Force field evolution during human blood platelet activation

Sarah Schwarz Henriques\(^1\), Rabea Sandmann\(^1\), Alexander Strate\(^2\), and Sarah Köster\(^1\)*

\(^1\)University of Göttingen, Department of X-Ray Physics and Courant Research Centre Nano-Spectroscopy and X-Ray Imaging, 37077 Göttingen,

\(^2\)University of Göttingen, Department of Transfusion Medicine, 37075 Göttingen, Germany

*sarah.koester@phys.uni-goettingen.de

Running title: Force fields in human blood platelets

Key words: cellular contraction, platelets, traction forces, force fields
Summary

Contraction at the cellular level is vital for living organisms. The most prominent type of contractile cells are heart muscle cells, a less well known example are blood platelets. Blood platelets activate and interlink at injured blood vessel sites, finally contracting to form a compact blood clot. They are ideal model cells to study the mechanisms of cellular contraction, as they are simple, bearing no nucleus, and their activation can be triggered and synchronized by the addition of thrombin. Here, we study contraction on the example of human blood platelets employing traction force microscopy, a single cell technique that enables time-resolved measurements of cellular forces on soft substrates with elasticities in the physiological range ~ 4 kPa). We find that platelet contraction reaches a steady state after 25 min displaying total forces of ~ 34 nN. These forces are considerably larger than what was previously reported for platelets in aggregates, demonstrating the importance of a single cell approach for studies of platelet contraction. Compared to other contractile cells, we find that platelets are particular, because force fields are nearly isotropic with forces pointing toward the center of the cell area.

Introduction

Blood platelets are responsible for clot formation in mammals in the early stages of wound healing (Michelson, 2007). When they activate at damaged blood vessel sites, they change their shape and interlink with each other through fibrin(ogen) and adhesive glycoproteins to form an early plug. In a final step, the platelet-fibrin clot retracts and thus solidifies the clot mass. This retraction is thought to bring the wound edges closer together and to re-establish normal blood flow by pulling the constricting clot mass against the vessel wall (Carr, 2003). Platelets, in particular, contribute to clot retraction by actively contracting and pulling on the fibrin links, a reaction which is believed to be achieved by myosin motors in combination with cytoskeletal actin fibers. Such an involvement has been shown for platelets by a rheological approach (Jen and McIntire, 1982) and by investigating myosin IIa knockout mice, where clot retraction is impaired (Léon et al, 2007).

Apart from being of great medical importance, platelets are an ideal model system for studying cellular contraction for two main reasons: They are simple cells without a nucleus and their activation, which occurs within minutes, can be triggered and synchronized by the addition of thrombin. Traditionally, research on platelet contraction has been closely linked to
clinical applications. Thus, a widespread method for studying platelet contractile forces and clot elasticity was developed by Carr et al. as a clinical measure for platelet function (Carr, 2003; Carr et al., 2007; Carr and Zeckert, 1991). It is based on the idea that contracting blood samples and platelet-rich plasma samples between a fixed and a freely supported surface change the gap size in a force-dependent manner. By monitoring the gap size and periodically calibrating the device with externally applied compressive forces, a time track of contractile forces of the sample is obtained. Knowing the number of platelets in the sample, the contractile force per platelet can be calculated. The resultant maximum contractile forces per platelet are found to be on the order of 0.3-0.4 nN 15-17 min after clotting is initiated (Carr et al., 2007; Carr and Zeckert, 1991). Using a similar device with a freely supported upper surface, but a rotationally oscillating bottom surface, Jen and McIntire performed rheology measurements on clot contraction and rigidity outside clinical applications and measured maximum contractile forces of 0.5 nN after 80 min clotting time (Jen and McIntire, 1982). Both methods are designed to study platelet contraction forces by measurements of large cell aggregates. Furthermore, the experimental conditions are difficult to control as the samples contain blood plasma and hence several clotting agents; in both methods external forces are applied to the sample, which may influence clot formation. In a recent approach, Liang et al. circumvent these problems by washing platelets in buffer prior to experiments and looking at contraction of microclots on a dense array of elastic microposts (Liang et al., 2010). They measure contractile forces per platelet of 2.1 nN 60 min after the initiation of clotting. However, platelets themselves release clotting agents upon activation and even in microclots the forces generated by individual platelets may add up nonlinearly to the overall contractile force. Single cell techniques are therefore needed to directly access platelet contractility. Lam et al. have performed AFM (atomic force microscopy) studies on single platelets between a fibrinogen-coated glass surface and a fibrinogen-coated cantilever (Lam et al., 2011) and measured average maximum contractile forces of 29 nN after 15 min. Even though this study is restricted to uniaxial contraction, the fact that their measured values are much higher than previously reported, highlights the importance of the single cell approach. Another promising single cell technique is traction force microscopy (TFM) (Dembo and Wang, 1999; Wang and Lin, 2007). Here, cells are placed on soft substrates with elasticities in the physiologically relevant range (~ 1-100 kPa). The substrate deforms in response to cellular contraction, which is visualized by help of marker beads that are incorporated into the top layer of the substrate. From the substrate's deformation, maps of local forces per unit area
$T(\vec{x}) = \frac{F(\vec{x})}{dA}$, so called traction forces, can be calculated for each individual cell. The experimental technique has been amply used to study contractile forces generated by both migrating cells, such as fibroblasts (Dembo and Wang, 1999; Pelham and Wang, 1999; Munevar et al., 2001; Beningo et al., 2001; Rajagopalan et al., 2004) or fish keratocytes (Oliver et al., 1999; Lee et al., 1994), and stationary contractile cells, such as airway smooth muscle cells (Butler et al., 2002; Tolic-Norrelykke and Wang, 2005). Average traction forces for the three cell types are typically on the order of $0.8-3.03 \cdot 10^3$ Pa, $10^2$ Pa and again $10^2$ Pa, respectively. Although usually single time shots are taken from the cells after incubation on the substrate overnight, it is possible to study the time evolution of traction force fields with TFM. Time sequences have, for example, been measured for fibroblasts (Munevar et al., 2001) and airway smooth muscle cells (Tolic-Norrelykke and Wang, 2005) with a spatial resolution of 3-4 µm or 2.7 µm, respectively, and a temporal resolution of 40 s.

