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SUPPLEMENTARY INFORMATION

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# Mechanics and contraction dynamics of single platelets and implications for clot stiffening

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## Supplementary Information

### Materials and detailed methods

Platelet isolation and labeling – Venus blood from healthy volunteers was drawn into ACD tubes (Becton-Dickinson) using a research protocol approved by the institutional review board of the University of California Berkeley. Platelet-rich plasma (PRP) was then obtained after centrifugation at 150 rpm for 15 min. PRP was then centrifuged at 900 rpm for 5 min, and the overlying platelet-poor plasma was then removed from the platelet pellet. Platelets were re-suspended in Hepes-Tyrodes buffer (138 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 5.5 mM glucose, 10 mM Hepes, 0.1% BSA, pH 7.4) at a cell density of $1\times10^6$/ml. Platelets were then fluorescently labeled with 5 µg/mL of CellMask Orange (Invitrogen) and were used for experiments within 3 hours of the blood draw. Immediately prior to experimentation, 1 mM $CaCl_2$, 1mM $MgCl_2$, and 1 U/mL of thrombin were added to the platelet suspension. For control experiments, 80 µM blebbistatin(-) (Sigma-Aldrich) was used to inhibit platelet myosin II and 2.4 µM eptifibatide (generously donated by Drs. Stephens, Andre, and Phillips of Portola Pharmaceuticals) was used to block the platelet glycoprotein IIb/IIIa receptor (integrin $\alpha_{IIb}\beta_3$).

AFM experiment setup and protocol – We used the side-view atomic force microscope to conduct experiments; instrument details can be found in Chaudhuri, et al., Nature Methods, 2009. Tipless gold-coated silicon nitride cantilevers were used for these experiments (MLCT-O, Veeco). The gold coating was removed from the silicon nitride cantilevers by brief immersion in aqua regia (3:1 mixture of HCL to $HNO_3$). Cantilevers and glass surfaces were piranha cleaned (3:1 mixture of $H_2SO_4$ to $H_2O_2$) and then incubated in 20 µg/ml fibrinogen (Sigma-Aldrich), or for experiments involving

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collagen, 20 μg/ml type I collagen (Chrono-Log), for 30 minutes at 37°C and then washed in degassed phosphate buffered saline before the experiment. Platelets remained adhered on the surface or cantilever in roughly equal proportions at the end of experiments suggesting that fibrinogen adsorption to the two surfaces was roughly similar. After immersing the chamber in the solution containing the platelets, the chamber was allowed to equilibrate for 5 – 10 minutes at room temperature. Then platelets near the surface, but still in suspension, were located. The platelets were positioned below the cantilever by moving the surface, and then the surface was stepped towards the cantilever in 1 μm increments until contact was made simultaneously between the platelet and both the cantilever and surface (See Supplementary Video #4). The platelet was then either allowed to freely contract and pull the cantilever towards the surface until contraction stalled or the isometric clamp was turned on after the platelet had contracted the cantilever ~150 nm below the zero deflection point. Images were taken every 30s to 60s and used to confirm contraction visually. After platelet contraction had remained stalled for ~5 min, the surface was ramped away from the cantilever at a speed of 500 nm/sec or 1,000 nm/sec to calculate the elasticity, extensibility, and adhesion force.

For each experiment, a single cantilever was exposed to only a single platelet in a given suspension. As each cantilever chip contains several cantilevers, an additional platelet measurement may be conducted before the majority of platelets settle on the glass surface, which prevents further experimentation. Therefore, typically 1, but at most 2, platelet experiments (each with a different cantilever) were conducted for each fibrinogen-coated cantilever/glass surface pair, which translates to 1 to 4 platelet experiments per blood draw.

