Supplementary Information

Fig. S1. **Gel showing proteins used in these experiments.** Lane 1 show tissue purified chicken smooth muscle alpha-actinin. Lane 2 shows expressed human fascin. Lane 3 shows tissue purified chicken muscle actin.

Fig. S2. **Rough structure models of bundles and crossed actin structures to examine the physical scale of these interactions.** A, steric interference should not prevent at least two alpha-actinin molecules (and possibly more) from connecting a single crossed filament pair. We make no claim that this conformation is real, nor that this model is atomically correct. We state only that sterics do not seem to rule out the possibility that such a confirmation could exist and the observed flexibility of the molecule would suggest a confirmation of this nature is possible. The actin filaments (pdb: 3B63) are models based on structures of monomeric actin. One filament on each panel is colored using a rainbow to show each separate monomer. The alpha-actinin structure (pdb:1SJJ) is a composite of partial structures overlayed on an electron microscopy map of the molecule. B, model of two actin filaments crosslinked by two alpha-actinin molecules. C, model of two actin filaments crosslinked by two fascin molecules (pdb: 1DFC) for comparison.

Fig. S3. **Alpha-actinin are stable in the absence of crosslinker in solution.** A-E, a two-filament bundle forms in the presence of 0.1 μM alpha-actinin. The tail of that bundle is then forms a loop, which zips up to form a second bundle. This crosslinking proteins hold the secondary bundle at a distinct angle from the first. One filament is highlighted by a red line, the other by a blue line. When the loop starts to close, the looped end of the blue line is highlighted in green to show the overlap. F-J, Crosslinking protein is removed from solution and buffer is flowed over the bent bundle system for 40 minutes. The topographic structure of this actin system is maintained over 40 minutes of observation, clearly showing that the crosslinkers remain bound to the filaments (Movie S10).

Fig. S4. **Fascin bundles are stable in the absence of crosslinker in solution and filament orientation preference is specific even when more than two filaments are present.** A, a single filament attached in the middle to a bead. The shorter of the free ends is marked with a red asterisk. B, additional filaments attach to the bead. At least three filaments attach to this bead in total. C, the filaments zip together into a bundle in the presence of 0.1 μM fascin, but the short end from the first filament is excluded from the bundle. All flow lanes except for the buffer are turned off at this point. E-H, the bundle does not dissociate over 40 minutes of buffer wash and the marked free filament end remains separate.

Movie S1. **Formation of single alpha-actinin crosslink.** This movie shows the formation of a single crosslink between two filaments in the presence of 1 μM alpha-actinin. Beads are 1 μM in diameter. This is true for all movies. This movie was sped up two times.

Movie S2. **Formation of alpha-actinin bundle.** This movie shows the formation of a bundle in the presence of 1 μM alpha-actinin. The filament on the left wraps around the top bead and extends back down. A second filament is strung between the two beads. All three sections of these two filaments form into a tight bundle. The topology of this bundle necessitates the presence of anti-parallel arrangements of filaments. Parallel bundling is also possible given this topology though not certain. This movie was sped up two times.

Movie S3. **Rotation of alpha-actinin crosslink.** This movie shows an alpha-actinin crosslink being stressed in multiple orientations. This is taken in the presence of 1 μM alpha-actinin. Movie is sped up twelve times.

Movie S4. **Fascin does not crosslink 90° crosses.** This movie shows a filament with a free end, extended by flow, diffusing over another filament. The crossing angle of these filaments is near 90°. This movie is taken in the presence of 1 μM fascin. No crosslink is observed. This movie was sped up two times.

Movie S5. **Fascin does not crosslink crossed filaments.** This movie shows a pair of crossed filaments in the presence of 1 μM fascin. The vertical filament is stationary, the horizontal filament is scanned up and down across the other. No crosslink is observed. This movie was sped up two times.

Movie S6. **Fascin does not crosslink anti-parallel filaments.** A single filament is wrapped around a dark bead. This filament is stretched out by flow. The bead is moved to cause more frequent interactions of the two ends of the filament. This movie is taken in the presence of 1 μM Fascin. The topology of this assay shows that the two filament ends are anti-parallel to each other. No crosslink is observed. This movie plays in real time.

Movie S7. **Fascin crosslinks parallel filaments.** This movie shows a bead with two attached filaments. One filament is wrapped around the bead and one is hanging off of it by an end. Towards the end of the movie one end of the wrapped filament bundles with the hanging filament. This bundle is seen clearly at the end of the movie as the bead is rotated. This movie is started just after attaching filaments in the actin channel. The bead and filaments are taken across the buffer channel and the bundling occurs when the filaments encounter the 1 μM fascin channel. Based on previous result we believe this is a parallel bundle. This movie was sped up four times.

