Supplementary Information

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Lin et al.: Fluctuations of intracellular forces during cell protrusion

Video Material

**Video 1:** Dual-channel FSM of X-Rhodamine-labeled F-actin (red) and eGFP-myosin regulatory light chain (green). Color-combined movie of F-actin and eGFP-myosin is shown in the rightmost panel of the video (yellow). Data of this movie is used in Figure 3 to analyze the co-localization of myosin-II and predicted contraction forces. The leading edge undergoes persistent protrusion in the bottom-right sector whereas other parts retract at the same time. Thus, differences in force organization can be analyzed simultaneously for protruding and retracting events. Streaks of strong fluorescent signals in the red channel indicate the location of actomyosin stress fibers. Notice the strong contraction in the region of lamella all the way to the perinuclear region at the top border of the movie. Width of the image: ~60 μm. Original frame rate: 0.1 frames/s. Replay rate: 12 frames/s.

**Video 2:** *Left panel:* Dual-channel FSM of X-Rhodamine-labeled F-actin (red) and eGFP tagged vinculin (green). *Middle panel:* Color-coded map of the predicted adhesion forces overlaid to vinculin channel. No overlay: no adhesion force. Green colors: low forces; red colors: high forces (see color bar in Fig. 4a). *Right panel:* Coupling of vinculin and F-actin speckle movements as quantified by the velocity magnitude coupling score (VMCS, see text). Red colors indicate strong coupling, yellow colors weaker coupling. The red curve in the left panel indicates the leading edge of cell protrusion. The leading edge undergoes persistent retraction in the top and bottom sectors whereas the middle sector maintains a protrusive state (boxes in middle panel). Predicted adhesion forces start to decrease when retraction starts, first in the bottom sector then in the top sector. The force decrease is synchronized with an increase of flow coupling. Notice the concurrent appearance of a no color region in the top and bottom sectors of the force map with full coupling (red colors) of F-actin and vinculin in the right panel. Image width: ~40 μm. Original frame rate: 0.1 frames/s. Replay rate: 1 frames/s.

**Video 3:** Definition of probing windows (white rectangles) along the leading edge. Original frame rate: 0.1 frames/s. Replay rate: 6 frames/s.

**Video 4:** *Leftmost panel:* FSM image time-lapse sequence of the protruding front of a Ptk1 epithelial cell (data analyzed in Figure 6). *2nd Panel (from the left):* Rates of F-actin assembly (red) and disassembly (green). *3rd Panel:* Predicted adhesion force magnitude (red: strong; green: weak) and boundary force (cyan vectors). *4th Panel:* Speed (red: fast; green slow) and direction (yellow vectors) of F-actin retrograde flow. The predicted force and flow panels are time-shifted by 20 sec relative to the F-actin turnover map to compensate for the delay of these events (see Figure 6).
Note 1: Flow driven by simulated intracellular forces

To aid the reader in understanding the principle force prediction by solving the inverse problem of how to get from network flow to force, we demonstrate by simulation the solution of the corresponding forward problem: Given a force distribution at the cell boundary and inside the cell, what is the resulting network flow? We show by separate simulations of each intracellular force type, i.e., boundary force $F_i$ at the leading edge $\partial \Omega_{LE}$, and adhesion and contraction forces $F_{II+III}$ inside the cellular region $\Omega$ (Fig. S1a), their specific effect on the resulting network flow.

To perform a realistic simulation, we assumed boundary, contraction, and adhesion forces throughout the field of view of an FSM movie for which we have shown the force reconstruction from experimentally measured flows (Figs 1 and 2). For all three examples, we held the interior boundary $\partial \Omega_{IN}$ stationary, assuming the cell as a whole does not move. In the scenario of Figure S1b, only contraction forces are applied ($F_{III} \neq 0$, red arrows). This generates almost uniform retrograde flow away from the leading edge (yellow arrows). In the scenario of Figure S1c, only boundary forces are applied ($F_i \neq 0$). In the absence of adhesions, this generates retrograde flow deep into the cell. In fact, the flow tapers off towards the bottom of the field of view only because of the boundary condition $u(x) \equiv 0$, on $\partial \Omega_{IN}$. In the scenario of Figure S1d, the retrograde flow generated by boundary and contraction forces is being resisted by adhesions. Adhesion forces are modeled as a viscous friction between the flowing network and the substrate, i.e. $F_{II}(x) = -\nu(x)u(x)$. The parameter $\nu(x)$ denotes the friction coefficient, which can vary in space (color-coding in Fig. S1d). Therefore, spatial variations in the adhesion force are caused by spatial variations in the friction coefficient or by spatial variations in the magnitude and direction of the flow field. This model implies that the network-substrate interface consists of transient bonds. The faster the F-actin slides over the cytoplasmic domain of adhesion complexes the higher is the probability for an interaction between the binding sites on both structures\(^1\). Qualitatively this behavior has been confirmed by indirect comparison of F-actin flow fields and traction force maps in moving keratocytes\(^2\).

The simulations in Figure S1b - d illustrate that adhesion sites modulate the rather uniform flows generated by polymerization and network contraction. In particular, they give rise to a gradient from fast to slow flow behind the protruding edge, a characteristic feature of actin dynamics in migrating epithelial cells. Given the specific effects the three forces have on flow organization, we expected that inversion of the procedure would allow us to reconstruct the force necessary to drive a measured flow field.
Figure S1: Simulation of network deformation induced by assumed boundary and intracellular force distributions. (a) Domain of simulation (Ω) with a free leading edge (\( \partial \Xi_{\text{LE}} \)) and a fixed interior boundary(\( \partial \Xi_{\text{IN}} \)). The interior boundary is fixed so that the whole cell does not move. (b) Simulated flow response (yellow) to an assumed contraction force field (\( F_{\text{III}} \), red). (c) Simulated flow response (yellow) to an assumed boundary force distribution (\( F_{\text{I}} \), cyan). (d) Simulated flow response to the assumed force distributions in b and c, plus an adhesion force distribution. Adhesion forces are modeled by viscous friction between network flow and substrate (see text). The assumed friction term is color-coded. Scale bar in b: 5 μm.
Note 2: Mechanical properties of the actin cytoskeleton

In vitro studies have shown that on length scales above the characteristic distance between filament cross-links and branches, F-actin networks behave like a viscoelastic continuum. The viscous contribution is frequency-dependent. It can be neglected for frequencies less than 1Hz at which high-resolution fluorescence microscopy experiments are typically performed. Although the relationship between force and network deformation becomes nonlinear at high strain rates due to strain stiffening, our qFSM data reveal that at the time scale of ~1 - 10 seconds, the intracellular strain induced by transient network deformation rarely exceeds 5%. Thus, for the purpose of force reconstruction on these time scales, in vitro F-actin networks can be approximated as a linear elastic material.

