugation at 750×g for 15 min at 4 °C and thin fibrils in the supernatant were collected by centrifugation at 2,500×g for 15 min at 4 °C.

Ultra-thin section electron microscopy of fixed myofibrils

The pellet of large myofibrils was re-suspended in HK buffer and treated either with 2 mM CaCl₂ and 2 mM ATP or 2 mM EGTA and 5 mM ATP for 5 min at room temperature. The
fibrils were then fixed with 1% glutaraldehyde for 1 hour at 4°C and stained with 1%

osmium tetroxide and subsequently with 1% uranium acetate. After dehydration in ethanol
and acetone, the samples were embedded in Quetol 812 resin (Nissin EM, Tokyo, Japan).

Ultrathin sections (60-nm or 200-nm thick) were cut using a ULTRACUT microtome
(Reichert Leica) and mounted onto Formvar-coated copper grid. For placing fiducial
markers, 15-nm gold particles (BBI Solutions, Cardiff, UK) were attached to both surfaces.

Images were recorded using a JEM-2100F microscope (JEOL, Tokyo, Japan) at University
of Yamanashi operated at 200 keV equipped with a F216 CMOS camera (TVIPS GmbH,
Gauting, Germany). The nominal magnification was set to 15,000× with a physical pixel
size of 8.6 Å/pixel. Tilt series images were recorded using EM-TOOLs program (TVIPS).

The angular range of the tilt series was from -60° to 60° with 2.0° increments and the target
defocus was set to 2 µm. Back-projection and subtomogram averaging were conducted as
described below in the cryo-EM section. For the subtomogram averaging of the thin-section
tomograms, one of the subtomograms was used for the initial reference.

Cryo electron microscopy of native myofibrils

The pellet of thin myofibrils was resuspended in HK buffer containing cytochrome c-
stabilized 15-nm colloidal gold \(^{31}\), and the protein concentration was adjusted to 0.02~0.05
mg/ml. 4 µl of the sample was mounted on freshly glow-discharged home-made holey
carbon grids and 1 µl of HK buffer containing either 2 mM CaCl\(_2\) and 5 mM ATP or 2 mM
EGTA and 5 mM ATP was applied to the sample. Grids were incubated for 1 min at room temperature, blotted for 10 seconds at 4°C under 99% humidity, and plunge frozen in liquid ethane using Vitrobot Mark IV (Thermo Fisher Scientific, Waltham, MA). Grid quality was examined using a JEM-2100F microscope. Grids frozen under optimal conditions were then used in the recording session.

Images were recorded using a Titan Krios G3i microscope at University of Tokyo (Thermo Fisher Scientific, Rockford, IL) at 300 keV equipped with a VPP, a Gatan Quantum-LS Energy Filter (Gatan, Pleasanton, CA) with a slit width of 20 eV, and a Gatan K3 Summit direct electron detector in the electron counting mode. The nominal magnification was set to 35,000× with a physical pixel size of 2.67 Å/pixel. Movies were acquired using the SerialEM software and the target defocus was set to 1-2 µm. The angular range of the tilt series was from -60° to 60° with 3.0° increments. Each movie was recorded for 1.5 sec with a total dose of 2.5 electrons/Å² and subdivided into 20 frames. The total dose for one tilt series acquisition is thus 100 electrons/Å². The VPP was advanced to a new position every 1-2 tilt series. When moved to a new position, the VPP was irradiated for 30-60 sec by illuminating a blank area before proceeding to the next tilt series acquisition.

Data processing

Movies were subjected to beam-induced motion correction using MotionCor2, and tilt series images were aligned, CTF corrected, and back-projected to reconstruct 3D
tomograms using the IMOD software package\textsuperscript{34}. Tomograms were 2×binned (pixel size of 5.34 Å) to reduce the loads of the calculation. Cross-sections of the Z-discs were displayed using the slicer option of 3dmod and the lattice points of the F-actins were manually picked to define the centers of the subtomograms. Volumes with 50×50×50 pixel-dimensions were cut out from 8×binned tomograms and were averaged using the PEET software suite\textsuperscript{35}.

