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Chapter 3

Orientation of the actin cytoskeleton determines cell shape and force exertion

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abstract

Characterization of cellular contractility is key to understanding many of the processes governing cellular function. With live-cell measurements of actin organization and cellular force exertion, we observed actin fibers to orient preferentially in the direction of force exertion. Since circular arcs provided a good fit to cortical actin in these fibroblast cells, we used the theoretical description of a mechanical equilibrium. We directly observed the local balance between internal stress, line tension, radius of curvature and external forces. However, the resulting internal stress did not give a constant result over different cells, as would be expected for a homogeneously contractile cell. We therefore developed an analytical model to describe the relationship between contractile stress and orientation of the actin cytoskeleton. Local stress fiber density and -force guidance were sufficient to explain our results. This yielded a quantitative description of the local force balance along the actin cortex. Our observed ten-fold increase in contractile stress is attributed to local actin orientation, showing that cell shape and force exertion are guided through directed contractility of the actin cytoskeleton.
3.1 Introduction

In recent years, the importance of the mechanical characterization of cellular behavior has attracted increasing attention [1, 2]. This interest has developed since processes like directed cell migration [3], stem-cell differentiation [4, 5] and metastatic development [6, 7] have been found to be controlled by a significant stiffness-dependent component. However, how cells probe the mechanical properties of the environment is largely unknown. The process of mechanosensing likely involves a step in which the cell pulls on its environment, followed by a biochemical stiffness-dependent readout. Cellular pulling forces in the nanoNewton range have been observed by various techniques [8–10]. These forces are generated by the actomyosin cytoskeleton. Actin with myosin contractility forms the active network allowing to pull on the environment via specific membrane-spanning proteins. For many cells and extended periods of the cellular lifecycle the cytoskeletal network was found to be fairly homogeneous. This observation has spurred the development of models that seek a quantitative description of cell-generated forces [11–13]. In particular, active solid theory [11] directly links internal forces to cell-shape and successfully describes observations that allow one to estimate the force exerted by the network.

However, for many cells, forces are highly directional and the organization of the cytoskeleton is far from homogeneous. Here we developed a description of cellular force generation building on those earlier models, including effects emerging from inhomogeneity in the network. To our surprise we found that the earlier result of a direct relation between cellular stress and cell shape [11, 14, 15] was fully recovered when the global cellular stress is interchanged by a local stress from an oriented stress-fiber (SF) network. We developed a model that takes relative SF density along the actin cortex and the fractional guidance along the network orientation into account. Using quantification of the network orientation, cortex curvature and a direct measurement of local cellular force exertion we validated our hypothesis. Further our model allowed us to obtain an estimation for the ratio between the stress developed from the cell’s homogeneous cytoskeletal network in comparison to the stress emerging from the oriented network. We showed for fibroblasts that the stress from the oriented SF network outgrows that of the homogeneous network by approximately one order of magnitude.
3.2 Methods

3.2.1 Cell biology

3T3 fibroblasts were cultured in high-glucose DMEM medium supplemented with 10% newborn bovine serum (Thermo Scientific), 2 mM glutamine and 100 µg/ml penicillin/streptomycin. Using virus transduction, a stable cell line expressing LifeAct-mCherry was created and kept in culture. Lentiviral particles using construct pRRL-Lifeact-mCherry (a gift of O. Pertz, University of Basel, Basel, Switzerland) were isolated from the supernatant of HEK293T cells. They were transiently transfected with third-generation packaging constructs and lentiviral expression vectors. 3T3 Fibroblasts were infected with the supernatant containing lentiviral particles in the presence of 4 µg/ml polybrene overnight. Cells were continuously selected using 1 µg/ml puromycin in culture.

After micropillar array preparation (see next section), cells were seeded at single cell density at approximately 100,000 cells per micropillar array. They were allowed to spread for 6 to 16 hours. Micropillar arrays were then inverted onto #0, 25 mm diameter, round coverslips (Menzel Glaser). The arrays were kept from floating using a support weight of glass during imaging.