Whether F-actin networks in living cells can be approximated as purely elastic is still a subject of intensive research. Currently, the published literature varies in opinion about whether cells behave more viscous-like or elastic-like in the frequency range typical for imaging. For 0.1 Hz, i.e. frame intervals of 10 s, several groups have estimated the cytoskeletal elastic modulus to be significantly larger than the viscous modulus. Others report the opposite, finding a large viscous modulus in the 0.1 Hz frequency range, and a dominant elastic modulus only towards higher (1 -10 Hz) frequencies. The variation between in vivo studies and their discrepancy from in vitro F-actin network results may partly be explained by challenges in defining the elastic and viscous contributions to F-actin mechanical properties in living cells.

Existing live cell rheological measurements do not probe F-actin specifically, but gauge the ensemble behavior of all species of cytoskeletal filaments, membranes, and the cytosol. Our measurements of F-actin network deformations, however, are molecularly specific, allowing us to neglect contributions by other cytoskeleton components, cytosol, or membranes. We therefore adhere to the more specific data available from in vitro networks, and consider F-actin networks as essentially elastic at the time scale of high-resolution live cell microscopy.

Even when neglecting viscous properties and focusing on values of the elastic modulus, published magnitudes range from <1 Pa, single Pa levels, up to several tens or even hundreds of Pa. The elastic modulus has also been found to vary between cell types, between individual cells, and between different regions of the same cell. Thus, in order to reconstruct absolute force levels, each experiment would require in situ calibration of the elastic properties. Currently, no tools exist to accomplish this at the spatiotemporal scale of microns and seconds.

The most critical distinction between an in vivo F-actin cytoskeleton and in vitro actin networks is the continuous assembly and disassembly of the former, leading to high plasticity of the material. Such a remodeling of the network continually relaxes the prestress of the network and resets its resting state. Accordingly, there is no defined relaxation state of the F-actin network in living cells for time scales that exceed the time...
scale of network remodeling. Rather, the F-actin network appears to flow as a viscous material over the time scale of network remodeling although its response at shorter time scales may be purely elastic. The consequences of material plasticity for the reconstruction of predicted forces from F-actin network deformation are illustrated in Figure 1d with the analogy of a system of linear springs. For long time scales, at which remodeling takes place, breaking (disassembly) of some springs and new formation (assembly) of others yield a change of the ensemble resting length (relaxation state) and/or the spring constant of the system. Therefore, Hooke’s law of a linear relationship between force \( F \) and spring extension \( u \)

\[
F = k \cdot u
\]

is no longer valid (\( L \) for resting length). As an example, if we were to apply Eq. (S1) to the spring extension between \( t_1 \) and \( t_2 \) in Figure 1d, implicitly assuming identical resting lengths \( L_1 \) in both time-points, we would significantly overestimate the force level at \( t_2 \) (arrow with dashed line), even when the ensemble spring constant were to remain approximately constant. On the other hand, on time scales \( \delta t \) significantly shorter than the remodeling process \( \delta t \ll t_2 - t_1 \), plasticity is not manifest. Consequently, Hooke’s law still holds for the spring system. Low plasticity conditions apply to the spring extension both between \( t_1 \) and \( t_1 + \delta t \) and between \( t_2 \) and \( t_2 + \delta t \) in Figure 1d.

Accordingly, forces are reconstructed by

\[
F_1 = k \cdot \left( \frac{u_1}{L_1} \right)
\]

and

\[
F_2 = k \cdot \left( \frac{u_2}{L_2} \right)
\]

respectively.

To describe the mechanical properties of such a plastic system at a specific moment, we introduce the concept of transient elasticity, which captures the linearity of the relationship between force development and transient F-actin network deformation over short time intervals. Using this concept, the local difference in the velocity of F-actin network flow recorded at a short time scale defines the network deformation induced by transient application of intracellular forces.

Clearly, it is critical to know the time scale of plasticity in order to know the time scale within which the assumption of transient elasticity is valid. Rates of F-actin turnover vary strongly between cell types and cell regions. Quantitative Fluorescent Speckle Microscopy (qFSM) provides precise information on F-actin assembly and disassembly rates in addition to F-actin deformations. This allows us to determine the timescale of network remodeling and to identify the time interval over which network deformations are non-plastic. For the epithelial cells studied in this work, the time scale of F-actin network remodeling is \( \sim 80 \) s. Sampling intervals of 5 - 10 s are one order of magnitude faster than the time scale of plastic network deformation. Thus, the short-term deformations of F-actin tracked by qFSM between two consecutive frames mainly result from transient variation of intracellular forces throughout the cell. By analyzing these changes over multiple time steps, it is possible to monitor the regulation of force generation on a relative scale. This was sufficient in this study to establish critical relationships between force fluctuations, cell morphological variations, and F-actin dynamics during epithelial cell protrusions. To define the force level on an absolute scale, additional measurements of the spatiotemporally variable elasticity of the F-actin cytoskeleton will be necessary.
Note 3: Mechanical coupling between lamellipodium and lamella