**Control experiments –** Control experiments were carried out to confirm normal platelet behavior in the AFM experiments. Platelet contraction is known to be a calcium-dependent process involving the integrin receptor for fibrin/fibrinogen (integrin $\alpha_{IIb}\beta_3$, a.k.a glycoprotein IIb/IIIa), which, via downstream outside-in integrin signaling, activates platelet cytoskeletal reorganization and sliding of myosin over actin filaments, among other processes (Coller and Shattil, Blood, 2008). To that end, we verified that contraction was completely inhibited in platelet suspensions without calcium or with the myosin II inhibitor blebbistatin and the platelet glycoprotein IIb/IIIa receptor antagonist eptifibatide. We found that

significant platelet adhesion, but no contraction, occurred with collagen-coated cantilevers and glass surfaces. Finally, use of cantilevers and glass surfaces coated with a thin fibrin gel revealed comparable platelet contraction to fibrinogen-coated surfaces. Supplementary Figure 4 represents a summary of all platelet contraction experiments including controls. In addition, pre-activation with thrombin may lead to myosin activation and in turn, platelet contraction, before AFM measurements can be made. To investigate the pre-activation of platelets before they were measured in the AFM, we conducted a subset of our experiments using a cell-permeant fluorescent indicator of cellular calcium mobilization and signaling (Fluo-4 AM, Invitrogen) as a marker of platelet activation. The major pathway of myosin activation in platelets is through calcium-mediated pathways and activation occurs at times of maximal calcium mobilization (Adelstein et al. Nature 1975, Takashima et al. J. Biochem 1988, Hartwig, Platelets 2007). Despite exposure to thrombin, platelets did not intensely fluoresce until contact with the fibrinogen-coated AFM cantilever and glass surface, and the average intensity of fluorescence increased by a factor of 4.96 ± 2.29 (n = 8) following contact with the cantilever and glass surface (Supplementary Figure 5). As platelet contact occurred, the fluorescence intensity of the constitutive cell membrane dye rose only by a factor of 0.85, likely due to scattering by the silicon nitride cantilever. When normalizing by the membrane dye channel, the fluorescence of the calcium dye increases by a factor of 6.24 upon platelet contact with the fibrinogen-coated surfaces. These data suggest that, in our system and despite exposure to thrombin, peak calcium mobilization occurs only after the platelets come into contact with fibrinogen-coated surfaces and, therefore, peak myosin activation likely occurs after contact as well.

**Data analysis** – For each measurement, the cantilever voltage signal was converted to deflection in Igor Pro 5 (Wavemetrics, Inc) using a voltage-deflection calibration constant that was determined at the beginning of each experiment. Maximum platelet contraction forces and times were selected manually due to some variability in the shape of the trace that precluded fitting the trace to a simple function. Average contraction rate was determined by dividing the maximum contraction force by the time to stall in each experiment. For the elasticity measurements, the stress and strain were calculated for the first 0.5 s of the rampout experiments. This relationship was fit with a line, and the best-fit slope of the line was used for the elasticity. The platelet dimensions were determined from images taken with the side-view imaging path, and the cross section of the platelet was assumed to be a circle in the estimation of platelet cross-sectional area that was needed to

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determine the stress. The adhesion force was defined as the maximum force that was exerted on the platelet during rampout experiments, and the extensibility was defined as the length of the platelet at the adhesion force divided by the initial length of the contracted platelet (determined from the side-view images). Mann-Whitney analyses (SPSS) were used to compare maximum contraction forces and contraction rates between different platelet populations and correlations were performed using Spearman correlations.

**Platelet imaging via confocal microscopy** – To measure the total surface area of contact and to determine platelet morphology in our AFM system, we imaged platelets using an inverted spinning disk confocal microscope (Zeiss Axio Observer) with a 100x 1.25 numerical aperture objective. Briefly, thrombin-activated, fluorescently labeled human platelets (Cellmask, Invitrogen) suspended in Hepes-Tyrodes buffer were positioned between a fibrinogen-coated AFM cantilever chip (Veeco) and fibrinogen-coated glass cover slip atop the microscope stage. Upon platelet adhesion to both surfaces, a z-stack of successive focal planes of the entire platelet was obtained using Metamorph imaging software. Three dimensional reconstructions were then obtained and the surface areas of contact to both the glass and cantilever surfaces were calculated using custom software written in Matlab (Supplementary Figure 1A). In addition, staining with Alexa Fluor 488-phalloidin (Invitrogen), which labels filamentous actin, shows that the majority of the f-actin within the platelet is present within the columnar connection between the two fibrinogen-coated surfaces (Supplementary Figure 1B). This gives further evidence that in our system actomyosin-mediated platelet contraction occurs primarily between AFM cantilever and glass surface.

