Self-Organization of Myosin II in Reconstituted Actomyosin Bundles

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ABSTRACT Cells assemble a variety of bundled actomyosin structures in the cytoskeleton for activities such as cell-shape regulation, force production, and cytokinesis. Although these linear structures exhibit varied architecture, two common organizational themes are a punctate distribution of myosin II and distinct patterns of actin polarity. The mechanisms that cells use to assemble and maintain these organizational features are poorly understood. To study these, we reconstituted actomyosin bundles in vitro that contained only actin filaments and myosin II. Upon addition of ATP, the bundles contracted and the uniformly distributed myosin spontaneously reorganized into discrete clusters. We developed a mathematical model in which the motion of myosin II filaments is governed by the polarities of the actin filaments with which they interact. The model showed that the assembly of myosins into clusters is driven by their tendency to migrate to locations with zero net actin filament polarity. With no fitting parameters, the predicted distribution of myosin cluster separations was in close agreement with our experiments, including a $-3/2$ power law decay for intermediate length scales. Thus, without an organizing template or accessory proteins, a minimal bundle of actin and myosin has the inherent capacity to self-organize into a heterogeneous banded structure.

INTRODUCTION

The actin cytoskeleton serves many purposes for cells, such as providing structural stability, guiding intracellular transport, and generating contractile force (1). To perform these diverse functions, the cytoskeleton adopts a variety of organizations which require component assembly and maintenance processes that can deliver components with tight spatial and temporal regulation (2–4). Cytoskeletal structures that exert contractile force are based on actin filaments, myosin II, and other components. Cells assemble these components into contractile networks and bundles (5) for physiological processes such as muscle contraction (6), cell division (7,8), cell migration (9,10), and tissue morphogenesis (11,12).

A common organizational feature of diverse contractile actomyosin structures is the presence of myosin II puncta. In nonmuscle cells, such puncta are aggregates of bipolar minifilaments, each containing $\sim$10–30 nonmuscle myosin II dimers (13,14). A second feature, demonstrated for some actomyosin organizations, is spatially organized actin filament polarity that is correlated with myosin positions. These features are most clearly seen in the myofibrils of striated muscle whose architecture, based on the sarcomere repeat unit, exhibits spatially periodic actin filament polarity and myosin II density (6). In muscle, myosin II thick filaments contract sarcomeres by pulling inward on actin filaments whose pointed ends orient toward the myosin located at the sarcomere center, whereas the barbed ends reside at the sarcomere boundaries. By comparison, actomyosin bundles in nonmuscle cells display a broad range of architectures. In stationary cells, stress fibers, contractile bundles of actin filaments and myosin II, display sarcomere-like structure, including alternating actin polarity and regularly spaced distinct myosin puncta with a period of $\sim$1 $\mu$m along the bundle (15,16). However, stress fibers in migrating cells display both graded and mixed actin filament polarity (15), and in fission yeast, the cytokinetic contractile ring contains irregularly organized myosin puncta and apparently random actin filament polarity (17,18).

Recent studies have addressed the processes used by cells to assemble and organize the components that constitute these actomyosin structures. Dorsal stress fibers are assembled by formin-mediated polymerization of actin filaments from focal adhesions, after which myosin puncta appear that displace the actin filament cross-linking protein $\alpha$-actinin (4). Once assembled, mature stress fibers and other actomyosin bundles undergo continuous actin polymerization from focal adhesions and insertion of nascent puncta of myosin II and $\alpha$-actinin (19,20). However, the mechanisms that underlie these processes of organization remain poorly understood.

Two broad strategies of assembly and organization of actomyosin structures can be envisaged: templating and self-assembly. In a templating strategy, preexisting templates bearing the blueprint of the structure to be assembled could act as molecular scaffolds to direct components to specific locations. In striated muscle the giant proteins titin, nebulin, and obscurin may assume such a role (21), and c-titin, an isoform expressed in nonmuscle cells, has been
identified in stress fibers, although its role is unknown. In a self-assembly strategy, components could spontaneously assemble themselves into organized structures. For example, to assemble its cytokinetic contractile ring, fission yeast uses a dynamic search-and-capture mechanism in which membrane-anchored components condense themselves into a tight ring by transient actin connections that myosin II pulls upon to draw the components together (8).

In vitro reconstitution of minimal actomyosin structures is a powerful approach to expose mechanisms of assembly, organization, and function. Some of the authors of this study recently reconstituted networks of actomyosin bundles tethered to substrate-bound beads (22). At a sufficiently high density of myosin, they observed spontaneous assembly of bundles that contained only actin filaments and smooth muscle myosin II thick filaments. Upon addition of ATP, the bundles contracted and became taut, generating ~500 pN of tension. This showed that myosin acting alone is capable of eliciting contraction of actin bundles on cellular length scales.