Protrusions of epithelial cells are composed of two dynamically and molecularly distinct, spatially in parts overlapping F-actin networks\(^\text{24}\). The lamellipodium covers a band of \(\sim 1\) to \(2\ \mu\text{m}\) subjacent to the protruding cell edge. Its F-actin network undergoes fast treadmilling under the control of Arp 2/3 and coflin, which leads to rapid retrograde flow of F-actin at the cell leading edge. The lamellipodium has been shown to be largely devoid of myosin-II (also confirmed by Video 1; and Fig. 3) and retrograde flow does not slow down myosin-II activity in inhibited by blebbistatin. In contrast, the lamella network flows under myosin-II mediated contraction. In the PtK1 cell studies presented here the flow rates in the lamella were 2 to 3 times slower than in the lamellipodium and F-actin turnover rates were substantially slower. Separation of speckles based on flow velocity and lifetime showed that there might be significant spatial overlap of these two F-actin networks\(^\text{24, 25}\). Thus, the question arises whether the F-actin cytoskeleton at the cell periphery can be modeled as one elastic network.

We argue for the purpose of intracellular force reconstruction at the length scale of 1 - 1.5 \(\mu\text{m}\) it is appropriate to make such an approximation if lamellipodium and lamella are mechanically coupled. Two observations from our force results suggest that they are indeed mechanically coupled. First, adhesion forces estimated from F-actin flow co-localize with distinct focal adhesion sites that are independently labeled and simultaneously imaged with the F-actin flow (Fig. 4). Given the spatially uniform gradient of the averaged flow from the lamellipodium/lamella region next to the leading edge to the lamella-only region, one would expect a uniform band of resistance forces at the junction of these regions that would yield adhesion forces regardless of the location of components of focal adhesions. Only if the two networks are mechanically integrated can one explain the precise co-localization of predicted adhesion forces with focal adhesions. Second, predicted boundary forces change in synchrony with predicted adhesion forces. This indicates that any variation of membrane tension due to the pushing of actin polymerization at the leading edge is instantaneously transmitted to focal adhesion sites. Such pure elastic response requires that the two networks are mechanically well-coupled.

To further test the assumption of two integrated networks, we performed a self-consistency test in which we removed the lamellipodium region from the image data, and thus constrained the force reconstruction to the lamella region only. If the assumption of coupled networks was invalid, one would expect fundamentally different force distributions between reconstructions with and without lamellipodium. Figure S2 shows a time sequence of the F-actin flow velocities (a) tracked by qFSM and both predictions of adhesion (b) and contraction/boundary (c) force transients are reconstructed with (upper panel) or without (lower panel) lamellipodium. Clearly, there is no fundamental difference between the two. Most importantly both reconstructions capture the same dynamic change of force transients over time (Fig. S2b, grey arrows).

The virtual boundary force reconstructed along the lamellipodium-lamella transition (gray, solid line Fig. S2) when the lamellipodium is removed reflects the stress inside the...
Figure S2: Self consistency test of the model assumption that the F-actin networks of lamellipodium (LP) and lamella are mechanical coupled. (a) F-actin flow velocities (yellow vectors) tracked by qFSM. (b) Predicted adhesion force transients displayed both as colormaps of force magnitude and as force vectors. Top row: Reconstruction with lamellipodium region. Bottom row: Reconstruction without lamellipodium region, i.e. a virtual cell boundary follows the transition between lamellipodium and lamella (gray dash-dotted and gray solid line in top and bottom rows, respectively).
Figure S2 (continued): (c) Predicted contraction (red vectors and colormap of force magnitude) and boundary forces (cyan vectors) reconstructed with (top row) or without (bottom row) lamellipodium region. Forces at the virtual boundary in bottom row indicate the network-internal stresses at the lamellipodium-lamella transition. Grey transparent arrows in a) highlight changes in predicted adhesion forces that are equally capture by reconstructions with and without lamellipodium. Circled areas are locations where relatively significant discrepancies between the two reconstructions are observed (see text). Scale bar: 5 µm.

F-actin network generated by F-actin polymerization at the cell edge. Thus, the virtual boundary force should approximately match the real boundary force. However, some significant differences are found along the virtual boundary (Fig. S2, circles). Closer examination of the two force maps showed that these mismatches localize in areas of either strong adhesion or contraction. The algorithm has no criterion to separate between boundary and domain (adhesion/contraction) forces and the resulting split is random. In contrast, when the boundary is defined by the actual cell edge, adhesion and contraction forces are by nature of the force distribution sufficiently distant from the cell edge to allow a reliable split between boundary and domain forces. Besides these differences, predicted force reconstruction in the lamella region is not distorted by removing the lamellipodium region. Therefore, we concluded that a simplified model of one network is sufficient for reconstructing relative changes in predicted force transients at a length scale of 1 µm.
Note 4: Continuum mechanical model to relate domain and boundary forces to network deformation

Under force application, a linear elastic network deforms as illustrated in Figure 1b (main text). Deformations of a two-dimensional network at a given point are measured by a 2x2 matrix referred to as the strain tensor. Mathematically, the strain tensor is defined as

\[ \varepsilon(x,t) = \frac{1}{2} \left( \nabla \mathbf{u}(x,t) + (\nabla \mathbf{u}(x,t))^T \right), \]

where spatial variation of the flow field \( \mathbf{u}(x) = [u_1(x); u_2(x)] \) at time \( t \) is analyzed by applying the gradient operator \( \nabla = \frac{\partial}{\partial x_1}; \frac{\partial}{\partial x_2} \) to both components of the flow vector at \( x = [x_1, x_2] \). Importantly, non-zero strain is observed only when the displacements in the immediate vicinity of \( x \) differ in either direction or magnitude or both. Spatially uniform displacements indicate that locally no forces are applied to the network. This is illustrated in Figure 1b where the flow vectors at the four corners of a rectangular network patch vary in direction and magnitude. Thus, in the next time-point the rectangle is deformed (solid polygon).