**Application of external load during platelet contraction** - *In vivo*, platelets and clots are subjected to a wide range of hemodynamic forces as well as forces exerted by nearby tissues during wound healing. To investigate how platelet contraction might respond to these dynamic physical environments and whether platelet contraction can withstand application of an external load, we increased the tension on individual contracting platelets by 3-10 nN. We observed that the platelet contraction persisted despite the increased load and that the contraction process still ceased within 10-15 minutes ($n = 3$), though stall force may be different due to the imposed increase in tension. After contraction stalled, platelets did not contract

further when the tension was reduced on the platelet, supporting the idea that platelet contraction is an irreversible process that occurs over a finite timescale once initiated, possibly due to ATP depletion.

**Estimated load sustained by individual integrin/fibrin pair during platelet contraction** - Upon contact with the fibrinogen-coated AFM cantilever and surface, the platelets only partially spread onto the two surfaces and the morphology changes from spherical to more columnar. From those images, the total surface area of platelet-fibrinogen contact were then calculated based on the cell membrane fluorescent dye: 3.84 ± 0.94 $\mu m^2$ (average contact area at top surface ± SD) + 6.51 ± 0.48 $\mu m^2$ (average contact area at bottom surface ± SD) = 10.35 $\mu m^2$ total surface area of contact, which is 26% of the total surface area of the approximately 40 $\mu m^2$ total area of thrombin-activated platelets (Senis, Blood, 2009). At the time of contact, assuming homogenous distribution of fibrinogen receptors on the cell surface and no redistribution, 26% of the 80,000 fibrinogen receptors on single platelets (Wagner, Blood, 1996) = 20,800 receptors would therefore be expected to be engaged. Each fibrinogen receptor would then carry a maximum load of approximately 0.91 pN (average maximum platelet contraction force - 19 nN / 20,800 receptors) during the contraction process in our system.

**The stiffness of the AFM cantilever presents a physiologically relevant *in vitro* stiffness** – Fibrin polymer networks have a bulk elasticity on the order of hundreds of Pa while the individual fibrin fiber has a bending elasticity on the order of MPa (Collet et al. PNAS 2005). It is not known over what length-scale platelets sense stiffness, but they must be sensing some stiffness in between these two extremes, likely closer to the bulk elasticity level. From the basic definition of elasticity, we can convert the stiffness of the cantilever into an equivalent elasticity as follows

$$E \equiv \frac{\sigma}{\varepsilon} \equiv \frac{F/A}{\Delta L/L} = \frac{k \cdot \Delta L/A}{\Delta L/L} = \frac{k \cdot L}{A} \qquad (1)$$

where $E$ = elasticity, $\sigma$ = stress, $\varepsilon$ = strain, $F$ = force, $A$ = contact area, $L$ = length, $k$ = cantilever stiffness. Using 18 and 43 pN/nm for the cantilever stiffness, 3 $\mu m^2$ for the area, and 2 µm for the platelet length, the equivalent elasticity is between ~10-30 kPa. Similar values for elasticity are calculated using a different method for estimating elasticity from probe stiffness

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(Mitrossilis et al. PNAS 2009). These values are all within the physiological range described above, and therefore the platelet contraction measurements made in our system are comparable to *in vivo* conditions.

**Stiffness dependence of work generated by platelets** – In our system, the external work, the work exerted by the platelet on the fibrinogen-coated cantilever and surface, generated by a platelet scales with force as a function of stiffness by the following equation:

$$W = F \cdot d$$
$$F = k \cdot d$$
$$\therefore W = F^2 / k$$

where $W$ is work, $F$ is force, $d$ is displacement, and $k$ is stiffness. We do not measure internal work, the work that is exerted to reorganize, stretch, and stiffen the platelet cytoskeleton. For a given stiffness cantilever, the external work scales with the square of the load. Supplementary Figure 3 shows how cumulative external platelet work scales with stiffness. Only two cantilever stiffnesses are included, as only internal work is exerted during isometric contraction. We see the same trend between external work generated by the platelet and stiffness of the microenvironment – specifically, work generated by a platelet increases with stiffness of the microenvironment.