Here, we reconstituted contractile bundles of actin filaments and skeletal muscle myosin II thick filaments to investigate the mechanisms of organization. In the presence of ATP, the bundles contracted and became taut over several seconds. Over a longer timescale of ~30 s, skeletal muscle myosin II, initially distributed uniformly along the bundles, self-organized into distinct puncta reminiscent of those in the actomyosin cytoskeleton of living cells. This demonstrates that myosin II self-organizes into a punctate spatial arrangement without templating and without the accessory proteins found in cellular actomyosin bundles. To understand the myosin II self-organization quantitatively, we developed a mathematical model based on the forces exerted by myosin II thick filaments on actin filaments. These forces propel myosin in the direction of local actin polarity in the bundle. The resulting motions aggregate myosin filaments at those bundle locations where the net bundle polarity vanishes. Without fitting parameters, our model successfully reproduces the broad distribution of distances between neighboring myosin puncta, \( d_{\text{myo}} \), observed in our experiments. In particular, our prediction that the distribution follows a power-law decay of \( \sim d_{\text{myo}}^{-3/2} \) on intermediate scales less than the filament length is in remarkably close agreement with our measurements. Our results suggest that polarity of the local actin network is an important regulator of myosin II organization, and that cells may employ a self-assembly strategy to organize components in actomyosin assemblies.

**MATERIALS AND METHODS**

**Buffers**

**Spin-down buffer**

20 mM MOPS, pH 7.4, 500 mM KCl, 4 mM MgCl₂, 0.1 mM EGTA, and 500 μM ATP.

**Wash buffer**

20 mM MOPS, pH 7.4, 50 mM KCl, 4 mM MgCl₂, and 0.1 mM EGTA.

**Assay buffer**

20 mM MOPS, pH 7.4, 100 mM KCl, 4 mM MgCl₂, 0.1 mM EGTA, 0.7% methylcellulose, 0.25 mg/mL glucose, 0.25% β-ME, 0.25 mg/mL glucose oxidase, and 35 μg/mL catalase.

**Protein preparations**

**Myosin thick filaments**

Rabbit skeletal muscle myosin II (Cytoskeleton, Denver, CO) is fluorescently labeled with Oregon Green (OG) 488 maleimide dye (Molecular Probes, Invitrogen, Carlsbad, CA), as described previously (13). Snap-frozen aliquots of OG-labeled and phosphorylated myosin are rapidly thawed. To isolate active myosin, myosin dimers are mixed with phalloidin-stabilized F-actin at a 1:4 myosin/actin molar ratio in spin-down buffer and centrifuged for 30 min at 100,000 × g. The supernatant contains myosin with low affinity to F-actin in saturating ATP (presumed to be enzymatically active), whereas the high-affinity binding fraction cosediments with the F-actin pellet (presumed to be enzymatically dead). Myosin protein concentrations are determined spectroscopically. Myosin thick filaments, which we estimate are typically no more than 0.5 μm in length (see Fig. S1 in the Supporting Material), are formed by diluting myosin in assay buffer, thus changing the salt conditions from 500 mM to 120 mM KCl and waiting 10 min at room temperature.

**Actin filaments**

Preparation of actin was described in a previous study (22). G-actin was generously supplied by Dr. David Kovar (University of Chicago, Chicago, IL.).

**Assembly of bundled F-actin networks**

Bead and coverslip preparation were described previously (22). Assembly of a network of bundles existing predominately within a single confocal plane is achieved using steps similar to those described in a previous study (22). First, a 10- to 20-μm-thick polyacrylamide gel is formed on a coverslip, and biotinylated BSA is covalently attached to the top surface. This substrate is largely inert to nonspecific myosin or actin binding (23). The substrate is loaded into a flow chamber customized for imaging with high-numerical-aperture objectives and small (~30 μL) exchange volumes. A dilute suspension of 3-μm-diameter neutravidin-coated beads in wash buffer is perfused into the flow chamber and incubated for ~10 min to allow the beads to sediment and bind to the biotinylated-BSA surface. Unbound beads are removed by further perfusion of wash buffer. Tetramethylrhodamine (TMR)-phalloidin-stabilized F-actin containing 10% biotinylated G-actin is gently sheared to a mean length of ~6.5 μm (22), diluted to 1 μM in assay buffer, and perfused into the chamber. Over 30 min, F-actin binds to the avidin beads, forming asters that likely contain actin filaments of random polarity (22). A majority of unbound F-actin is removed by perfusion of two chamber volumes of assay buffer. The remaining bead-bound F-actin provides sites to template the assembly of actomyosin bundles. Upon perfusion of assay buffer containing myosin thick filaments in an ADP background, a bundled network forms over 30 min. The chamber is then washed with one volume of assay buffer, and contraction is initiated via perfusion of assay buffer with 100 μM ATP. Microscopy was described previously (22). All images shown are inverted contrast with low-pass filtering. We have observed myosin self-organization qualitatively in numerous experiments over several years; quantitative results presented in this article were obtained from two independent experiments, one containing 261 total actomyosin bundles and the other containing 118 total bundles.
**Image analysis**