Network deformations create tension, which balance the forces applied to the network. The relationship between network deformation and tension for an elastic material is given by

\[ \sigma_{ij} = \sum_{k,l=1}^{2} c_{ijkl} \varepsilon_{kl}, \quad i, j = 1, 2. \]

In this equation, \( \sigma \) denotes the 2x2 stress tensor and \( c \) the elasticity tensor of order 4. The structure of Eq. (S3) resembles the structure of Eq. (S1), which describes the force-extension relationship for an elastic spring. Instead of a scalar force \( F \), Eq. (S3) contains the stress tensor \( \sigma_{ij} \); instead of a scalar extension \( u/L \), the strain tensor \( \varepsilon_{ij} \); and instead of a scalar spring constant \( k \), the elasticity tensor whose coefficients \( c_{ijkl} \) define the material properties of the 2D network. For the simplest case of a linear elastic, locally isotropic 2D network, \( c_{ijkl} \) depends on two parameters \(^{26}\). One widely used pair of elastic parameters consists of Young’s modulus \( E \) and the Poisson ratio \( \gamma \). Together, they are complete in defining the local properties of the linear elastic F-actin network. For F-actin networks the Poisson ratio is approximately constant in space and time \( (\gamma \sim 1/3)^{27} \), leaving Young’s modulus as the only free variable of the model. Expressing \( c_{ijkl} \) in terms of these two parameters, the stress tensor at any location \( x \) is calculated from the flow vector \( \mathbf{u}(x) \) by substitution of (S2) into (S3):

\[ \sigma(x,t) = \frac{E(x,t)\gamma(x,t)}{(1-2\gamma(x,t))(1+\gamma(x,t))}(\nabla \cdot \mathbf{u}(x,t)) I + \frac{E(x,t)}{2(1+\gamma(x,t))}(\nabla \mathbf{u}(x,t) + (\nabla \mathbf{u}(x,t))^T). \]

\( I \) is the 2x2 identity matrix, and \( \nabla \cdot \mathbf{u} \) denotes the divergence of the flow vector. Eq. (S4) incorporates the notions of transient elasticity and network deformation by defining all terms as time-dependent variables. In addition, the material properties are represented by space-dependent variables, accounting for the spatial heterogeneity of the network. Currently, there is no method to measure absolute values of Young’s modulus in the...
lamellipodium and lamella networks that matches the sampling resolution of $u(x,t)$.
Thus, $E(x,t)$ in Eq. (S4) had to be defined on a relative scale assuming that variations in $E(x,t)$ are mainly associated with variations in network density. Accordingly, we set $E(x,t)$ proportional to the speckle intensity averaged over $1 \mu m^2$ (see Supplementary Note 6).

The balance between network stresses and the intracellular forces $F_\parallel$ and $F_\bot$ exerted on the network inside the domain $\Omega$ (Fig. 1a) is defined as
\[
\nabla \cdot \sigma = F_\parallel - F_\bot, \quad \text{in } \Omega.
\]

The balance between the boundary force $F_\parallel$ and network stresses along the cell edge $\partial \Omega_{LE}$ is given by Neumann boundary conditions:

\[
\sigma n = F_\parallel, \quad \text{on } \partial \Omega_{LE}
\]
where $n$ is the outward normal to the cell edge.

An important conclusion from these equations is that the forces at a specific position are defined by the local network deformation. What local means depends on the sampling density of flow vectors based on which strain tensors are calculated by discrete spatial differentiation (Eq. S2). In the case of qFSM, the spatial sampling of flow vectors is diffraction-limited (~500 nm distance between speckles) allowing calculation of strain tensors with submicron resolution. In practice, the force reconstruction requires regularization to filter noise in the speckle flow fields (see Fig. 2b in main text). Thus, effectively the resolution of our predicted force maps is in the range of $1 - 1.5 \mu m$ (see also Supplementary Note 7).
Note 5: Solution of the inverse problem to relate measured network flows to predicted boundary and domain forces

Given an experimentally measured F-actin flow field \( \mathbf{u}_D(x,t) \) boundary forces \( \mathbf{F}_i \) and domain forces \( \mathbf{F}_{\text{II+III}}(x,t) \) are predicted by solving Eqs. (S4) – (S6) simultaneously in all locations of \( \Omega \). To define the solution, additional Dirichlet boundary conditions have to be introduced for the leading edge

\[
\mathbf{u} = \mathbf{h}, \quad \text{on } \partial \Omega_{\text{LE}},
\]

and the interior boundaries

\[
\mathbf{u} = \mathbf{h}, \quad \text{on } \partial \Omega_{\text{IN}},
\]

Here, \( \mathbf{h} \) denotes the flow velocities at the boundary.

Because of measurement noise in \( \mathbf{u}_D(x,t) \) no meaningful force field exists that strictly satisfies Eqs. (S4 – (S8). Thus, the force reconstruction must be further constrained by minimizing the difference between the measured flow field and the flow field produced by the predicted domain and boundary forces.

To define the objective functions of the minimization problem we computed two forward maps, \( A \) and \( B \), between the force field inside the cell and along the cell boundary, respectively, and the resulting network flow. Let \( L_\Omega \) and \( L_{\partial \Omega} \) denote the function spaces for the domain forces and the boundary forces, respectively, and \( H \) the function space for the flow vectors. Based on this definition, the forward map \( A \) from the domain force space to the flow vector space

\[
A : \mathbf{F} \in L_\Omega \mapsto \mathbf{u} \in H
\]

and the forward map \( B \) from the boundary force space to the flow vector space

\[
B : \mathbf{F} \in L_{\partial \Omega} \mapsto \mathbf{u} \in H
\]

are given by the solution \( \mathbf{u} \) to the partial differential equations (PDE) (S4) and (S5), subject to the following two homogenized boundary conditions:

HA. The solution \( \mathbf{u} \) satisfies the Dirichlet boundary conditions (S7) and (S8) with boundary flow \( \mathbf{h} \equiv 0 \) on \( \partial \Omega_{\text{IN}} \) and \( \partial \Omega_{\text{LE}} \).