We find an external work of ~6 fJ is generated by a single platelet in an environment with an stiffness of 43 pn/nm. Though the working cycle and efficiency of myosin II in platelets have not been directly measured, taking similar numbers from skeletal muscle shows amount of work is reasonable given the timescale of contraction seen here. Assuming that 24 – 38 zJ are generated per myosin stroke, 5 - 20 strokes/ second (based on data from myosin II activity in skeletal muscles from Piazessi et al., *Cell*, 2007), and 12,000 myosins per platelet (Hartwig, *Platelets*, 2006), over a timescale of 500 seconds (the average timescale for contraction to reach completion in platelets), the overall energy potentially generated is 720 fJ – 4600 fJ, or more than 2 orders of magnitude greater than 6 fJ. This large difference may be related to the fact that platelets are less organized than sarcomeres in skeletal muscle, requiring significant internal work to reorganize the platelet cytoskeleton but ultimately resulting in similar stall forces once increasing force aligns the actin fibers within the platelets.

**Platelets-rich clots may be mechanically analogous to particle filled elastomers –** According to our measurements, platelets are two orders of magnitude stiffer than platelet-free clots and one order of magnitude stiffer than platelet-rich clots. The measured high elasticity and large adhesion forces may allow platelets to reinforce the mechanical properties of the clot directly, as in particle filled elastomers. A common example is tire rubber, in which stiff carbon black particles are used to enhance the mechanical properties of the soft rubber matrix (Callister, *Materials Science and Engineering: An Introduction*, 2003). In these composite materials, the embedded particle increases the elasticity of the material overall by directly bearing some of the load based on the particle occupying a certain volume fraction of the material, acting as a multi-point crosslinker in the matrix, and reducing the mobility and deformation of the matrix around each particle (Bergstrom, *Rubber Chemistry and Technology*. 1999) Within this context, the high stiffness and adhesion to the fibrin matrix of the contracted platelet may allow the platelet to directly bear load, restrict local deformations of the fibrin matrix, and serve as a crosslinking center within the fibrin gel.

**Implication of results to the platelet aggregation –** Knowledge of single platelet contraction behavior has potential implications for the physical mechanism of platelet aggregation at the site of vascular injury, which is one of the initial events of hemostasis and occurs even before the formation of the fibrin polymer mesh. Soluble monomeric fibrinogen is the known bridging molecule that mediates these platelet-platelet interactions, and recent work indicates that platelet actomyosin contraction occurs within aggregates even in the primary hemostatic plug (Ono, *Blood*, 2008). Thus the results for single platelet contraction shown here are directly applicable to platelet contraction within platelet aggregates as well. Our data suggests that as platelets aggregate and contraction begins, they will sense the high elasticity of neighboring contracted platelets and rapidly contract to form a stiff and compact aggregate.