The timescale for myosin II to reach its steady-state distribution is defined by the first image in which myosin ceases visible motion. The spacings between myosin II puncta are measured at steady state using ImageJ software (http://rsb.info.nih.gov/ij). The myosin intensity profile along a 10-pixel (1-μm)-wide line drawn over the bundle is measured, and the separation between adjacent puncta, $d_{\text{myo}}$, is defined as the distance between adjacent fluorescence-intensity peaks. Total bundle length, $L$, is determined by the length of a straight line drawn along the entire bundle, between bead attachment points or branch points with other bundles.

**RESULTS**

**Assembly and contraction of reconstituted bundles of actin and skeletal muscle myosin II**

To examine mechanisms of organization in actomyosin assemblies, we reconstituted bundles of actin filaments and skeletal muscle myosin II in vitro using the technique described previously for smooth muscle myosin II (22). After allowing avidin-decorated beads to bind to a biotinylated coverslip, partially biotinylated F-actin filaments of length $6.5 \pm 4.0$ μm (mean ± SD, $n = 200$) (22) were perfused into the solution. These TMR-phalloidin-stabilized actin filaments bound to the beads to form F-actin asters (Fig. 1 A, i and ii) that likely have random polarity (22). After wash steps, only a dilute F-actin background remained in solution.

Thick filaments of Oregon Green (OG)-labeled rabbit skeletal muscle myosin II, estimated to be $0.5 \mu$m in length (Fig. S1), were then perfused into the flow chamber with concentration 0.56 μM. Because nucleotides were absent, the myosin heads are bound with high affinity to F-actin either in rigor or with ADP (24). Over ~30 min, a branched network of actomyosin bundles formed that was tethered between beads (Fig. 1, A (iii) and B), after which the myosin remaining in solution was washed out. These bundles were wavy, indicating that they lacked tension (Fig. 1 B), and were stable over periods of >1 h.

Upon addition of 100 μM ATP to initiate the catalytic activity of myosin II, two dramatic changes occurred. First, the actomyosin bundles contracted and became taut. The only exceptions were some bundles $\leq 6.5$ μm long, the length of the constituent actin filaments. In many cases, the bundles ruptured (41% of 379 total bundles ruptured within 8 s of ATP perfusion), apparently under their own tension (Fig. 1 B). Second, in 65% of the 222 bundles that did not rupture, myosin II, initially distributed relatively uniformly along the bundles, self-organized into clearly distinct puncta flanked by regions with background levels of fluorescence on either side (Fig. 1 B). The remaining 35% of bundles failed to form distinct puncta (Fig. S2), although some of these nevertheless developed an inhomogeneous distribution of myosin (Fig. S2).

**Myosin II self-organizes into puncta after bundle contraction**

We next characterized the self-organization of skeletal muscle myosin II into puncta. Before ATP perfusion, myosin II was distributed approximately uniformly along the bundle axis (Fig. 2, A–D). Upon perfusion of ATP, bundles rapidly contracted and became taut over ~2–8 s (Fig. 2, A and C). Concurrent with and after contraction, myosin evolved into distinct clusters over ~30 s, after which the myosin distribution was stable (Fig. 2, A, C, and D, and see Fig. 5 B). Thus, bundle contraction and myosin self-organization occurred on different timescales, and the myosin motions persisted for a considerable time after the bundles had become taut. With time, the actin became...
somewhat more concentrated at the locations of the myosin puncta, but actin remained visible along the entire bundle. This is as expected, given that the bundle remained intact.

The emergence of distinct myosin puncta could be caused by myosin aggregation due to translation along the bundle axis, or by selective dissociation of myosin at specific locations along the bundle. To distinguish between these possibilities, we tracked the myosin intensity profile along the bundle as a function of time after ATP perfusion (Fig. 2, A and B, and Movie S1). This revealed that the total amount of myosin in bundles decreased by ~30% over 14 s (Fig. 2, A and B), consistent with measurements of bundles containing smooth muscle myosin II (22). Despite this net loss of myosin, there was an increase in myosin concentration over time at the locations of emerging myosin puncta concurrent with a depletion of myosin in flanking regions (Fig. 2 B). Furthermore, peaks in the myosin intensity profile translated along the bundle axis (Fig. 2, A and B, and Movie S1). These observations argue against a selective dissociation mechanism and strongly suggest that myosin...
self-organization occurs by aggregation of myosin due to translation along the bundle.