3.2.2 Force measurement

Cellular traction forces were quantified using micropillar arrays as previously described [10]. Briefly, PolyDiMethylSiloxane (PDMS) micropillars with 2 µm diameter, 6.9 µm height and 2 µm spacing were produced using replica-molding from a silicon wafer etched with reactive ion etching. Only the tops of the micropillars were micro-contact printed with 50 µg/ml Fibronectin and 10 µg/ml Fibronectin-Alexa405. The labeled Fibronectin was imaged to ensure a homogeneous substrate and to determine the micropillar center. Through calibration of the bulk PDMS stiffness, exact micropillar dimensions and Finite Element simulations, we obtained a precise force-deflection calibration [10]. From the deflection fields we obtained a pillar localization precision of 30 nm, corresponding to 500 pN. All analysis was performed using specifically designed software (Matlab, Mathworks).
3.2 Methods

3.2.3 Microscopy

Live-cell measurements were performed in overnight time-lapse measurements on a confocal spinning-disk setup based on an Axiovert 200 body (Zeiss) with a home-made focus-hold system. For imaging, a 405 nm laser (Crystalaser) and a 561 nm laser (Cobolt) were controlled with and Acousto-Optic Tunable Filter (AA Optoelectronics) and coupled into the confocal spinning-disk unit. Collimated 850 nm LED illumination was coupled into the backport of the microscope and aligned to reflect of the glass-water interface back out the backport via a dichroic mirror. Using a reference detector before incoupling and a high sensitivity detector (Thorlabs) after reflection, the sample was kept at constant distance from the objective. Simultaneously, the temperature was kept at 37 °C with constant 5% CO2 concentration in a stage-top incubator (Tokai Hit, Japan). In this configuration, time-lapse movies were recorded overnight on live cells on inverted micropillar arrays.

3.2.4 Cellular curvature fitting

On spread cells, a custom-made algorithm in Matlab was used to determine curvature of the actin cortex. For a two-dimensional description (the cells are spread on a surface), the line tension $\lambda$ is equal to the force along the curved actin cortex. The local mechanical equilibrium at this curve can be described by the balance of the surface tension $\sigma$. This is given by the line tension $\lambda$ and the radius $R$ through $\sigma=\lambda/R$. When two arcs emanated from one site of force exertion, we decomposed the force vector along the tangent of the two. A combined measure of the curvature and the line tension thus gives the local surface tension.

3.2.5 Actin stress fiber orientation

Orientation and coherence of the actin cytoskeleton was determined using the OrientationJ plugin in ImageJ. This plugin makes orientation and coherence maps based on the local gradient of an image. Analysis was done using a Gaussian gradient with a window of 5 pixels. Only pixels with a local coherence above a threshold of 0.4 (on a scale 0-1) were used. The results from the ImageJ analysis was imported into Matlab (Mathworks) and further image processing was done in specifically designed Matlab scripts.
For the comparison between cortical actin orientation and the cell edge only the pixels in an arc with a radius between 10 and 50 pixels (approximately 5 µm into the cell) beyond the fitted arc were taken into account. The average orientation of the edge was determined using the coordinates of the endpoints of the edge. The resulting relative orientation of the actin with reference to the cell edge was determined to be the difference between the actin orientation and the edge orientation.

3.3 Results

3.3.1 Actin stress-fibers co-orient with local force exertion

Mouse 3T3 fibroblasts that were transformed with the actin label LifeAct-mCherry were spread on inverted micropillar arrays and observed on a high-resolution microscope. This approach allowed us to simultaneously observe cell shape, organization of the cortical actin and of the actin SFs, as well as to analyze the forces the cells applied to the substrate. Figure 3.1A shows a 3T3 fibroblast expressing LifeAct-mCherry (red) as it was spread on micropillars coated with labeled fibronectin (blue). Actin SFs inside the cell and the cortical actin fibers that stretch along the cell’s perimeter were clearly visible. Forces, as determined from the deflections of the micropillars, are indicated by white arrows. They extend in a range of 0-10 nN as observed previously for fibroblasts [8–10]. These live-cell measurements provided simultaneous information on cellular force exertion and the actin cytoskeletal organization.

Already from the raw image a strong parallel correlation between SF orientation and the direction of the cellular force is obvious. To further quantitate this observation we analyzed the SF arrangement. The local orientation of the actin cytoskeleton was quantified by means of a structure-tensor approach that involves image thresholding and erosion [16] (figure S1A). Subsequently, the orientation-map was analyzed for areas of structural coherence (coherence map, figure S1B) that finally yielded an orientation map as shown in figure 3.1B. The actin meshwork orientation ranged from -90° to +90° with respect to the x-axis of the figure. Subsequently we quantified the direction of the cellular forces with respect to the direction of the SF directional map. The analysis yielded that forces largely align with the direction of the SFs. Figure 3.1C shows co-orientation (0±20)° of the actin cytoskeleton with the direction
3.3 Results

Figure 3.1
The orientation of actin stress fibers coincides with the direction of force exertion. (A) A 3T3 Fibroblast expressing lifeAct-mCherry (red) shows a polarized SF structure on top of micropillars with labeled fibronectin (blue). Cellular contractile forces are directed inward, but co-orient with the orientation of stress fibers (Arrow scalebar lower left corresponds to 20 nN, scalebar lower right corresponds to 10 µm). (B) Orientation map of actin fluorescence as depicted in (A), color coding from -90° (blue) to +90° (red). (C) Histogram of orientation difference of SFs relative to orientation of force exertion. Orientations of SFs and force exertion coincide within a distribution of standard deviation of 20° (24 cells, 997 orientations).

of force exertion for 997 displaced pillars in 24 cells. Our orientation analysis on the local scale of several micrometers corroborates earlier observations of directional cytoskeletal contractility [13] on the scale of the whole cell.