We adapt TFM to the extremely small size of human blood platelets (diameter 2-5 µm (Michelson, 2007)) to study the mechanisms underlying platelet contraction and identify aspects that are common to other cells generating contractile forces. We find that platelet contraction reaches a steady state, where platelets pull nearly isotropically toward the center of the cell area and, as for migrating cells (Munevar et al., 2001; Oliver et al., 1999) and smooth muscle cells (Tolic-Norrelykke and Wang, 2005), forces seem to be strongest along the edge of the cell body. In the steady state the traction forces are on the order of $\sim 3.3 \cdot 10^3$ Pa, corresponding best to values measured for fibroblasts. Total forces are in the range of $\sim 34$ nN, which is similar to and slightly larger than previously reported from other single cell techniques (Lam et al., 2011). Our findings can be satisfactorily explained by actomyosin activity. However, further contributions to force generation, possibly including actin depolymerization and induction of fluid flows, cannot be excluded.

Results
We isolated human blood platelets from platelet concentrates, seeded them on elastic polyacrylamide substrates with incorporated fluorescent marker beads and followed platelet contraction with traction force microscopy for 60-92 min. We used linear elasticity theory to calculate traction force fields for the cell area $A$ from the measured bead displacements. As
input, the cell area $A$ and the bead displacements were extracted from the microscopy recordings. Whereas the area $A$ was obtained from phase contrast images with a combination of a Laplacian filter and thresholding, we identified the bead displacements in corresponding fluorescence images by particle tracking. In particular, we searched for each bead in a frame $i$ along the projected line of motion known from frame $i-1$. A sketch of the steps involved in data analysis is shown in Fig 1. A comparison of this automated contour tracking and manual contour tracking is shown in figure S1 in supplementary materials. In the following description of the results, we will use the notation $\bar{X}$ to describe a time average of any variable $X$ and $\langle X \rangle_N$ to describe the corresponding ensemble average.

**Temporal evolution of contractile forces**

As a result of data analysis we obtain movies of local forces $\vec{F}(\vec{x})$ per unit area $dA$, i.e. of traction forces $\vec{T}(\vec{x},t) = \frac{\vec{F}(\vec{x},t)}{dA}$. A qualitative inspection of these movies (see Movie_1 in supplementary materials) provides an impression of the characteristics of platelet contractile forces. We observe that already after a few minutes a stable force field emerges. To quantitatively assess how long it takes platelets to establish a stable force field, we therefore analyze the time track of the total force $\vec{F}_{tot} = \int_{A} \vec{T}(\vec{x}) dA$ generated after cell adhesion to the substrate. An example of a typical time track is shown in Fig. 2A. It shows that the forces increase at the beginning of platelet activation ($t_{tp} < 1798$ s) before the cell reaches a steady state at $F_{tot}^{St} \sim 40$ nN, in which contractile forces are balanced against elastic forces.