To characterize the steady-state organization of myosin II in the bundle after the myosin motions were complete, we measured the final distribution of separations between adjacent myosin puncta, $d_{\text{myo}}$, defined as the distance between adjacent peaks in the myosin II intensity profile measured along the bundle (Fig. 2D). In contrast to the periodic myosin distribution in sarcomeric bundles, the spacings in reconstituted bundles were broadly distributed, with a peak at ~1.3 $\mu$m (Fig. 2E) and a mean and SD of $<d_{\text{myo}}>$ = 2.0 ± 1.5 $\mu$m.

Next, we examined whether the broad distribution of separations between myosin II aggregates was a consequence of the broad distribution of total bundle lengths, $L = 11.5 \pm 5.0$ $\mu$m (mean ± SD) (Fig. 2E, inset). Plotting myosin cluster separation versus bundle length revealed no such correlation (Fig. 2F). This strongly suggests that the statistics governing myosin location along a bundle depend only on local bundle properties rather than on global properties such as total bundle length.

**Model of self-organization dynamics of skeletal muscle myosin II in actomyosin bundles**

To establish the mechanisms underlying myosin II self-organization, we developed a mathematical model based on the tendency of myosin II to migrate toward the barbed ends of actin filaments (Fig. 3). The model assumes that the motion of myosin II thick filaments depends only on the polarity of neighboring actin filaments. To test this hypothesis, we solved the model by evolving the positions of myosin II thick filaments in simulated bundles, and then compared the simulation results with our experimental measurements.

**Actin filaments in simulated bundles**

Simulated bundles of length $L$, with ends at $x = 0$ and $x = L$, were generated by defining the positions and orientations of all actin filaments in the bundle, each of which has length $l_{\text{act}}$. Because the experimental bundle-assembly process was unsupervised and random (Fig. 1A), actin filaments in simulated bundles were randomly positioned (filament $i$ centered at location $X_i$) in the range $-l_{\text{act}}/2 < x < L + l_{\text{act}}/2$ and had random orientations, with polarity $P_i$, where $P_i = +1$ ($-1$) for actin filaments with barbed ends oriented in the $+x$ ($-x$) direction (Fig. 3A). The mean number of actin filaments in the cross section is $n_{\text{act}} \approx N_{\text{act}}l_{\text{act}}/(L + l_{\text{act}})$, where $N_{\text{act}}$ is the total number of actin filaments in the bundle. Since we observe actin along the entire bundle and bundles become taut before myosin II self-organizes (Fig. 2A), we assume that the random actin organization is static during myosin II self-organization. We simulated 27,000 bundles with lengths $L$ taken from the experimentally measured distribution (Fig. 2E, inset) and with values of $l_{\text{act}} = 6$ $\mu$m (22) and $n_{\text{act}} = 5$ (Fig. S3), consistent with experimental measurements (Table 1).

**Myosin II dynamics**

Myosin II thick filaments of length $l_{\text{myo}} = 0.5$ $\mu$m (Fig. S1 and Table 1) were placed with an initially uniform distribution along the bundle axis (Fig. 3A), consistent with our experiments (Fig. 2, A–D). The mean number of thick filaments in cross section is $n_{\text{myo}} \approx N_{\text{myo}}l_{\text{myo}}/(L + l_{\text{myo}})$, where $N_{\text{myo}}$ is the total number of thick filaments in the bundle.

At time $t = 0$ (perfusion of ATP), the position of each thick filament begins to evolve according to the properties of its interactions with neighboring actin filaments. 1), Myosin exerts forces on actin filaments in the direction of their pointed ends. 2), The force a thick filament exerts on an actin filament is proportional to the extent that they overlap, as observed in striated muscle (25). For simplicity, we ignore molecular details of the myosin thick filament, such as the bare zone in the center of thick filaments that contains no myosin heads. Assuming a linear force-velocity relation, the force acting on thick filament $j$ due to its interaction with actin filament $i$ is
TABLE 1  Model parameter values