3.3.2 Cortical stress fibers suggest a homogeneous contractility

Next to the cytosolic SFs we analyzed the cortical SFs that align along the perimeter of the cell and contribute to the cellular integrity. Many of those cortical fibers were described by circular arcs to high accuracy as exemplified in figure 3.2B. For a mechanical system to display a deformation along a circular-arc one would predict a homogeneously distributed tension to act on the system [17]. The observation of a circular arc-like cell perimeter has been reported earlier and has led to the homogeneous active solid theory of a cell [11]. In active solid theory the cell’s contractility is characterized by an isotropic stress $\sigma$ acting on the cortical actin leading to an outer circular arc of radius R. At the point of intersection between two arcs the cell exerts a force $F$ on the environment that gives the line tension $\lambda$ along the arc (see schematic figure 3.2A).
Indeed, our measurements showed that the highest forces emerged on pillars that were at the intersection of those circular arcs (see figure 3.2C). We therefore treat such a circular arc as a local mechanical equilibrium. This equilibrium follows Laplace's law, where internal contractile stress $\sigma$ follows from the arc-radius $R$ divided by the line tension $\lambda$:

$$\sigma = \frac{R}{\lambda} \quad (3.1)$$

Qualitatively our data followed the expected behavior for an increasing radius of curvature compared to spanning distance $d$. Previously [11], this behavior was explained by introducing an elastically-modified line tension. Even though the tension-elasticity model explains the observed variation in $R$-$d$ dependence, it does not take a potential guidance of contractility along an inhomogeneous network into account. In our measurements we were able to directly quantify the line tension $\lambda$ through the force exerted on the substrate. Independantly, we quantified the radius of curvature $R$ by fitting a circle as depicted in figure 3.2B. Resulting from equation 4.1 we obtained a direct measure of the local contractile stress $\sigma$, which should be constant for a homogeneously contractile system. However, we observed variations between different circular arcs, ranging from 0.1 to 1 $\mu$N/$\mu$m.

### 3.3.3 Contractile stress increases depending on the local stress fiber orientation

The shortcomings of the description of a cell as homogeneous system, together with our observation of the co-orientation of forces with the SF network motivated us to develop a cellular model that includes the orientation information. We use the observation that the SF orientation is nearly constant along each circular-arc. In our model, we take two distinct mechanisms into account that have consequences for both cell shape and force exertion. The first effect relates to the effective density of SFs at the cell periphery. When we assume the average distance $\delta$ between SFs to be constant, the density along the cortex depends on the local actin orientation $\theta$. We propose that a more perpendicular orientation of SFs causes a higher local network density at the edge and thus a higher curvature:

$$\frac{1}{R} = \frac{1}{\delta} \sin(\theta) \quad (3.2)$$
**3.3 Results**

Cortical actin can be described by circular fits corresponding to an internal contractile stress. (A) Active solid theory describes how a continuum is contracted by an internal contractile stress \( \sigma \). In a local mechanical equilibrium along the cortex the stress is given by the radius of curvature \( R \) and line tension \( \lambda \). (B) Certain cellular edges can be fitted well by a circle, indicating an inward pulling force that is distributed over the entire cortex. (C) Micropillar deflections quantify the local force generated by the cell. The force is directed along the cortical actin and is large at the extremities of a circular arc, but mostly absent along the arc. The measured force gives a direct measure for the line tension \( \lambda \). (scalebar in lower right in B and C indicates 10 \( \mu \)m, force scalebar in lower left in C indicates 10 nN)

Further we assummed that the force generated by SFs is transmitted through the cortical actin fibers. The second effect is described by the tangential part of the tension, \( \lambda_{\tan} = \lambda \sin(\theta) \), which is the component of the force transmitted in the SFs. This reduced force is homogeneously distributed along the cortical actin. It should be noted that our model, although local directional information is included, still fulfills the strong requirement of a locally homogeneous medium that leads to circular-arc deformations of the cortical actin. As is schematically depicted in figure 3.3A, we assume forces exerted by the oriented SF network to be transmitted through a finer actin meshwork giving rise to the shape of the actin cortex. Finally, when the SFs are fully parallel along the edge we assume a basal level of homogeneous contractility \( \sigma_0 \). Taken together, the effect the contractile stress along the cell periphery can be described as:

\[
\sigma = \frac{\lambda_{\tan}}{R} = \frac{\lambda}{\delta} \sin^2(\theta) + \sigma_0
\]  