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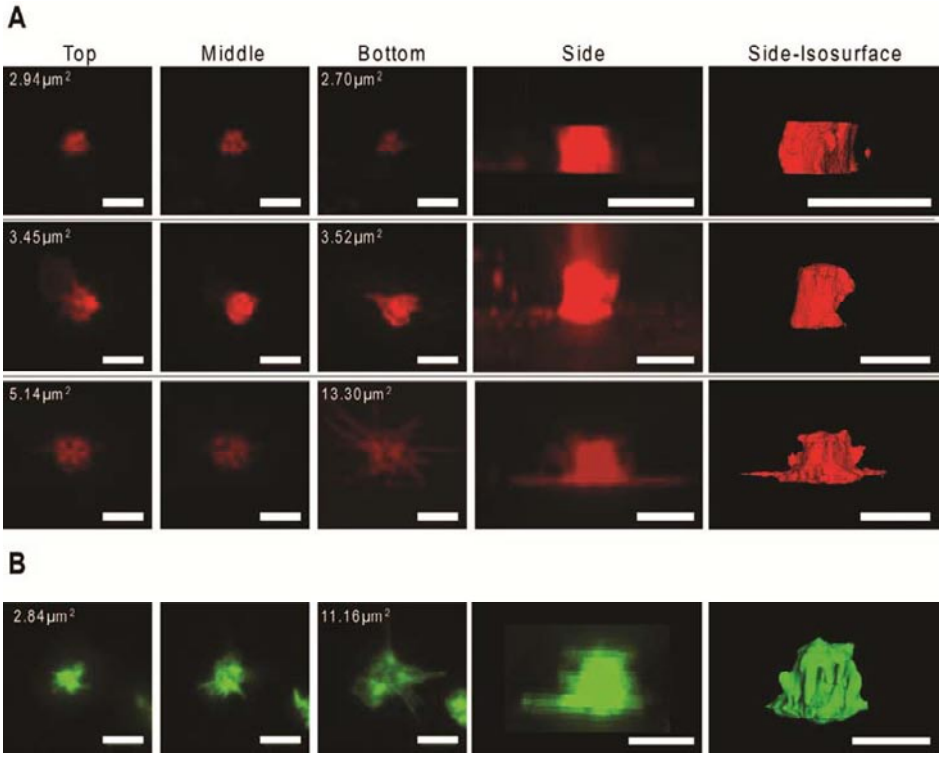
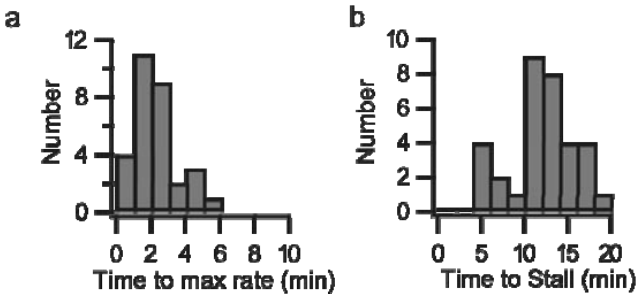
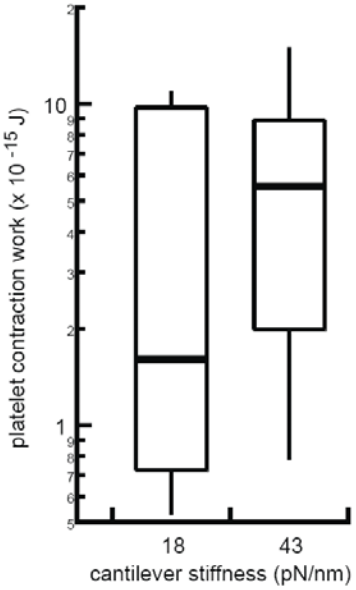
## Supplementary Figures




**Supplementary Figure 1.** High resolution imaging of platelet morphology via confocal microscopy. (A) Different focal planes of z-stacks of three typical thrombin-activated, membrane-labeled platelets positioned between a fibrinogen-coated AFM cantilever and fibrinogen-coated glass cover slip. Each row of images are from the same platelet and selected images include the plane of contact between the platelet and the cantilever (Top), a plane in the middle of the platelet between the two surfaces (Middle) and the plane of contact between the platelet and the glass surface (Bottom). In addition, 3D reconstructions of all planes enabled imaging of the platelet from the side (Side), simulating the platelet morphology visualized in our system. A 3D isosurface rendering of a side-view reconstruction (Side-Isosurface) is also displayed for each platelet. Platelet area in each plane is also displayed. Scalebar = 2 μm (B) Fluorescently-labeled phalloidin staining reveals the structure and morphology of the platelet cytoskeleton in our system.




**Supplementary Figure 2.** Histograms of the timescales for platelet contraction, showing the distributions of times for platelet to reach maximum contraction rates (a) and for platelet contraction to be stalled (b). Contraction rates were obtained from contraction-time curves by using a Savitzky-Golay filter.