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$l_{\text{act}}$</td>
<td>Length of the actin filaments</td>
<td>6 $\mu$m</td>
</tr>
<tr>
<td>$l_{\text{myo}}$</td>
<td>Length of reconstituted skeletal muscle</td>
<td>0.5 $\mu$m</td>
</tr>
<tr>
<td>$n_{\text{act}}$</td>
<td>Mean number of actin filaments in cross section</td>
<td>5</td>
</tr>
<tr>
<td>$n_{\text{myo}}$</td>
<td>Mean number of myosin II thick filaments in cross section</td>
<td>2</td>
</tr>
<tr>
<td>$L^*$</td>
<td>Bundle length</td>
<td>3.6–26.5 $\mu$m</td>
</tr>
</tbody>
</table>

Fitting parameter

$y_{\text{myo}}^0$ | Unloaded velocity of skeletal muscle myosin II | 1.1 $\mu$m/s |

*Consistent with previous measurements by Thoresen et al. (22).
†Estimated from fluorescence images of myosin puncta. The lower bound of myosin puncta width sets the upper bound of thick filament length (Fig. S1).
‡Quantitative fluorescence imaging shows that bundles have $\sim$1–6 actin filaments in cross section (Fig. S3).
§Chosen to be $>1$ to be consistent with initial myosin staining along the entire bundle (Fig. 2, A and C). Results are insensitive to the value of $n_{\text{myo}}$ for $n_{\text{myo}}$ $>$ 1.
*Measured in Fig. 2 E, inset. Simulations and numerical calculations were performed on simulated bundles having the measured distribution of bundle lengths.
| Used as fitting parameter so that the simulation matches the observed time-scale for the myosin II distribution to reach steady state (Fig. 5 B). |

where $w_{ij}$ is the length of myosin-actin overlap, $f_i$ is the myosin stall force at full overlap, $v_{\text{myo}}$ is the velocity of thick filament $j$, and $v_{\text{myo}}^0$ is the unloaded sliding velocity of skeletal muscle myosin II. As the actin bundles are thin ($n_{\text{act}}$ $\approx$ 1–6; Table 1), we assume that a thick filament interacts equally with all actin filaments with which it overlaps and that it exerts a total force proportional to the number of overlapping filaments. The sum of forces acting on a given thick filament, due to interactions with all neighboring actin filaments, must vanish, $\sum_j f_{ij}^\text{myo} = 0$. Applying this condition to Eq. 1 gives the following result for the myosin thick filament velocity:

$$v_{\text{myo}} = v_{\text{myo}}^0 \sum_i w_{ij} P_i = v_{\text{myo}}^0 \langle P \rangle.$$  \hspace{1cm} (2)

Here, $\langle P \rangle$ denotes the mean actin polarity experienced by a myosin thick filament weighted by the amount of overlap with each actin filament. Thus, the dynamics of each myosin II thick filament are determined only by its unloaded velocity and the polarities of neighboring actin filaments (Fig. 3 B).

During the simulation, for each time step of duration $\Delta t$, the position of each myosin thick filament was updated according to Eq. 2. We used $\Delta t = 0.067$ s, so that during one time step a myosin moves a maximum of $v_{\text{myo}}^0 \Delta t = 75$ nm (Table 1), much less than the relevant simulation length scales $L$, $l_{\text{act}}$, and the mean separation between actin filament ends, $l_{\text{step}}$ (see below). The myosin II profile along the bundle was determined by calculating the number of thick filaments at each bundle location. To mimic imaging of the bundles in fluorescence microscopy (Fig. 2, A and C), the 1D myosin density profile was then convolved with a Gaussian of SD 0.15 $\mu$m to represent the point spread function.

The model predicts that myosin II migrates to zeros of actin polarity, and the steady-state distribution of myosin II puncta separations is a power law

The dynamical simulations described above follow the time evolution of myosin II thick filament locations and capture the entire self-organization process. However, the final myosin II distribution generated by the self-organizing dynamics can be directly computed by noting that in steady state, myosin will be located at bundle positions where the mean polarity vanishes, $\langle P \rangle = 0$, since at these locations the myosin velocity vanishes (see Eq. 2). Because myosin thick filaments are much shorter than the actin filaments ($l_{\text{myo}} << l_{\text{act}}$), the mean actin polarity, $\langle P \rangle$, experienced by a myosin thick filament centered at some location $x$ along the bundle is approximated by the mean polarity of all actin filaments that intersect the bundle cross section at $x$, which we name $p(x)$. Thus, $p(x) = P_{\text{tot}}(x)/N_{\text{tot}}(x)$, where $P_{\text{tot}}(x)$ is the sum of the polarities of all actin filaments that cross $x$ and $N_{\text{tot}}(x)$ is the number of such filaments. Then, to determine the statistics of separation between adjacent myosin puncta in steady state, we calculated the statistics of the separations of adjacent zeros of $P_{\text{tot}}(x)$ (Fig. 3 C).