HB. The solution \( \mathbf{u} \) satisfies Eq. (S5) with \( \mathbf{F}_{\text{II+III}} = 0 \), the Neumann boundary condition (S6) on \( \partial \Omega_{\text{LE}} \), and the Dirichlet boundary condition (S8) on \( \partial \Omega_{\text{IN}} \), where the boundary flow \( \mathbf{h} \equiv 0 \) on \( \partial \Omega_{\text{IN}} \).

Because of the homogenization of the boundary conditions both maps \( A \) and \( B \) are linear and of infinite dimensions. Thus, assuming a continuous force field, the resulting flow field can be approximated by calculating and summing up the contributions of elementary force field samples distributed throughout \( \Omega \) and along \( \partial \Omega \). The more force field elements are sampled the more accurate will be the approximation of the resulting flow.
Using the linear forward maps $A$ and $B$ the inverse mappings of flow to the domain force $F_{\text{II+III}}$ and of flow to boundary force $F_1$ are obtained by least-squares minimization of

\begin{align}
F_{\text{II+III}} &= \arg \min_{F \in \Omega} \left( \| u_{\text{II+III}} - AF \|_2^2 + \alpha \| F \|_2^2 \right), \\
F_1 &= \arg \min_{F \in \partial\Omega} \left( \| u_1 - BF \|_2^2 + \alpha \| F \|_2^2 \right).
\end{align}

Here, $u_{\text{II+III}}$ and $u_1$ denote the homogenized flow fields. These are derived from the measured flow field $u_D$ so that they satisfy the homogenization conditions $HA$ and $HB$, respectively (see summary of algorithms below). The operator $\| \cdot \|$ denotes the vector $L_2$ norm (Euclidean length). The objective functions (S11) and (S12) are comprised of two terms. The first term defines the $L_2$ norm of the difference between the homogenized, measured flow vectors and the flow field obtained by application of the forward mapping $A$ and $B$ to a candidate force field $F$. Minimization of these terms by variation of $F$ within the cell and along the cell edge yields predicted domain and boundary forces that optimally match the measured flows. Minimization of the first term alone may generate very large force values in locations where measurement noise causes sharp flow gradients. To lower the influence of noise on the force prediction, a second term penalizes irregularly large force magnitudes via the $L_2$ norm of the force vectors. This extension of the objective function is referred to as Tikhonov regularization\textsuperscript{28}. Minimization of the second term alone yields a zero force field. Simultaneous minimization of both terms balances between matching predicted forces to the measured flow fields and limiting the influence of measurement noise. The balance is controlled by the regularization parameter $\alpha$ ($> 0$). The choice of values for $\alpha$ depends on the signal-to-noise ratio of the flow measurements and the characteristics of the flow field. Several strategies have been proposed to identify the “optimal” range of $\alpha$ values. We applied the L-curve method\textsuperscript{29}, an a posteriori strategy that does not require prior knowledge of the noise level. $\alpha$ values identified by the L-curve method span about two decades. Within this range, lower $\alpha$ values provide fine spatial details in the predicted force fields at the cost of more noise, while a high $\alpha$ values yield smoother force field with less spatial detail but also

Numerical solutions to (S11) and (S12) are found in finite subspaces $L_m^{(n)}$ and $L_n^{(n)}$ that approximate $L_\Omega$ and $L_{\partial\Omega}$ by $m$ and $n$ basis functions, respectively. Let $A_m$ and $B_n$ denote the finite matrix representations of $A$ and $B$. The discrete solutions to (S11) and (S12) are then given by inversion of two linear equations:

\begin{align}
(\alpha I + A_m^T A_m) F_{\text{II+III}} &= A_m^T u_{\text{II+III}}, \\
(\alpha I + B_n^T B_n) F_1 &= B_n^T u_1.
\end{align}

Many choices of basis functions in $L_m^{(n)}$ and $L_n^{(n)}$ are possible. The main requirement is that the length scales of the functions match the resolution of the flow measurements. We used the shape functions of finite element meshes as the basis functions in $L_m^{(n)}$, and $B$-splines as the basis functions in $L_n^{(n)}$. Given these bases, $A_m$ and $B_n$ were calculated.
column by column. The \( j^{\text{th}} \) column of \( A_m \) was obtained by solving Eqs. (S4) and (S5) subject to the homogenization conditions HA using the \( j^{\text{th}} \) basis function in \( L^{(m)}_\Omega \) as the domain force. The \( k^{\text{th}} \) column of \( B_n \) was obtained by solving Eqs. (S4) – (S6) subject to the homogenization conditions HB using the \( k^{\text{th}} \) basis function in \( L^{(n)}_{\partial \Omega} \) as the boundary force.

In summary, predicted domain forces \( F_\text{II+III} \) and boundary forces \( F_1 \) were obtained by execution of two algorithms.

**Inverse Algorithm A:**

- Establish the finite subspace \( L^{(m)}_\Omega \) of the function space of domain forces.
- Use each of the \( m \) basis functions in \( L^{(m)}_\Omega \) once as the domain force \( F_\text{II+III} \) and solve Eqs. (S4) and (S5) subject to HA. Each flow solution evaluated at the positions of flow measurements defines one column of the finite forward map \( A_m \).
- Calculate \( u_\text{II+III} \) by subtracting from the measured flow field \( u_0 \) the flow solution to Eqs. (S4) and (S5) that satisfies the Dirichlet boundary condition Eqs. (S7) and (S8) with \( h \equiv u_D \) on both \( \partial \Omega_{\text{LE}} \) and \( \partial \Omega_{\text{IN}} \) and an assumed domain force \( F_\text{II+III} \equiv 0 \) in \( \Omega \).
- Solve Eq.(S13) using the regularization parameter \( \alpha \) as determined by the L-curve method.