**Supplementary Figure 3.** Distribution of work exerted by platelets pulling against cantilevers with stiffnesses of 18 and 43 pN/nm. Medians, quartiles, and 90/10 levels are shown.

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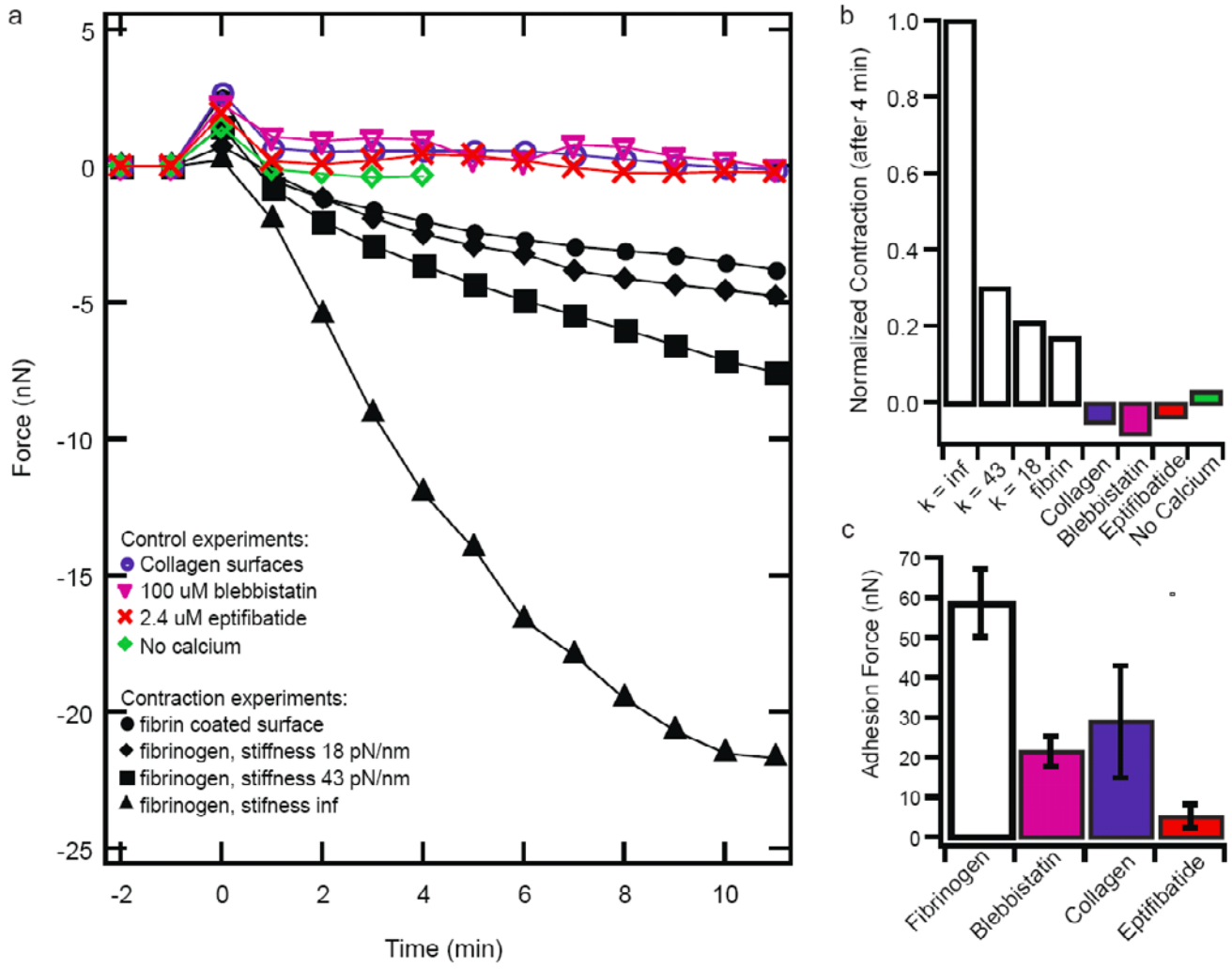
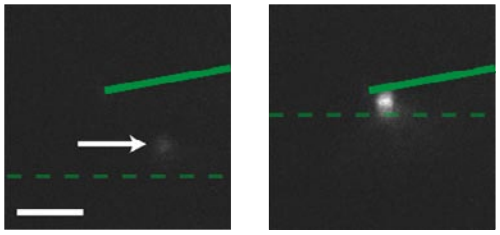