To compare typical values for $F_{tot}^{St}$ and $t_{tp}$ for a statistical ensemble, we fit linear functions to both force increase and steady state region in the time tracks $F_{tot}(t)$ and set the turn point $t_{tp}$ between the two regions as an additional fit parameter. Histograms of the relative abundance of $F_{tot}^{St}$ and $t_{tp}$ for an ensemble of 14 cells from 4 different cell preparations are shown in Fig. 2. We find that on average platelets display a steady state total force of $\langle F_{tot}^{St} \rangle_N \sim 34$ nN which is reached $\langle t_{tp} \rangle_N = 25$ min (1500 s) after cell adhesion to the substrate. Furthermore, the distribution for $F_{tot}^{St}$ is asymmetric and has an extending tail towards larger values. This observation is likely due to the different sizes of platelets. In fact, looking at $F_{tot}^{St}$ plotted against the time-averaged cell area $\bar{A}$ in the steady state (Fig. 3A), we see that larger platelets indeed display larger total forces. To obtain a parameter that is independent of the cell area,
we also plot for each cell the steady state value $T^S$ of the traction force $\langle |\vec{T}| \rangle_A$ averaged over the cell area $A$ (Fig. 3B). The ensemble-average is $\langle T^S \rangle_N \sim 3.3 \cdot 10^3$ Pa.

Spatial distribution of contractile forces

Analyzing the movies of traction forces $\vec{T}(\vec{x}, t)$ of individual cells, we observe that in the steady state, forces are largest at the periphery of the cell, while the focus of the traction force fields is near the center of the cell area. To quantitatively describe the spatial distribution of the traction force fields we analyze the divergence minima $\min_{\vec{x}}(\nabla \vec{T}(\vec{x}))$ after noise reduction for each individual time frame. For each time frame in the steady state we plot the position of $\min_{\vec{x}}(\nabla \vec{T}(\vec{x}))$ relative to the center of the cell area ($x_1 = 0, x_2 = 0$). The graphs we thus obtain for each cell, however, differ visibly (see Fig. 4A, B). In some cases, the position of $\min_{\vec{x}}(\nabla \vec{T}(\vec{x}))$ scatters only little in time around its average position. In other cases, the point $\min_{\vec{x}}(\nabla \vec{T}(\vec{x}))$ moves in time on a circle or semicircle around the center of the cell area. Consequently, we consider the average distance $\bar{d}$ of $\min_{\vec{x}}(\nabla \vec{T}(\vec{x}))$ to the center of the cell area in the steady state to represent platelet contraction. For each cell, we normalize this average distance $\bar{d}$ by the radius $R$ of the average cell area and compare the normalized values. The radius $R$ we obtain by calculating the time-average $\bar{I}$ of the binarized images $I(t)$ of the single cells in the steady state, performing thresholding and determining the mean distance between the center of the cell area and the cell contour in the resulting binarized image. The final histogram of normalized distances $\bar{d}/R$ is displayed in Fig. 4D. It has a peak below 0.5, showing that on average contractile forces in platelets point inwardly toward a focus near the center of the cell area. For comparison a circle with radius $R$ is also included in Fig. 4A and B. Note, however, that this does not denote the actual cell outline but rather gives an idea of an average (temporal and spatial) cell size.

The movies of traction forces $\vec{T}(\vec{x}, t)$ from individual cells further indicate that the force fields generated by contracting platelets are nearly isotropic. We therefore approximate the force fields as an overlay of two force dipoles and look at the dipole moments $D_{\pm}(t)$ of the major and minor axis of contraction to describe the degree of anisotropy of the fields. For each cell, we fit a constant to the steady state regime in the time tracks of $D_{\pm}$. As an example,
figure 5 shows the histogram summarizing the steady state values, $D_{-St}^{sl}$, for the minor contractile axis. We find again an asymmetric distribution, here with an average of $\langle D_{-St}^{sl} \rangle_{N} \sim -20 \text{nN} \cdot \mu \text{m}$. The extending tail to larger values of $|D_{-St}^{sl}|$ again correlates with the cell area. Interestingly, when calculating the ratio between the steady state force dipole moment of major $\left( D_{+St}^{sl} \right)$ and minor $\left( D_{-St}^{sl} \right)$ contractile axis for each cell, we obtain a histogram with a prominent peak around 1.25. This confirms that the platelets pull nearly isotropically towards the center of the cell area: the difference between major and minor axis of contraction is small.

Discussion

We study cellular contraction on the example of human blood platelets using traction force microscopy (TFM) with the aim to conclude upon mechanical principles of force generation and transduction. In particular, our goal is to identify features that platelets share with other cell types generating contractile forces.