Movie S8. **Alpha-actinin bundles do not dissociate.** This movie depicts an anti-parallel bundle of two filament ends that were formed in the presence of 0.1 μM alpha-actinin. Once the link was formed the bundle was moved into the buffer lane of the flow cell and all other flow lanes were turned off before the movie was started. The movie depicts the same bundle over the course of forty minutes. The bundled section has increased brightness and rigidity as compared to the single filament. The bundle is not seen to dissociate. The movie is sped up two times.

Movie S9. **Fascin bundles do not dissociate.** This movie depicts a bundle of two filaments that were formed in the presence of 0.1 μM fascin. Once the link was formed the bundle was moved into the buffer lane of the flow cell and all other flow lanes were turned off before the movie was started. The movie depicts the same bundle over the course of forty minutes. The filaments are not seen to dissociate. The bundled section begins at the bead and is approximately 2.5 μm in length, as seen by the brightness and rigidity of that section. The bead is repeatedly moved to show the stiff bundle. At the beginning of the movie one filament is quite long, extending well past the bundle end. Between the first and second section of the movie the longer of the two filaments either breaks or depolymerizes back to the bundling point, but the bundle is unaffected. The movie is sped up two times.

Movie S10. **Alpha-actin bundles and crossed structures are stable in the absence of competitive agents.** This movie shows the assembly of a bundle of filaments using 0.1 μM alpha-actinin. Once the bundle is formed it is spun around and a loop is formed. That loop is then compressed by additional crosslinking. The movie is sped up two times.

Movie S11. **Continuation of Movie S10.** After the completion of movie S10, all flow except for the buffer was turned off. The bundled and looped structure was then washed for 40 minutes, and no dissociation was observed. The kink in the bundle does not change, implying that alpha-actinin is stable even when crosslinking structures other than two aligned filaments. The movie is sped up two times.

Movie S12. **Fascin Bundle of multiple filaments is stable in the absence of competitive agents.** Here multiple filaments are formed into a tight bundle. The first filament to be attached the bead, was attached in the middle. Fascin is a polarity selective crosslinker, and this is again observed, as the short end of the first filament never integrates into the bundle. The movie is sped up two times.

Movie S13. **Continuation of Movie S12.** The bundle is washed with buffer for forty minutes. The structure is stable over the first thirty minutes. In between the thirty and forty minute time points some breakdown occurs. It is unclear if this is due to bundle dissociation, filament disruption or some other process. This is the only example of this behavior we have observed. Even if separation is taking place, the time scale of this behavior implies that *in vivo* simple crosslinker dissociation cannot explain bundle dynamics and some other behavior must regulate crosslinker turnover. Stable bundle behavior like this led us to examine the mechanism of displacement of crosslinkers by competition. The movie is sped up two times.