**Supplementary Figure 4.** Control data for platelet contraction and adhesion. (a) Averaged contraction traces for platelets in the indicated conditions. Individual traces were binned to 1 minute time slots and averaged for all traces for cantilever and the glass surface coated with collagen (n = 4), with fibrinogen but the addition of 100 uM blebbistatin (n = 4) or 24 uM eptifibatide (n = 4), Calcium removed (n = 4), or surfaces coated with fibrin (n = 1). Averaged traces from experiments under conditions of different stiffnesses are also shown for comparison. Platelet contraction occurred only when surfaces were coated with fibrinogen/fibrin whereas in all control conditions, the force did not deviate significantly from 0. (b) Relative platelet contraction, which data from (a) is normalized to isometric clamp conditions at 4 minutes. (c) Adhesion

force between the platelet and the cantilever or surface is shown for different conditions indicating that platelet adhesion to fibrinogen-coated surfaces depends on actomysoin and the glycoprotein IIb/IIIa receptor. Error bars indicate standard error of the mean of the population.



**Supplementary Figure 5.** Intracellular calcium dye confirms maximum activation of platelet occurs upon contact with the fibrinogen coated glass surface and the fibrinogen coated cantilever surface. Example sideview AFM images of thrombin-exposed platelets stained with a cell-permeant fluorescent calcium mobilization indicator, before and after contact with the glass surface (indicated by green dotted line) and cantilever surface (indicated by solid green line). Dye intensity increases by a factor of 4.8 +/- 2.0 (n = 8) following contact with the two surfaces. This suggests that, in our system and despite exposure to thrombin, peak calcium mobilization occurs only after the platelets come into contact with fibrinogen-coated surfaces and therefore, peak myosin activation likely occurs after contact as well. Scale bar is 10 μm.

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**Supplementary Video Captions**

Supplementary Video 1 – Typical platelet contraction experiment using a side-view AFM. A fluorescently-labeled human platelet contracts near instantaneously upon contact with a fibrinogen-coated AFM (top) cantilever and fibrinogen-coated glass surface (bottom), pulling the two surfaces together over a time scale of approximately 15 minutes.

Supplementary Video 2 – Platelet contraction under a distance (isometric) clamp. To measure the maximum contraction force of an individual platelet in the sideview AFM system, a feedback algorithm was used that places the platelet under isometric contraction, which is equivalent to an infinitely stiff microenvironment. This algorithm modulates the position of the surface during platelet contraction such that the distance between the cantilever and surface is held constant throughout the experiment.

Supplementary Video 3 – Measurement of single platelet elasticity, extensibility, and adhesion with a sideview AFM. After platelet contraction was completed, the fibrinogen-coated glass surface (bottom) was moved away from the fibrinogen-coated cantilever at a constant rate to determine the elasticity and extensibility of the platelet, as well as the strength of adhesion between the platelet and the fibrinogen-coated surface or cantilever. As the surface was moved away, the force of attachment between the platelet and the surface increased and the platelet elongated.

Supplementary Video 4 – Capturing of platelet floating above glass surface using the side-view AFM. This video depicts the positioning of a fluorescently-labeled human platelet that is observed above a glass surface (reflection of platelet off of glass surface is also seen) just underneath an AFM cantilever. Once platelet is positioned just below the AFM cantilever, the surface is moved into the platelet, initiating contact between the platelet and the two surfaces (the AFM cantilever surface, and the glass surface). The side-view imaging path was essential for this precise control of platelet contact to the two surfaces, and thus activation of the platelet.