For a range of myosin cluster separations, this leads to a power law distribution with an exponent of $-3/2$, as follows. The total bundle polarity $P_{\text{tot}}(x)$ changes only at the locations of actin filament ends. Now a bundle of $n_{\text{act}}$ filaments in parallel contains on average $2n_{\text{act}}$ ends per length $l_{\text{act}}$, and the average spacing between actin filament ends is therefore $l_{\text{step}} = l_{\text{act}}/2n_{\text{act}}$ along the bundle axis, with $l_{\text{step}} = 0.6$ $\mu$m from Table 1. Thus, after each distance $l_{\text{step}}$ along the bundle, a new filament end is encountered and the total polarity changes by $\pm 1$, depending on the polarity of the actin filament and whether the end is barbed or pointed. Because the actin filament orientation is random, the polarity changes are random and the total polarity $P_{\text{tot}}(x)$ follows a random walk as a function of $x$. Here, $x$ is a time-like variable, and the polarity changes randomly after a “time step” of duration $l_{\text{step}}$. This random walk behavior only occurs over distances $x < l_{\text{act}}$, because the polarity changes caused by the two ends of a single filament are correlated. Thus, for length scales larger than the step size $x >> l_{\text{step}}$, the polarity variation along the bundle axis is mathematically analogous to the positional variation of a diffusing particle with diffusion constant $1/l_{\text{step}}$. Hence,
the probability distribution of the polarity at \( x \), given the
polarity at \( x = 0 \), is approximately Gaussian (26):

\[
G(P_{\text{tot}}, x | P_{\text{tot}}(0)) = \frac{1}{\sqrt{4\pi l_{\text{step}}}} e^{-\frac{(P_{\text{tot}} - P_{\text{tot}}(0))^2}{4l_{\text{step}}^2}}, \quad (l_{\text{step}} < x < l_{\text{act}})
\]

(3)

The distribution of separations between adjacent zeros of
\( P_{\text{tot}}(x) \), \( f_{\text{zero}}(x) \), is the distribution of distances from one
polarity zero, \( P_{\text{tot}}(0) = 0 \), to the next zero, \( P_{\text{tot}}(x) = 0 \). To obtain
\( f_{\text{zero}}(x) \), we first calculate the probability density,
\( F(P_{\text{tot}}, x) \), that the polarity is \( P_{\text{tot}} \) after “time” \( x \), given
that it initially vanished, and given that the polarity was
never zero during this period. This conditional probability
is again the solution to a diffusion problem, but now with
an absorbing boundary condition, \( F(0, x) = 0 \). The solution
is related to the Gaussian of Eq. 3 but is now the sum of one
Gaussian with \( P_{\text{tot}}(0) = 0^+ \), and a second, negative mirror-
image Gaussian with \( P_{\text{tot}}(0) = 0^- \) to enforce the boundary condition at \( P_{\text{tot}}(0) = 0 \) (26). This amounts to taking the
derivative of the Gaussian with respect to \( P_{\text{tot}} \), evaluated
at \( P_{\text{tot}}(0) = 0 \). Integrating this result for \( F(P_{\text{tot}}, x) \) over all
values of \( P_{\text{tot}} \) gives the total “survival probability” that no
polarity zero is encountered after distance \( x \). Taking minus
the derivative of the survival probability with respect to \( x \)
then yields the desired result, the probability density that
a second polarity zero is encountered after a distance \( x \):

\[
f_{\text{zero}}(x) = \frac{C}{l_{\text{step}}} \left( \frac{x}{l_{\text{step}}} \right)^{-3/2} \left( l_{\text{step}} < x < l_{\text{act}} \right),
\]

(4)

where the constant \( C \) fixes the normalization. This is the
distribution of separations between zeros in the actin
polarity. In addition to being valid only when \( x \) is much
larger than the “time step” \( l_{\text{step}} \), so that the continuous
“diffusion” limit is valid, it is only valid provided \( x \) is
much less than the filament length, \( l_{\text{act}} \). This is because on
scales of \( x > l_{\text{act}} \), the free Gaussian statistics of Eq. 3 are
no longer valid, as the changes in polarity that occur at
the two ends of a given actin filament are correlated. Note
that elsewhere we replace \( x \) with the notation \( d_{\text{myo}} \) to
represent separations between myosin puncta.

Thus, for separations less than the filament length, the
probability distribution of myosin separations follows a
power law with exponent \(-3/2\). This exponent is independent
of model parameter values, and is the signature of the
essential mechanism proposed by our model, namely,
that myosin is driven to zeros of the total bundle polarity.
Comparison of the predicted exponent to experiment thus
constitutes a stringent test of the model. To determine the
complete distribution for all separations, including those
beyond \( l_{\text{act}} \), and to account for finite bundle-size effects
and corrections due to the distribution in filament-end
separations (assumed above to be fixed and equal to \( l_{\text{step}} \)),
we simply measured the zeros in the actin distribution of
our simulated bundles (Fig. 3 C).