Contractile forces and force generating mechanisms in single platelets

We measure an average total contractile force in the steady state of 34 nN, which corresponds well to a previous report on single platelet experiments, where a force of 29 nN (Lam et al., 2011) was obtained from AFM measurements. By contrast, compared to previous studies on cell aggregates our values for the total contractile forces per platelet are at least one order of magnitude higher. For example, rheology measurements on clot contraction yield a maximum contractile force per platelet of $\sim 0.5 \text{nN}$ (Jen and McIntire, 1982). Even force measurements on smaller cell ensembles, namely microclots on elastic pillars, display contractile forces per platelet as low as 2.1 nN (Liang et al., 2010). This discrepancy is all the more striking, if we consider that the traction forces we measure with TFM are, if biased at all, an underestimate. If distant neighboring cells pull on the substrate in opposite directions to the cell under consideration, the substrate deformation and hence the traction forces appear reduced. It has to be taken into account, however, that a direct comparison between single platelets and platelets within clots in vitro or even in vivo is not possible. In vivo, platelets at the bottom of the clot, which adhere first, are bound to von-Willebrand-factor and collagen; fibrinogen is incorporated in the thrombus as well. Comparing the forces that we measure for individual platelets with the force per platelet from clot measurements can therefore only be an estimate.
One possible explanation for the discrepancy between our results and results from experiments on cell aggregates is that the aforementioned experiments on cell aggregates probe contraction only along selected directions. As our measurements show, however, platelet contraction is nearly isotropic. Consequently, all contractile forces along the directions that are not measured on cell aggregates are not included in the total contractile force per platelet. Possibly, this fact also explains why our single cell experiments using TFM yield slightly higher forces compared to Lam et al.'s single cell approach with AFM, which is restricted to uniaxial contraction. Importantly, the difference between our results and the AFM results are small compared to the differences between single cell and cell aggregate measurements. Therefore, it seems likely that the main reason for the difference in the total forces is due to complex platelet interaction, where forces generated by single platelets may add up nonlinearly to the overall force of the cell aggregate.

The question arises whether platelets are able to generate contractile forces as high as 34 nN with regard to their contractile apparatus. It is generally believed that actomyosin is the force generator in platelets (Jen and McIntire, 1982; Léon, 2007; Pollard et al., 1977). A single platelet contains on average 12,000 myosin II motors (Michelson, 2007), each of which can effectively produce in vivo about 1.3 pN per myosin head (Finer et al., 1994). Thus, depending on the synchronized action of the myosin heads, the total force that can be generated by actomyosin is 15.6 - 31.2 nN, which is in good agreement with our results. In fact, for isolated myosin motors forces as high as 7 pN were measured (Finer et al., 1994), leading to an estimate for the upper limit of the total forces of 84 nN. Our results are below this upper limit and we conclude that our measured total forces are feasible for the contractile apparatus of platelets.

Although actomyosin contraction seems to be the main force generating mechanism in platelets, additional contributions to force generation cannot be ruled out. These contributions possibly originate from mechanisms similar to those that lead to protrusion forces in migrating cells (Li et al., 2005): actomyosin contraction may induce an internal plasma flow in the cell pointing inwardly, which may in turn enhance contraction. Another contribution to the total traction force might arise from actin depolymerization at the cell center producing forces in a manner resembling the thermal ratchet models for actin polymerization (Peskin et al., 1993; Mogilner and Oster, 2003). The significance of these mechanisms for platelets is still unclear but with regard to the movies taken during TFM measurements, we assume that
at least intracellular plasma flow may contribute to the high contractile forces which we measure.

Principles of cellular contraction

We find that platelets exhibit high traction forces of \(~ 3.3 \cdot 10^3\) Pa in the steady state. Compared to fibroblasts (Dembo and Wang, 1999; Munevar et al., 2001; Rajagopalan et al., 2004), fish keratocytes (Oliver et al., 1999) and airway smooth muscle cells (Tolic-Norrelykke and Wang, 2005) generating traction forces of 0.8-3.03 \(\cdot 10^3\) Pa, \(10^2\) Pa and again \(10^2\) Pa, respectively, platelets are among the strongest cells measured by TFM. Although actomyosin is recognized as the main generator for contractile forces in all of the above mentioned cell types (Li et al., 2005; Gunst and Tang, 2000; Pollard et al., 1977), different mechanisms of actomyosin rearrangement may account for the differences in traction forces and, not surprisingly, the arrangement of the force fields. According to our findings, stationary platelets generate isotropic contractile force fields. By contrast, migrating cells such as fibroblasts and fish keratocytes as well as stationary, contractile smooth muscle cells generate polarized force fields (Munevar et al., 2001; Oliver et al., 1999; Tolic-Norrelykke and Wang, 2005). We assume that actomyosin in platelets on soft substrates reorganizes into more isotropic patterns compared to migrating cells and stationary smooth muscle cells that have a clear direction of motion or action.

Interestingly, we reveal that contraction forces are strongest at the edge of the platelet body - a phenomenon shared with other cells types (Munevar et al., 2001; Oliver et al., 1999; Tolic-Norrelykke and Wang, 2005). We conclude that the reorganization of focal adhesion sites and cytoskeletal structures in cells is driven by general principles, although differing in details. Regarding the role of platelets in primary haemostasis it makes sense that they pull nearly isotropically to homogeneously reduce clot size during clot retraction.