Figure S1

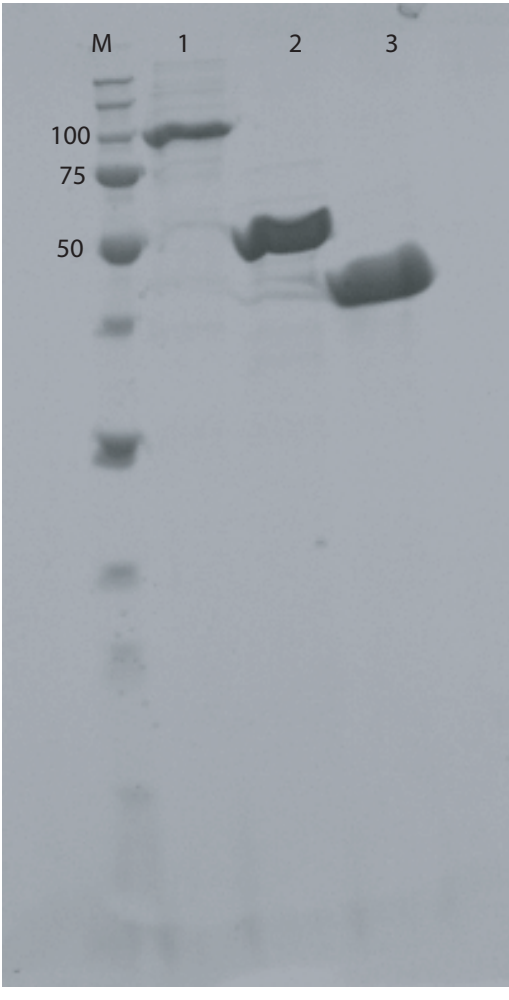


Figure S2

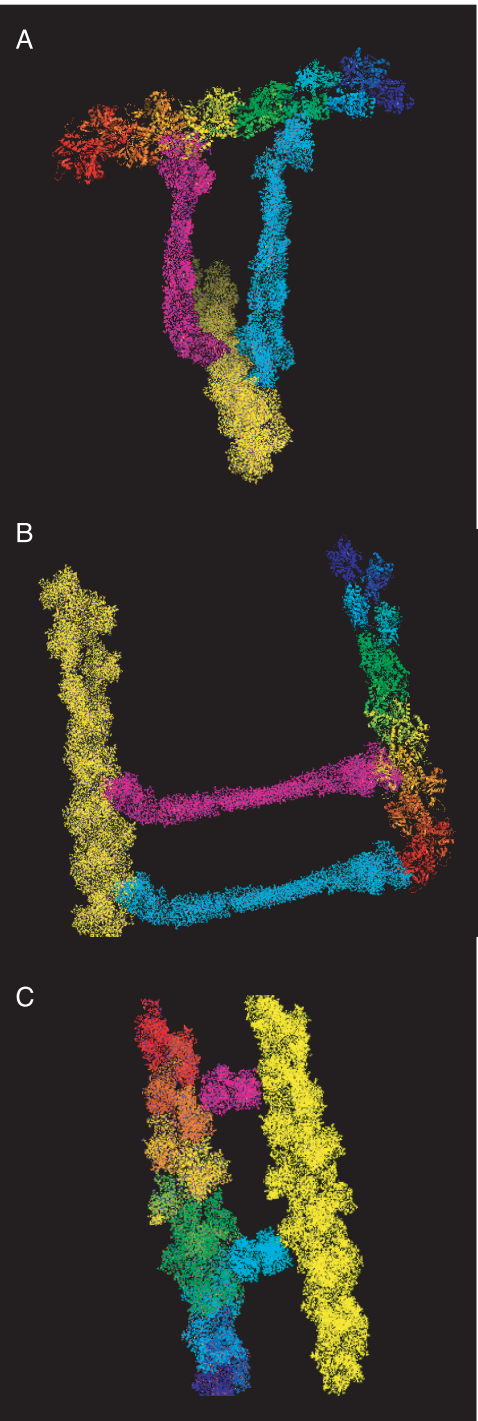
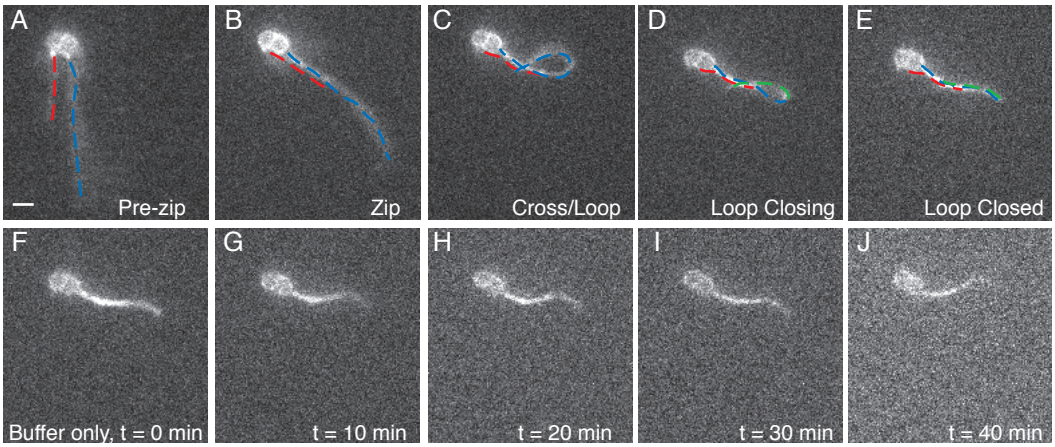


Figure S3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I
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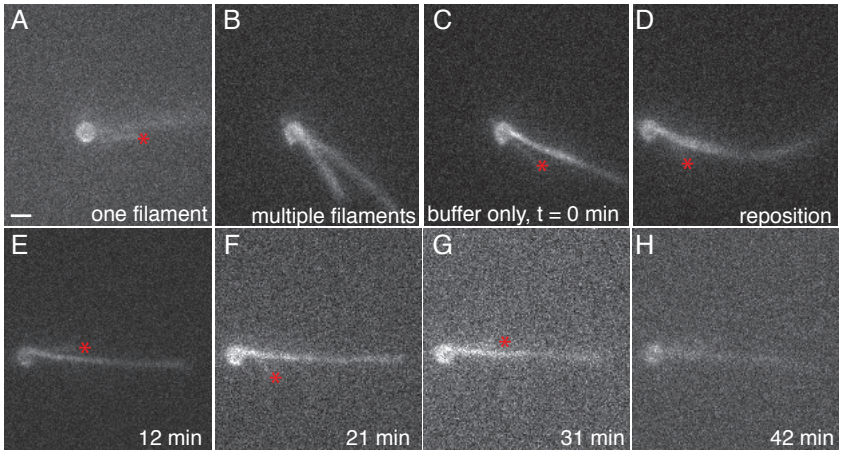
Figure S4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