**Inverse Algorithm B:**

- Establish the finite subspace \( L^{(n)}_{\partial \Omega} \) of the function space of boundary forces.
- Use each of the \( n \) basis functions in \( L^{(n)}_{\partial \Omega} \) once as the boundary force \( F_1 \) on \( \partial \Omega_{\text{LE}} \) and solve Eq. (S4) – (S6) subject to HB. Each flow solution evaluated at the positions of flow measurements defines one column of the finite forward map \( B_n \).
- Calculate \( u_1 \) as the difference between two flow solutions to Eqs. (S4) and (S5): For both solutions, the domain force \( F_\text{II+III} \) obtained by the Inverse Algorithm A is used in Eq. (S5). Both also satisfy the Dirichlet boundary condition Eq. (S8) with \( h \equiv u_D \) on \( \partial \Omega_{\text{IN}} \). The first solution additionally satisfies the Dirichlet boundary condition Eq. (S7) with \( h \equiv u_D \) on \( \partial \Omega_{\text{LE}} \). The second solution satisfies the Neumann boundary condition Eq. (S6) on \( \partial \Omega_{\text{LE}} \) with a free edge, i.e. \( F_1 = 0 \).
- Solve Eq. (S14) using the regularization parameter \( \alpha \) as determined by the L-curve method.
Note 6: Decomposition of predicted domain forces into adhesion and contraction forces

To separate the contributions of contraction and adhesion to the predicted domain forces, we relied on two assumptions:

I. in adhesive regions, force vectors are directed anti-parallel to the flow; and
II. in contractile regions, force vectors are directed parallel to the flow.

To implement these criteria, we designed the so-called cone rule (Fig. 2c). The cone rule divides the directions about the local flow vector into a negative cone and a positive cone, respectively. To determine the division line between the two force domains (bold black line in Fig. 2c), we first identified two narrow cones with opening angles of 45º (contraction) and 18º (adhesion). These openings were derived from the spread of two clusters of angles between predicted forces and measured flow vectors (Fig. 2d), the first centered at 0º, the second at 180º. Predicted force vectors falling into one of the narrow cones are classified as contraction-dominant and adhesion-dominant, respectively. The difference in spread reflects that, by nature, adhesion forces strictly oppose the flow direction, while contraction forces do not necessarily align with the flow direction. The division line between the two narrow cones is then defined such that the two clusters associated with the narrow cones have minimal overlap (103.5º in Fig. 2d). The sector between the 18º narrow cone and the division line is referred to as the mixed zone. Here, forces are predominantly adhesive, but contraction makes a significant contribution. To further separate the contributions of adhesion and contraction, predicted forces in the mixed zone are decomposed into projections onto the two narrow cones (Fig. 2c). No such separation is calculated for predicted forces in the sector between the 45º narrow cone and the division line. Although contributions from a weak coupling of the F-actin network to adhesion sites cannot be excluded, contraction force vectors alone can form angles greater than 45º with the direction of F-actin flow. In this case, the effect of contraction is change in flow direction rather than increases flow speed (circles in Fig. 3c).
Note 7: Error analysis of force reconstruction on simulated flow data

We tested the performance of force reconstruction on flow data that was simulated assuming an input force field. We analyzed the error in the predicted force field by adding normally distributed noise of increasing levels to the simulated flow field. The analysis shows that our inverse algorithm is robust and reliable up to a noise level of ~90% (definition of relative noise level: see Eq. (S16) below).

The force field used as an input for the simulations (Fig. S3a, red arrows overlaid on a color coded map of force magnitude) consists of two categories of focal adhesions (small, I and big, II), two closely apposed medium-size focal adhesions (III), and a weak (IV) and a strong (V) contraction zone. This force field design allowed us to test the performance of force reconstruction in detecting weak forces and small FAs, and in separating close FAs. The boundary force (cyan arrows) was designed such that its magnitude modulation was independent of the magnitude modulation of adhesion forces. The purpose of this design item is to demonstrate that our experimental finding of spatiotemporally coupled boundary and adhesion forces (see main text) is not by construction of the mechanical model, but presents a significant cell mechanical behavior.

Noise was added to the simulated flow field according to

\[
u_{\text{noise}}(x) = u_E(x) + \eta_1 \mathbb{E}(\|u_E(x)\|) \mathbb{R}(x) + \eta_2 \mathbb{A}(x),\]

where \(u_E\) is the exact, simulated flow vector without noise (yellow arrows in Fig. S3a), \(u_{\text{mean}} = \mathbb{E}(\|u_E(x)\|)\) is the average length of the flow vectors in the field, and \(\mathbb{R}\) (relative noise) and \(\mathbb{A}\) (absolute noise) are random vectors with two independent, normally distributed components with standard deviation equal to 1. The levels of the two noise types \(\eta_1\) and \(\eta_2\) are denoted in percentages of the individual and the average flow vector length, respectively.

To measure the combined level of the two noise types, we use the relative difference between the recovered flow field \(u_i\) (produced by the predicted force field) and the noise-perturbed input flow field \(u_{\text{noise}}\):

\[
\eta = \frac{\|u_i - u_{\text{noise}}\|}{\max(u_{\text{tiny}}, \|u_i\|)}
\]

A threshold, \(u_{\text{tiny}}\) (25% of the mean flow velocity), is used in Eq. (S16) to avoid systematic bias of the residual statistics caused by very slow flow vectors.

Our analysis shows that the regularization scheme increasingly underestimates force magnitude as the noise level is elevated (color bars in Fig. S3a and b). Regularization, however, is necessary because force reconstruction is sensitive to noise. The ill-posed nature of the inverse problem is evidenced by two observations in Figure S3c and d: i)
The reported error in the predicted force field is significant even in absence of noise, i.e. \( \eta_1 = \eta_2 = 0 \). ii) The error increases sharply at small noise levels (< 10%) but stabilizes at higher noise levels. The reconstruction error at zero noise is associated mainly with the different treatment of adhesion resistance in the forward simulation and in the inverse reconstruction. In the forward simulation, the predicted adhesion resistance force is modeled as a friction term, i.e. \( F_{\eta}(x) = -v(x)u(x) \) where the friction coefficient \( v(x) \) is a known input. In the inverse reconstruction, we first reconstruct the domain force \( F_{\text{II-III}} \) and then separate \( F_{\text{II}} \) from \( F_{\text{III}} \) by the cone rule (see Supplementary Note 6).