Averaged subtomograms of thin-section tomography were used for the initial reference, and the alignment was repeated three times for 8×binned, twice for 4×binned, once for 2×binned tomograms. The coordinates and the subtomograms with 200×200×200 pixel-dimensions (5.34 Å/pixel) were imported to Relion-3\textsuperscript{36} and further refined using conventional 3D refinement and 3D multi-body refinement. Reconstruction scheme was summarized in Fig. S2. The numbers of tomograms and subtomograms used for the final reconstructions were as follows; EGTA+ATP state: 43 tomograms and 8,854 subtomograms; Ca+ATP state: 43 tomograms, and 12,535 subtomograms. The averaged maps will be available at the EM Data Bank (www.emdatabank.org) upon publication.

For model building of the α-actinin and the F-actin, the crystal structure of the α-actinin (PDBID: 4D1E)\textsuperscript{3} and the F-actin model (PDBID: 6KLL)\textsuperscript{24} were fitted to the refined maps using UCSF Chimera\textsuperscript{37}. 

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Acknowledgements

This research is partially supported by Platform Project for Supporting Drug Discovery and Life Science Research (BINDS) from Japan Agency for Medical Research and Development (AMED) under Grant Number JP19am0101115. Computational resource of SGI Rackable C1102-GP8 (Reedbush-U/H/L) was awarded by "Large-scale HPC Challenge" Project, Information Technology Center, the University of Tokyo. This work was supported by the Takeda Science Foundation (to T.O.) and the Naito Foundation (to T.O.).

Author contributions

T.O. designed the research; T.O., and H.Y. analyzed data and wrote manuscript.

Competing interests: Authors declare no competing interests.
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Figure 1
Figure 2
Figure 4

A

EGTA+ATP

Ca+ATP

B

EGTA+ATP

Ca+ATP

1st eigenvector
2nd eigenvector

1st eigenvector
2nd eigenvector

(+)
(-)

(+)
(-)

5°
3°

47 Å
50 Å

47 Å
50 Å

Figure 4
Figure 5
Figure 6
Figure 1

Electron microscopy of epon-embedded cardiac myofibrils. (A-B) Slices of tomograms showing the cross-sections of the Z-discs in the EGTA+ATP and the Ca+ATP states. Black and white of the images were inverted. (C-D) Slices of averaged subtomograms showing the actin lattices. The square lattice of the EGTA+ATP state and the diamond-shaped lattice of the Ca+ATP state were shown.

Figure 2

Cryo-electron microscopy of native cardiac myofibrils. (A) Low-magnification image of a thin myofibril in the EGTA+ATP state. Broken box indicates the recording area in B. (B) 0° tilt image of the Z-disc. (C) Slice view of the reconstructed tomogram. Red broken line indicates the position of the cross-section in D. (D) Cross-section of the tomogram of the Z-disc. (E) Lattice points visualized by enhancing the contrast of the cross-sectional view. These lattice points were manually picked for extraction of the subtomograms.

Figure 3

Subtomogram averaging of the repeat unit of the Z-disc. (A, B) Averaged subtomograms composed of the central F-actin (gray), the opposite-polarity F-actins...
(yellow), and the α-actinin molecules (orange). Eye symbols on the top views indicate the direction of the side views on the right. (+): barbed end; (-): pointed end. Top views are seen from the pointed end of the central F-actin. (C, D) Combination of the six shifted maps with the original map. Coordinates were re-centered to the neighboring six F-actins (four opposite polarity F-actins, and two parallel F-actins) and additional 3D refinements were conducted based on the new coordinates. The resulting shifted maps were then superimposed by aligning one F-actin of the shifted map with the corresponding F-actin of the original map, as indicated by the red arrows. (E, F) Composite maps created by combining seven maps. The EGTA+ATP and the Ca+ATP states show the basket-weave and the diamond-shaped lattice conformations, respectively. Both monomers of the α-actinin dimer (orange and green) were fully visualized in the combined maps. Eye symbols on the top views indicate the direction of the side views on the right.

Figure 4

Multibody refinement of the F-actin and the α-actinin. (A) Composite maps composed of one central F-actin (gray), four opposite-polarity F-actins (yellow), sixteen α-actinin monomers (orange and green). The α-actinin crystal structures (PDB 4D1E) were fitted into the α-actinin maps (red and green models). Eye symbols indicate the direction of the side views on the right of the respective top views. (+): barbed end; (-): pointed end. (B) Visualization of the first and the second eigenvectors (Fig. S1C). In both states, the first
eigenvector represents tilting of the central F-actin about the center of the filament, and the second eigenvector represents rotation of the opposite-polarity F-actin about the central F-actin.