Clinical applications

Besides improving the general understanding of contractile force generation and transduction in cells, TFM studies may finally lead to new concepts of platelet function. The method may also become a useful new tool to study platelet function in patients. We observe that contractile forces of platelets reach a steady state after 25 min (see Fig. 2), which is strikingly similar to the time reported on clot retraction (Carr and Zeckert, 1991; Carr, 2003; Carr et al., 2007). Therefore the contractile forces measured with TFM may reflect platelet function
during primary haemostasis. Currently, platelet function is mainly examined using semi-quantitative aggregometric or flowcytometric methods revealing the potential to aggregate or the expression of platelet-specific antigens before and after activation in vitro. Platelet dysfunction is difficult to diagnose with the current methods as there is still a considerable lack of standardization and well-established cut-off values with these methods. The measurement of contractile forces with TFM could become a promising quantitative approach to screen for both inherited diseases with platelet dysfunction such as Glanzmann’s thrombastenia or sticky platelet syndrome and acquired platelet dysfunction including the anti-platelet effect of drugs such as cyclooxygenase-2 (COX-2) inhibitors (Aspirin) or thienopyridins (Clopidogrel, Prasugrel). For blood banks, another interesting application of TFM could be the quality control of platelet concentrates since there is no single test available so far to accurately assess the quality of platelet concentrates after production or after storage. We believe that further studies based on TFM of platelets will provide deep insight in the physiology and pathophysiology of platelets including adhesion, activation, shape change, aggregation and clot retraction and we see potential chances for the clinical application of TFM at least in specialized laboratories.

Concluding remarks

In conclusion, our results show that human blood platelets are strong cells with total contractile forces in the range of 34 nN which is significantly higher than for experiments on cell aggregates and demonstrates the importance of the single cell approach. Future experiments on small clusters of a few platelets could show how exactly platelets influence each other and shed light on this interesting point. Platelets are particular as a contractile system with regard to the near isotropy of traction force fields measured, where the focus of the force field is a point near the center of the cell area. It is well possible that these force fields adapt to shear stress in the blood flow; combining TFM with microfluidics might be a suitable approach to study such influences (Das et al, 2008). Average traction forces are on the order of $3.3 \cdot 10^3$ Pa and are therefore higher than for migrating fish keratocytes or stationary contractile smooth muscle cells, albeit in the same range as for migrating fibroblasts. Considering these differences in traction forces, we conclude that the mechanism to generate traction forces may depend on the cell type, possibly involving fluid flows and cytoskeletal disassembly processes apart from the actomyosin mechanism. Interestingly, it has been found that myosin contractility is required for maintenance of platelet structure during
spreading (Calaminus et al, 2007). Future experiments, investigating the role of actomyosin in platelets, could be performed by adding blebbistatin or the rho kinase inhibitor Y27632. Such experiments may shed light on the question if actomyosin is the only force generating mechanisms in platelets and if not, to which extend it is involved.

However different the magnitude of traction forces are, we find that platelets share common traits with other cells: traction forces are higher along the edge of the cell body, similar to previous TFM studies on fibroblasts, fish keratocytes and airway smooth muscle cells (Munevar et al., 2001; Oliver et al., 1999; Tolic-Norrelykke and Wang, 2005). This indicates that force transduction in cellular contraction is driven by general principles and confirms previous results that focal adhesion sites for platelets are denser at the edge of the cell (Nachmias and Golla, 1991).

Materials and Methods

Subjects and platelet isolation

Experiments were conducted in agreement with national and regional laws and according to the ethical vote of the Ethic Committee of the University of Göttingen, votum 11/11/09. Platelets were collected from healthy, volunteer donors with a negative history for anti-platelet or pain killer medication for at least 7 days prior to donation at the Blood Donation Center of the University Clinic of Göttingen. Platelets were harvested using the apheresis system of Trima Accel (Caridian BCT Europe, Garching, Germany) or prepared from buffy coats pooled from 4 donors with the same AB0 blood group using Fenwal blood donation bags with CPD stabilizer and Fenwal OptiPress II (Fenwal Europe SPRL, Mont-Saint-Guibert, Belgium). Platelet isolation from leucocyte-depleted platelet concentrates and TFM experiments were performed between day 4 and 7 after donation. To isolate platelets, we added aggregation-inhibiting Prostaglandin PGE$_1$ at a final concentration of 2.7 µg/ml (Cayman Chemical Company, Ann Arbor, MI, USA) and performed 3 centrifugation steps of 20 min at 480 x g and 21 °C. Between centrifugation steps we resuspended the pellets in warm Pipes-Saline-Glucose (PSG; 37 °C) to which we added PGE$_1$ as before. After the final centrifugation step we resuspended the pellet in warm medium M199 (37 °C; Lonza, Basel, Switzerland) and diluted the cell suspension to final platelet counts of 5-9 · 10$^7$ cells/ml.