The error of force reconstruction as shown in Figure S3c is measured for boundary forces, and for regions with nonzero and zero domain forces. Errors in the reconstructed boundary and the nonzero domain forces are calculated as the mean relative error, i.e.

\[
E = \text{mean}_{x \in \Omega_{\text{x0}}} \left( \frac{\|F_R(x) - F_i(x)\|}{\|F_i(x)\|} \right), \Omega_{\text{x0}} = \{x : \|F_i(x)\| \neq 0\}.
\]

Errors in the region of zero domain force are calculated relative to the average magnitude of nonzero forces:

\[
E = \text{mean}_{x \in \Omega_0} \left( \frac{\|F_R(x) - F_i(x)\|}{\text{mean}_{x \in \Omega_{\text{x0}}} \|F_i(x)\|} \right), \Omega_0 = \{x : \|F_i(x)\| = 0\}.
\]

In Eqs. (S17) and (S18), \( F_R \) denotes the reconstructed force field, \( F_i \) the input force field to the simulation and \( \text{mean} \) the mean force magnitude in \( \Omega_{\text{x0}} \), the region of nonzero input forces.

A significant portion of the reconstruction error as measured in Eq. (S17) originates from the systematic underestimation of force magnitude. Thus, we further performed an error analysis on the relative distribution of reconstructed forces. This indicates the accuracy with which force relations between different cellular locations can be reconstructed. We define relations in a predicted force field in terms of i) magnitude, ii) direction, and iii) the location of nonzero force vectors (Fig. S3d). To measure the error in the relations between force magnitudes, we first scaled the mean magnitude of estimated forces uniformly in the region with nonzero forces to the mean magnitude of the input forces. Accordingly, the relative error of force relations is defined as

\[
E_M = \text{mean}_{x \in \Omega_{\text{0}}_0} \left( \frac{\rho \|F_R(x)\| - \|F_i(x)\|}{\|F_i(x)\|} \right), \rho = \frac{\text{mean}_{x \in \Omega_{\text{x0}}} (\|F_i(x)\|)}{\text{mean}_{x \in \Omega_{\text{x0}}} (\|F_R(x)\|)}
\]

(Fig. S3d top panel). To measure the error in the force direction we calculated the angle between the reconstructed and the input force vectors divided by \( \pi \) (Fig. S3d lower panel).

To measure the relative error of force location, we first defined the location of significant reconstructed forces by setting a threshold below which forces are statistically
insignificant. Misidentified force locations occur either i) in areas with false negatives (blue dots in Fig. S3b), or ii) in areas with false positives (grey dots in Fig. S3b). The error is then defined as the area ratio between misidentified force locations and $\Omega$. Clearly, the error depends on the selection of the significance threshold. Figure S3d (lower axis) shows the relative error of force location when the threshold is varied from 20% to 50% of the mean magnitude of reconstructed forces in $\Omega_{\mu0}$. The error curves indicate that a threshold of 50% provides optimal identification of force location for input noise levels above ~15%. For input noise levels below 15% thresholds down to 30% can be used. More restrictive threshold (<30%) are not preferred even with input noise levels close to zero.

The results in Figure S3b indicate that the separation of nonzero and zero forces gets increasingly blurred by the force reconstruction as the noise level increases. In the example shown, predicted force locations obfuscate the distinction of closely apposed FAs when the combined noise level is above ~50%. The analysis also shows that above noise levels of 50% caution must be applied as to the interpretation of small patches of adhesion or contraction forces. However, overall these tests confirmed that force location, direction and magnitude on a relative scale can be predicted from noisy measurements of F-actin network flow.
**Figure S3**: Error analysis of predicted forces based on simulated flow data. (a) *Upper panel*: Assumed input force field (red arrows) and corresponding simulated flow field (yellow arrows). The input force field is governed by one small (I), one large (II) and two medium size adhesions (III). The latter are closely apposed to each other. In addition, input forces are generated by one weak (IV) and one strong (V) contraction zone and boundary forces (cyan arrows). The boundary force field is designed such that its maxima are shifted relative to the maxima of adhesion forces. This design is intended to demonstrate that the co-localization of predicted boundary and adhesion forces in real experimental data is not by construction of the continuum mechanical model. *Lower panel*: Force field reconstructed from the simulated flow vectors without adding noise. The regularization step, which is required for the reconstruction of forces from noisy flow fields, yields small errors in the reconstructed force location and underestimated force magnitudes even for noise-free flow fields. (b) Force fields reconstructed from noise-
perturbed flow fields at noise levels 5%, 36%, and 90%. Noise levels are determined by posterior estimation of the flow residual, defined as the relative difference between the noise-perturbed input flow field (green) and the recovered flow field (yellow) produced by the reconstructed forces (see Eq. S16). 

*Lower right panel:* Comparison of input (green) and recovered (yellow) flow vectors at a residual level 90%. Blue and grey dots in A and B represent false negative and false positive force locations, respectively (see text). (c) Relative error of the force reconstruction according to Eqs. (S17) and (S18). This error is dominated by underestimation of absolute force levels associated with the requirement for regularization. (d) *Top axis:* Error of force relations in terms of relative magnitude (upper two thick lines) and force direction (lower two thin lines). Red corresponds to the predicted domain force whereas cyan corresponds to boundary force. *Bottom axis:* Relative error in reconstructed force location. To measure the relative error of force location, a threshold is used to cut off insignificant forces. Grey, black, green and blue curves in the lower axis correspond to thresholds of 20%, 30%, 40% and 50% of the mean force magnitude of the nonzero force region. False positive and false negative force locations in a and b are calculated by using thresholds of 30% and 40%, respectively. Scale bar in a: 5 μm.
Note 8: Validation of force reconstruction by co-localization of contraction forces with eGFP-myosin-II and adhesion forces with eGFP-vinculin

In epithelial cells, contraction forces are generated by myosin-II motors\textsuperscript{30}, while adhesion forces are transmitted to the extracellular matrix in nascent adhesion complexes and focal adhesions\textsuperscript{31}. Therefore, it is expected that regions of dominant contraction forces co-localize with myosin-II. Regions of dominant adhesion forces are expected to co-localize with the protein components of adhesion sites\textsuperscript{32}.