**Figure 5**

Conformational changes in the \(\alpha\)-actinin. (A) Top view of the actin-actinin cross-bridging complex. Gray: central F-actin; yellow: opposite-polarity F-actin; orange and green: \(\alpha\)-actinin monomers. The maps of the EGTA+ATP and the Ca+ATP states are light- and deep-colored, respectively. Eye symbols indicate the direction of the side views in B and C. (B) The \(\alpha\)-actinin dimer twisted about its long axis and the opposite-polarity F-actin showed a \(\sim13^\circ\) turn. (C, left) The \(\alpha\)-actinin dimer showed a \(\sim21^\circ\) swing, which translocates the opposite-polarity F-actin by \(\sim80\) Å. (C, right insets) Close-up views of the interface between the central F-actin and the actin-binding domain of the \(\alpha\)-actinin. Eye symbols indicate the direction of the close-up views. The F-actin models (PDB 6KLL) were fitted into the central F-actin maps.

**Figure 6**

Angular mismatches between the helical and the lattice symmetry. The rotation angles of the F-actin helix and the square/diamond-shaped lattice of the Z-disc were compared. The \(\alpha\)-actinin molecules (transparent red) regularly bind to the central F-actin. The \(\alpha\)-
actinin-bound actin monomers are colored red or blue. The one-start helix of the F-actin with 27.5 Å-rise and 166.6°-twist can be regarded as a two-start helix with 55 Å-rise and

26.8°-twist. The “helical angles” are the rotation angles calculated from the helical symmetry of the F-actin relative to one of the actin monomers designated as “0°”. The “lattice angles” correspond to the rotation angles of the opposite polarity F-actins around the central F-actin. In the EGTA+ATP state, the lattice angles are an arithmetic progression with common difference of 90°. In the Ca+ATP state, by contrast, the rotation between the two-neighboring opposite-polarity F-actins is either 80° or 100°. Although the square lattice favors the one-start helix model because of the nearly 90°-turn per seven monomers (asterisks), there are discrepancies in other positions (e.g. 90° versus 107.2°). On the other hand, the diamond-shaped lattice favors the two-start helix model (yellow stars).
Ca+ATP

Glutaraldehyde-fixation
OsO₄-Uranium acetate staining
4 tomograms
450 subtomograms
5 tomograms
538 subtomograms

Initial references for cryo-tomography

43 tomograms
12,925 subtomograms
43 tomograms
16,685 subtomograms

21.36 Å/pixel
10.68 Å/pixel
5.34 Å/pixel
PEET
PEET
Relion

8,854 subtomograms after duplicates removal
12,535 subtomograms after duplicates removal

Multibody refinement

Masks
Bodies
Masks
Bodies

Figure S2
Supplemental materials

Supplemental figure 1

(A) Related to Fig. 2. Comparison of the sarcomere lengths between the two ionic states. Sarcomere lengths (Mean ± SD, n = 25 for each state) were determined by measuring the distances between the midpoints of the two neighboring Z-discs. The observed length change was in agreement with the previous study \(^\text{17}\). The asterisk indicates statistical significance (p< 0.001, Students’ t-test). (B) Related to Fig. 4A. Fourier shell correlation plots calculated using Relion-3 post-processing. Resolutions of the averaged tomograms were limited to 31-32 Å (black line, actin+actinin). Multibody refinements improved the resolutions up to 23-25 Å. Opposite actin: the opposite-polarity actin; actinin 1: the \(\alpha\)-actinin molecules in orange that bind to the central F-actin; actinin 2: the \(\alpha\)-actinin molecules in green that bind to the opposite-polarity F-actin. (C) Related to Fig. 4B. Contributions of the eigenvectors to the variance. The first and the second eigenvectors explained nearly 40% of the variance in both states.

Supplemental Figure 2

Summary of the reconstruction scheme.
Supplemental movie 1

Movie representation of Fig. 4A.

Supplemental movie 2

Movie representation of Fig. 4B.