Preparation and characterization of substrates
Polyacrylamide (PAA) substrates were prepared according to references (Rajagopalan et al., 2004) and (Brandley and Schnaar, 1988). Briefly, a polymerization solution was mixed containing 8% monomeric acrylamide (Bio-Rad, Hercules, CA, USA); 0.04% crosslinker bis-acrylamide (Bio-Rad); 50 mM HEPES; 13.4 mM catalyzer TEMED (Bio-Rad), 10 mM acrylic acid NHS-ester (Sigma-Aldrich, St. Louis, MO, USA), 2 µl/ml red fluorescent marker beads (0.1 µm diameter, 2% solids, excitation 580 nm, emission 605 nm; Invitrogen, Carlsbad, CA, USA) and 0.05% ammonium persulfate to initiate polymerization. 5 µl-drops of the solution were added to coverslips that were pretreated with 3-aminopropyltrimethoxy silane and glutaraldehyde (Dembo and Wang, 1999) and the drops were covered by round cover slip (10 mm diameter) to obtain flat substrates. After one hour of polymerization, we removed the round coverslips and covalently bound a fibrinogen protein layer to the surface of the substrate via the NHS ester by incubation at 4 °C overnight with 0.1 mg/ml protein solution (Calbiochem - Merck KGaA, Darmstadt, Germany; human plasma). The remaining hydrolyzed NHS-ester was removed with 50 mM ethanolamine solution, which was left on the substrates for one hour at room temperature. Substrates were stored in MilliQ and washed 3 times with cell medium M199 (Lonza) shortly before the experiments.

Substrate elasticity was measured using a Physica MCR 501 rheometer (Anton Paar, Graz, Austria). The polymerization process was recorded over 2 hours using a flat top plate (25 mm diameter) at a frequency of 1 Hz and a deformation amplitude of 1%. Following the time record, a frequency sweep from 0.01 Hz to 100 Hz at 1% amplitude was performed. Finally, an amplitude sweep from 0.01% to 500% at 1 Hz was performed. Fitting the linear regions in all three measurements yielded an elastic modulus of 4 kPa for our substrates.

Traction force microscopy experiments and data analysis

Forces per unit area were measured using TFM. We placed platelets on elastic PAA substrates in a stage top incubator (Tokai Hit, Shizuoka-ken, Japan) with a humid atmosphere containing 5% CO₂ at 37 °C. Platelet contraction was visualized via fluorescent beads in the substrates, where the bead density determined the spatial resolution (~0.8 µm). The beads were then traced over time by fluorescence microscopy at 100x (Olympus UPLFLN 100xO2PH: phase contrast, oil immersion; NA = 1.3) magnification using an inverted light microscope (Olympus IX 81) equipped with a highly sensitive Hamamatsu ORCA-R2 CCD camera (Hamamatsu, Herrsching am Ammersee, Germany). Simultaneously, we recorded phase contrast images to obtain the cellular boundary and identify the cell-substrate contact area.
For image acquisition and microscope control we used the Olympus CellR software. From the information on bead displacements $\mathbf{d}(x')$, the cell-substrate contact area $A$ and the elastic properties of the substrate, we calculated the traction forces $\mathbf{T}(\mathbf{x})$ based on linear elasticity theory by solving the following inversion problem (Dembo and Wang, 1999): 

$$
d_j(x') = \int_A G_{ij}(x' - x) T_i(x) \, dx_1 \, dx_2
$$

where the Green's function $G_{ij}$ was taken to be Boussinesq's solution for semiinfinite, elastic media (Landau and Lifshitz, 1986). Traction force fields were calculated for each frame in a total record of 60-92 min with 10 s intervals between consecutive images.

Data analysis comprised three steps: Bead traction, cell contour tracing and calculation of traction forces. Traction forces were calculated by the program LIBTRC kindly supplied by Micah Dembo (version 2.4). This program received input from the bead traction and cell contour tracing performed by MATLAB scripts (version 7.9.0.529 (R2009b) with Image Processing Toolbox; The MathWorks Inc.). Before the images were processed, however, we corrected both fluorescence and phase contrast images for variations in illumination by a high pass filter. We then adjusted the contrast and reduced noise by a Gaussian filter, a median filter and a Wiener filter.

For the bead traction, we used a particle tracking algorithm, where the beads were identified among the local intensity maxima in a frame $i$ according to projection lines of the bead motion in frame $i-1$. We opted for this approach rather than using a correlation technique, because for platelets the bead patterns change too rapidly to be reliably traced over time. Calculated bead displacements were corrected for an overall drift in the images by subtracting the maximum of the histogram of bead displacements. Following bead traction, we performed cell segmentation to obtain the cell contour from the phase contrast images. For that purpose, we used both thresholding and a Laplacian filter in combination with again a high pass filter and a selective mean filter (Sigma Filter Plus plugin for ImageJ). Unconnected pseudopodia were linked with the cell body by a final dilation erosion operation in MATLAB. In cases where the bead traction or the cell contour tracing failed, manual corrections were carried out.