Fig S4a/b confirms co-localization of the predicted contraction force transients with eGFP- myosin-II regulatory light chain, especially within the central and posterior portion of the lamella. Some anterior regions display weak contraction forces despite the relatively strong myosin-II signals (area marked by a rectangle). The actin signals indicate an elevated density of stress fiber ends in this region (Fig. S4c), which are known to innervate into focal adhesions\textsuperscript{33}. Thus, in these areas contraction and adhesion forces are likely to cancel one another, as reflected by low predicted forces.

Fig. S4d displays the corresponding adhesion and boundary force predictions. The cell edge is in a state of retraction in the left half of the field-of-view while it is in a state of protrusion in the right half of the field-of-view (Fig. S4c, Video 1). The protruding sector is associated with strong predicted adhesion forces in a ~5 μm-wide band next to the cell edge. No such forces are predicted in the retracting sector. This provides mechanical evidence that cell protrusion requires the formation of adhesive bonds between cytoskeleton and ECM, which is regulated on a scale of single microns.

To test the co-localization of predicted adhesion force transients with adhesion complexes, we relied on previous reports which showed high traction forces are accompanied with high fluorescence intensity of eGFP-vinculin\textsuperscript{34, 35}. Indeed, areas of high eGFP-vinculin intensity co-localized with areas of increased adhesion forces (Fig. 3a, main text, Top, Middle and Bottom boxes). Together, these experiments established the co-localization of contraction and adhesion force transients with molecular components of the corresponding force generating machineries, further validating the proposed model for intracellular force reconstruction.
**Figure S4:** Validation of force reconstruction by colocalization of predicted contraction forces with myosin-II distribution in lamellipodium and lamella. (a) Localization of eGFP-myosin regulatory light chain. (b) Predicted contraction forces (red arrows overlaid to color-coded map of contraction force magnitude). Bars highlight matching details in myosin-II and force patterns. Colocalization is weak in white boxes. Ellipses mark contraction forces that form an angle greater (left) and less (right) than $45^0$ to the flow direction (see panel d). (c) Localization of X-rhodamine-F-actin speckles (see Video 1 for dual-color time-lapse sequence of F-actin and myosin-II). Color-coded lines indicate movement of the cell edge (blue: early time-points; red: late time-points). (d) F-actin flow field (yellow arrows). Predicted adhesion forces (red arrows) and boundary forces (cyan arrows). Scale bar in a: 10 μm.
Note 9: Effect of spatial variation in network elasticity

Absolute force levels could be estimated only if the material properties were known in every location of the sample. For live cells this information is inaccessible at the resolution scale of qFSM flow data. However, to study how forces are dynamically regulated during morphogenic events, the reconstruction of absolute force magnitudes is unnecessary. It is sufficient to know where and when forces develop, and how the directions of forces in neighboring locations deviate. These data could be directly derived from the flow field if the F-actin elasticity was spatially homogeneous or spatial variations could be assessed from, e.g., the fluorescence intensity. Clearly, the assumption of a spatially homogeneous F-actin network seems overly simplified. Thus, we asked to what extent predicted force distributions in lamellipodium and lamella still reflect meaningful force relationships, even when homogenous elasticity of the F-actin network had to be assumed due to a lack of more complete information.

To address this question, we compared force distributions reconstructed with different assumptions of network elastic properties. We recorded a dual-color qFSM movie of X-rhodamine actin (red) and GFP-vinculin (green, Fig. S5a). The X-rhodamine signal shows significant spatial variation in intensity, indicating, for instance, the position of stress fibers. Presumably, stress fibers and other regions of higher F-actin density are stiffer than the rest of the network. To test how such heterogeneity affects force reconstruction, we calculated a map of heterogeneous Young’s modulus assuming it is proportional to the F-actin concentration indicated by the X-rhodamine fluorescence (Fig. S5b). The resulting range of moduli varied over one order of magnitude, which is in good agreement with the stiffness variation across lamellipodium and lamella reported by high resolution AFM mapping. Predicted force fields were then reconstructed, first assuming homogenous elasticity (Fig. S5c and d) and then assuming spatially variable elasticity (Fig. S5e and f). The two force fields display very similar patterns. In both cases, the adhesion-dominant regions (Fig. S5c and e) colocalize well with the location and intensity of the GFP-vinculin signal (Fig. S5a, green). Close comparison of the predicted force vectors (zoom-in views of Fig. S5c and e) suggests that the distributions of both the direction and relative magnitudes are similar. Thus, the outcome of the predicted force reconstruction is relatively insensitive to elasticity variations. A possible explanation for this robust behavior is the high resolution of the qFSM-measured flow data, which warrants determination of stable flow gradients with submicron resolution (see Supplementary Note 4). At this length scale variations in the average network elasticity are small compared to the magnitude of the flow gradient. Consequently, the numerical solution to the inverse problem formulated by Eqs. (S11) and (S12) is dominated by the strain tensor for which high quality information is available.
Figure S5: Sensitivity of the force reconstruction to spatial heterogeneity in network elasticity. (a) X-rhodamine F-actin and eGFP-vinculin distribution. Circles highlight locations of adhesion sites with stress fibers. (b) F-actin flow (yellow vectors) and predicted adhesion forces (red vectors) overlaid on color-coded adhesion force magnitude map. (c) F-actin flow (yellow vectors) and predicted contraction forces (red vectors) overlaid on color-coded contraction force magnitude map. Forces in b and c are reconstructed by assumption of spatially homogeneous network elasticity. (d) Elasticity field derived from the F-actin intensity. Values vary over one order of magnitude. (e, f) Predicted adhesion and contraction forces, reconstructed by assumption of spatially variable network elasticity. Scale bar in b: 5 μm.
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