Model reproduces experimentally observed steady-state myosin II distributions

To compute model-predicted probability distributions of
separations between adjacent myosin II puncta, we evolved
our bundle simulation to steady state according to Eq. 2 and
then calculated the separations between peaks in the myosin
intensity profile (Fig. 4 A), analogous to the procedure we
used for our experimental measurements (Fig. 2 D). Using
the parameter values in Table 1, model predictions were in
remarkable agreement with the experimental observations,
capturing the shape of the probability distribution with no
fitting parameters (Fig. 4 B, red line).

In particular, the experimental data were in close agreement
with the model’s predicted inverse 3/2 power-law decay for myosin separations greater than the small cutoff
scale \( l_{\text{step}} \) (0.6 \( \mu \)m, using Table 1) but smaller than the filament
length \( l_{\text{act}} \) (6 \( \mu \)m, Table 1). To test this, we fitted a power law
to the experimental data for separations \( d_{\text{myo}} \) in the range
\( a_{\text{step}} < d_{\text{myo}} < l_{\text{act}}/\alpha \), choosing the constant \( \alpha = 1.5 \) to be
greater than unity, as the power law is an asymptotic pre-
diction valid, in principle, only for scales well inside the
window bounded by \( l_{\text{step}} \) and \( l_{\text{act}} \) (Fig. 4 C, thick blue line).
Within this window of 0.9 \( \mu \)m \(< d_{\text{myo}} < 4 \mu \)m, we found
a best-fit exponent of \( -1.51 \pm 0.14 \) (SE) (Fig. 4 C).

We then confirmed that in our simulated bundles, the
steady-state myosin locations indeed coincide with the
zeros in total actin filament polarity (locations where
\( P_{\text{tot}} = 0 \)). We found that the distribution of separations
between zeros in \( P_{\text{tot}} \) from our simulations (Fig. 4 B, dashed green line) closely tracked the distribution of myosin
separations in the simulation (Fig. 4 B, solid red line) as expected.
The small differences between these two distributions
arise because 1), the simulation includes finite-size
myosin II thick filaments, and 2), to mimic optical effects,
the simulations include a point-spread function that smears
the myosin distribution and obscures the smallest separa-
tions between myosin clusters.

The organization of myosin II depends on bundle
thickness and actin filament length

Next we used our model to predict how the spatial distribu-
tion of myosin II clusters would vary as the properties of
the actomyosin bundles are varied. We calculated the mean
distance between adjacent myosin II puncta, given by loca-
tions where the net actin polarity vanishes in simulated
bundles, as a function of both the mean number of actin
filaments in cross section, \( n_{\text{act}} \), and the actin filament length,
\( l_{\text{act}} \) (Fig. 4 D). From the distribution of myosin puncta
separations that we calculated exactly, Eq. 4, the mean separa-
is
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The mean distance between adjacent myosin clusters increases linearly with the actin filament length, \( <d_{\text{myo}}> \sim l_{\text{act}} \), and decays with increasing numbers of actin filaments in the bundle, \( <d_{\text{myo}}> \sim n_{\text{act}}^{-1/2} \) (Fig. 4 D). Both of these trends could be tested by future experiments.

**Dynamics of myosin II self-organization**

We next used our simulation to investigate the time course of myosin II self-organization. Kymographs of the myosin distribution in simulated bundles showed evolution from an initially uniform distribution to the final punctate distribution (Fig. 5 A), similar to experimentally measured kymographs (Fig. 2, A and C).

Our experiments showed that the time for the myosin distribution to reach steady state was 31.0 ± 17.2 s (mean ± SD) (Fig. 5 B, upper). In the simulations, we defined the myosin self-organization time as that by which all myosin II motion had ceased. We fitted the simulated mean self-organization time to the measured value, using the unloaded velocity of skeletal muscle myosin II as the fitting parameter. This procedure yielded \( v_{\text{myo}}^0 = 1.1 \mu m/s \) (Fig. 5 B, lower), on the same order as, but approximately threefold lower than, the value measured in in vitro gliding assays, \( ~2.8 \mu m/s \) (27.28). Simulated bundles displayed a broad distribution of myosin self-organization times, whose standard deviation of 16.3 s was in close agreement with our experimental measurements (Fig. 5 B).

Thus, the experimental data are consistent with our model in which the velocity of a myosin II aggregate depends only on the polarities of the actin filaments in its neighborhood and the unloaded sliding velocity of the myosin.