Furthermore, the cell contour of each time frame was used to derive the time track of the cell centroid position and therefore to determine the time of adhesion of the cell. To that effect, the distance curve of the cell centroid to its initial position was fitted to an exponential function $y(t) = a(1 - e^{-bt})$. The time of adhesion was taken to be the time, where the difference between fit and saturation value $a$ was on the order of the fit residues and set as $t = 0$ s.
From the information on bead displacements and the cell-substrate contact area $A$ obtained from the cell contour, we calculated traction force maps $\mathbf{T}(\mathbf{x})$ for each time frame using LIBTRC, which additionally provides the mean traction force

$$\left\langle \frac{\mathbf{F}}{dA} \right\rangle_A = \left\langle \mathbf{F} \right\rangle_A = \frac{1}{A} \int_A \mathbf{T}(\mathbf{x}) \, dA$$

the total force

$$F_{\text{tot}} = \int_A |\mathbf{T}(\mathbf{x})| \, dA$$

and the force dipole moments $D_\pm$ of the major and minor axis of contraction as the Eigen values of the matrix $M$

$$M_{ij} = \frac{1}{2} \int_A (x_i - x_a)(T_j - \langle T_j \rangle_A) \, dA + \frac{1}{2} \int_A (x_j - x_a)(T_i - \langle T_i \rangle_A) \, dA$$

where $i=1,2$ and $(x_{c1},x_{c2})$ is the cell centroid position.

Acknowledgments

The authors thank Florian Rehfeldt and Christina Jayachandran of the Third Institute of Physics at the University of Göttingen for their help with the rheology measurements and Jens Nolting of the Department of X-Ray Physics for technical assistance. The LIBTRC program was kindly provided by Micah Dembo from Boston University (MA, USA). We would like to thank Joachim Riggert and Tobias Legler from the University Clinic Göttingen for providing us with platelet concentrates and for fruitful discussions of our results. This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG) in the framework of SFB 937 and the Excellence Initiative and by a doctoral fellowship to Sarah Schwarz Henriques by the International Max Planck Research School for Physics of Biological and Complex Systems in Göttingen.

References


Figure legends

Figure 1: Sketch delineating the steps involved in data analysis: Phase contrast and fluorescence images are taken simultaneously during TFM recording over a time period of 60-92 min. Bead displacement maps are calculated from the marker beads in the fluorescence images by a particle tracking approach, where each bead in a frame \( i \) (empty, dotted circles) is searched for among the local maxima in a frame \( i \) (full circles) along the projected lines of motion known from frame \( (i-1) \). In parallel, the cell contour is extracted from the phase contrast images by performing cell segmentation with a combination of a Laplacian filter and thresholding. From the bead displacement maps traction forces are finally calculated for the cell area \( A \) known from the cell contour (contour drawn manually in this example – see figure S1 in the supplementary material). All scale bars represent 2 µm.

Figure 2: Platelets reach a steady state and display large contractile forces: A: Time course of the total force \( F_{tot} \) generated by a single blood platelet after cell adhesion to a polyacrylamide substrate plotted on a logarithmic scale for a typical traction force microscopy data set. Linear fits to both force increase region (< 1798 s) and steady state region (≥ 1798 s) are displayed as solid black lines. From the linear fits, the turn point \( t_{tp} \) between the two regions of force generation and the total steady state force \( F_{tot}^{st} \) are obtained. The inset and panel B show the statistical distribution of values for \( F_{tot}^{st} \) and \( t_{tp} \). (n=14 cells.)

Figure 3: Total contractile force is dependent on cell area: A: Steady state total force \( F_{tot}^{st} \) generated by individual blood platelets (error bars show the fit error) plotted against the time-averaged cell area \( \overline{A} \) (s.e.m. is smaller than the symbols). B: Distribution of the traction force \( T^{st} \) averaged over the cell area \( A \) at the steady state. (n=14 cells.)

Figure 4: Platelets pull toward a point near the center of the cell area: A, B: Two examples of the position of the divergence minimum \( \min_x \left( \nabla \cdot \overline{T}(\hat{x}) \right) \) for each time frame of the movie with respect to the center of the cell area \((x=0,y=0)\) at the steady state after noise reduction, C: Typical image of the steady state traction force field \( \overline{T}(\hat{x}) \), where the reference arrow shown in the bottom left corner represents 10 kPa, D: Distribution of the average...
distance $\overrightarrow{d}$ for $\overrightarrow{T}(\bar{x})$ relative to the center of the cell area at the steady state. $\overrightarrow{d}$ is normalized for each cell by the average distance $R$ between the center of the cell area and the cell contour. (n=14 cells.) Scale bar in C: 2 µm.