(3.3)

From our extended model predictions can be made for the cellular forces and concomittant cell shape which are confirmed by our experiments. First, following equation 4.2, the curvature should scale linearly
Actin orientation and cell shape

with $\sin(\theta)$, depending on the orientation of the local actin network. The orientation of the actin cytoskeleton we defined along a region $\sim 5 \mu m$ into the cell along the curved actin cortex. In figure 3.3B the arc curvature $(1/R)$ is shown as a function of SF orientation along the arc. Indeed, the data follow the predicted linear increase characterized by a slope of $\frac{1/R}{\sin(\theta)}=(0.12\pm0.01) \mu m^{-1}$ ($R^2=0.8218$). Hence, the curvature of cortical actin scale linearly with the direction of internal actin orientation.

In our experiments, we further quantified the local cellular force exertion and thus the local internal stress at a curved actin cortex. We quantified the line tension as the tangential force along the curved actin from the deflection of a pillar (see figure 3.3A). When we take this line tension into account and test our model, we observe a striking correspondence in figure 3.3C. Both curvature and force exertion together depend on the orientation of the actin network, which determines the local contractile stress. It is important to note here that we have three independent measurements for network orientation, curvature and line tension and all come together to corroborate our model. For a parallel orientation of actin SFs, i.e. $\sin(\theta)=0$, the fit to our model gives a basal contractile stress $\sigma_0=(0.08\pm0.02) nN/\mu m$. The contractile stress increases with more parallel SF orientation scaling with $\sin^2(\theta)$ at a rate of $\frac{\lambda_5}{\delta}=(0.86\pm0.07) nN/\mu m$ ($R^2=0.8179$). Our quantitative model explains the increase in contractile stress from the local SF orientation over one order of magnitude.

3.4 Discussion and conclusion

Contractility of the actin cytoskeleton generates significant pulling forces which are at the basis of cellular function. We observed that local cellular force exertion co-orients with the actin cytoskeleton. The forces exerted along the edge were dominant at the extremities of curved edges. But the actin cortex is circular, implying a force distribution along these arcs. In this work, we have expanded on active solid theory to describe a model that takes inhomogeneous and directed contractility into account. To validate our model describing network spacing and force bearing SFs, we quantified actin organization, curvature of the cortex and cellular force exertion in live fibroblast cells.

Actin curvature increases with a locally more perpendicular actin cytoskeletal organization in a linear relation. When the forces exerted
3.4 Discussion and conclusion

Figure 3.3
Local cortical actin curvature and force exertion increase depending on local actin orientation. (A) Schematic depiction of our model, indicating a relation between external force applied, curvature, SF orientation leading to contractile stress $\sigma$. $\theta$ is the angle of actin fibers relative to the cell cortex. Curvature and contractile stress are predicted to increase with $\sin(\theta)$. (B) Experimental results (blue) show a good fit to the predicted linear increase in actin cortex curvature. In our model, this is attributed to decrease in SF spacing due to the angled oriented network. The linear fit (red line) describes an increase in curvature of 0.12 $\mu$m$^{-1}$. (C) Internal stress $\sigma = \lambda / R$ increases even more pronounced with larger $\theta$. This can be explained by a co-operative effect of decreased local spacing and force exertion carried over SFs. The fitted model (red line) shows a quadratic increase from a contractile stress baseline $\sigma_0 = 0.08$ nN/$\mu$m with 0.86 nN/$\mu$m. This behaviour explains an increased contractile stress over one order of magnitude depending on SF orientation.

are taken into account an even more dramatic increase emerges. The measured values correspond well to the predicted theory, indicating that the orientation of actin SFs matters for both the density and the force transmission. From a baseline of homogeneous contractile stress of 0.08 nN/$\mu$m, perpendicular orientation of the actin cytoskeleton coincides with a ten-fold increase in contractility. These results imply a re-distribution of contractility along cortical actin and a strong dependence on a polarized actin structure of the contractile stress. The theoretical framework presented here provides a quantitative and qualitative description of the active mechanical directed behaviour of a single cell.
3.5 Supplemental figures

Figure S1

(A) Orientation map of a single cell and the surrounding noise. Orientation range over 180 degrees, with color coding from -90 (blue) to +90 (red) degrees. (B) Coherence map of the same cell with an arbitrary scaling from 0 (blue) to 1 (red). Only sufficiently coherent structures above a threshold of 0.4 are taken as significant values.