**DISCUSSION**

We combined mathematical modeling with quantitative measurements of reconstituted actomyosin bundles to identify a mechanism that organizes myosin II into heterogeneous clusters similar to those observed in the actomyosin cytoskeleton. Our results demonstrate that in bundles containing only actin filaments and thick filaments of skeletal muscle myosin II, myosin has the inherent ability to self-organize into puncta in the absence of a template or other components. The experimental data were consistent with our model in which the dynamics of myosin II are determined only by the polarities of nearby actin filaments (Fig. 3). When the total actin polarity is nonzero, the forces are proportionally unbalanced and myosin translates along

\[
\langle d_{\text{myo}} \rangle \approx \int_{l_{\text{act}}}^{l_{\text{act}}} \frac{x f_{\text{zero}}(x) dx}{l_{\text{act}}} \sim \frac{l_{\text{act}}}{n_{\text{act}}^{1/2}} \tag{5}
\]

where we use \( l_{\text{step}} \ll l_{\text{act}} \) and the fact that the true distribution falls off exponentially for \( x > l_{\text{act}} \). Thus, the mean distance between adjacent myosin clusters increases linearly with the actin filament length, \( <d_{\text{myo}}> \sim l_{\text{act}} \), and decays with increasing numbers of actin filaments in the bundle, \( <d_{\text{myo}}> \sim n_{\text{act}}^{-1/2} \) (Fig. 4 D). Both of these trends could be tested by future experiments.
reached steady state in 31.0 s between x = 0 and x = 25 μm. In experiments (Fig. 2 A) well before myosin self-organization was complete (30 s) (Fig. 5), and because actin was visible along the entire bundle at all times (Fig. 2 A). These assumptions are corroborated by the ability of the model to account quantitatively for the experimentally measured separations of myosin puncta, without fitting parameters (Fig. 4, B and C). However, in the absence of specific cross-linking molecules, it is not known how the actin filaments maintain bundle integrity. This could be due to a combination of nonspecific interactions and the cross-linking activity of myosin thick filaments. In addition to a set of apparently static actin filaments that our results suggest are responsible for the myosin self-organization, we observed a dynamic component to the actin density that correlated with the myosin puncta locations (Fig. 2 A). This presumably corresponds to a mobile fraction of un-cross-linked actin filaments; we speculate that, being free to translate, this fraction would not support force and would therefore not affect the myosin dynamics.

Here, we assembled bundles containing bipolar thick filaments of skeletal muscle myosin II. Previously, some of the authors of this article reported contraction of reconstituted bundles containing side polar (29) smooth muscle myosin II thick filaments (22). Interestingly, smooth muscle myosin in reconstituted bundles is also able to self-organize into puncta (Fig. S4 and see Fig. 2 of Thoresen et al. (22)), suggesting that the mechanisms of contraction and self-organization of reconstituted bundles do not depend on the detailed thick filament composition and structure. However, while bundles containing skeletal muscle myosin II contracted and became taut within 2–8 s after perfusion of ATP (Fig. 2, A and C), smooth muscle myosin bundles contracted over a longer ~20 s timescale (22). This is consistent with the slower working velocity of the smooth muscle myosin motor, 0.2–0.4 μm/s (30), compared to >1 μm/s for skeletal muscle myosin II (27,28).

Similar to actomyosin bundles reconstituted in vitro, stress fibers in cells have a punctate distribution of myosin II (16), exhibit complex myosin dynamics after stimulation (16), and recoil after rupturing spontaneously (31) or by laser ablation (32,33). Several studies attempted to infer the mechanisms that underlie stress fiber kinetics from their behavior after stimulation or during recoil. These studies implicated factors, in addition to myosin contractility, such as internal and external frictional and elastic forces, as well as elastic stress-dependent actin depolymerization (34–37).

Although these previous studies addressed kinetics in already-assembled stress fibers, the work presented here addresses component motions that lead to assembly of the stress fiber itself. The self-assembly mechanism described here may help cells to organize contractile actomyosin structures, as the punctate myosin distributions in reconstituted bundles are similar to those in cellular actomyosin bundles (4,16). Presumably, templating mechanisms also
contribute to organization in cells, as sarcomeric contractile structures contain scaffolding molecules such as titin (21). The myosin distribution that we observed was more random than the tightly periodic distribution in striated muscle, with a broad distribution of spacings between myosin regions (Fig. 2E) that is reminiscent of more disordered bundled architectures in the cell, such as the cytokeratin ring and randomly organized stress fibers (15,17).

SUPPORTING MATERIAL
Four figures and one movie are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)00921-6.

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