Figure 5: **Platelets pull nearly isotropically, the difference between major and minor contractile axis is small**. Distribution of the force dipole moment $D_{St}$ corresponding to the minor axis of contraction in the steady state extracted from traction force maps $\overrightarrow{T}(\bar{x},t)$. The inset shows a histogram for the ratio between the force dipole moments of the major ($D_{St}^{+}$) and minor contractile axis ($D_{St}^{-}$) at the steady state for the individual cells. (n=14 cells.)
fluorescence images → bead traction → bead displacement maps → traction force map

phase contrast images → cell segmentation → Laplacian thresholding → cell contour

Projected line of motion
A

$t_{tp}$

force increase

steady state

$F_{tot}^{St}$ [nN]

$0$ $10$ $100$ $1000$

t [s]

$0$ $1000$ $2000$ $3000$ $4000$ $5000$

$F_{tot}^{St}$ [nN]

$# cells$

$0$ $20$ $40$ $60$ $80$

B

$# cells$

$0$ $2$ $4$ $6$

$0$ $1000$ $2000$ $3000$ $4000$ $5000$

t [$s$]
A

![Graph showing the relationship between $F_{SI}$ and $A_{\text{cell}}$.](graph_A)

$F_{SI}$ [nN]

$A_{\text{cell}}$ [µm²]

B

![Histogram showing the distribution of $T_{SI}$ for different groups of cells.](graph_B)

$T_{SI}$ [Pa]

# cells

<table>
<thead>
<tr>
<th>$T_{SI}$ [Pa]</th>
<th>Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2000</td>
<td>2</td>
</tr>
<tr>
<td>2000-4000</td>
<td>4</td>
</tr>
<tr>
<td>4000-6000</td>
<td>2</td>
</tr>
<tr>
<td>6000-8000</td>
<td>1</td>
</tr>
</tbody>
</table>
Supplementary Material

Supplementary material consists of one movie, which has a reproduction time that is 100 x (real time), and one figure illustrating how cell contours were traced during data analysis. The movie is encoded with the Xvid codec and displayed vector arrow heads were scaled with the MATLAB function adjust_quiver_arrowhead_size.m by Kevin J. Delaney (BMT Scientific Marine Services Inc, Escondido, CA, USA).

Movie 1: Evolution of traction forces. Phase contrast images (taken during a traction force microscopy (TFM) recording of human blood platelet activation and contraction) are shown together with the traction forces (cyan arrows) that the platelet generates. Traction forces were calculated for the cell area (enclosed by manually drawn blue line – see figure S1) based on the substrate deformation caused by platelet contraction and using linear elasticity theory. To better display the force fields, the images were magnified by a factor of ~ 2.7. The time \( t \) during platelet activation is shown in blue, where \( t = 0 \) s marks the point of adhesion of the platelet to the substrate. A reference arrow is displayed in the upper left corner representing 10 kPa. For reasons of exceeding movie size, we have cut out the last few frames of the steady state traction force fields and show only the first 2/3 of the TFM recording, which, however, includes the main features of traction force field evolution and spatial distribution.

Figure S1: Sketch of cell contour tracing during data analysis: Traction force calculation for a TFM data set is restricted to the cell area known from the cell contours, which are determined prior to force calculation. For the data set in Movie 1 of the supplementary material the cell contours were determined manually for each of the 471 phase contrast images of the corresponding TFM recording. For that purpose, each frame \( i \) was compared both to the previous frame \( i-1 \) and the following frame \( i+1 \), to better estimate pseudopod positions and to reduce the influence of noise (see left-hand side of figure). Due to the time consuming nature of the procedure, however, the cell contours in all the other TFM data sets (total sum of frames for all 14 cells that were evaluated: 6007) were analyzed automatically by a self-written MATLAB script with a few manual corrections where the automatic contour tracing failed. The script initially removes noise with a median, a Wiener and a Gaussian filter. Then it uses a Laplacian filter on each phase contrast image and combines the result with the binarized phase contrast image \( BI \) obtained from thresholding (see right-hand side of figure). In a final step, the cell body of the binarized image \( BI \) is dilated to find hitherto
unconnected pseudopods in the Laplacian-filtered image and these pseudopods are finally connected to the undilated cell body by morphological closing. To estimate how efficient the automatic cell contour tracing is, the script was also used for the data set in Movie 1: figure S1 compares the result for both the automatic and manual contour tracing for the phase contrast image shown at the top. All images have the same size and the scale bar in the top image corresponds to 2 µm. In the final result of calculated steady state traction forces $T_{St}^s$, total forces $F_{tot}^s$, force dipole moment $D_{St}^s$ and ratio between force dipole moments $D_{+St}^s / D_{-St}^s$, we find that for the TFM data set shown here the automatic contour tracing has a deviation relative to the manual contour tracing of $\Delta T_{St} / T_{St} = +6.4\%$, $\Delta F_{tot}^s / F_{tot}^s = -12.8 \%$, $\Delta D_{-St}^s / D_{-St}^s = +21.2\%$ and $\Delta(D_{+St}^s / D_{-St}^s) / (D_{+St}^s / D_{-St}^s) = -1.4\%$, respectively. We estimate that for all our other TFM data sets the errors introduced by the automatic contour tracing are of the same order of magnitude.
phase contrast image → automated contour tracing + Laplacian thresholding → final cell contours

manually traced contour → automated contour tracing → final cell contours