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Chapter 2

The nanoscale architecture of force-bearing focal adhesions

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Focal adhesions and forces

abstract

The combination of micropillar array technology to measure cellular traction forces with super-resolution imaging allowed us to obtain cellular traction force maps and simultaneously zoom in on individual focal adhesions with single-molecule accuracy. We achieved a force detection precision of 500 pN simultaneously with a mean single-molecule localization precision of 30 nm. Key to the achievement was a two-step etching process that provided an integrated spacer next to the micropillar array that permitted stable and reproducible observation of cells on micropillars within the short working distance of a high-magnification, high numerical aperture objective. In turn, we used the technology to characterize the super-resolved structure of focal adhesions during force exertion. Live-cell imaging on MCF-7 cells demonstrated the applicability of the inverted configuration of the micropillar arrays to dynamics measurements. The smallest structural features of focal adhesions, however, could not be resolved by diffraction-limited microscopy. Forces emanated from a molecular base that was localized on top of the micropillars. What appeared as a single adhesion in conventional microscopy were in fact multiple elongated adhesions emanating from only a small fraction of the adhesion on the micropillar surface. Focal adhesions were elongated in the direction of local cellular force exertion with structural features of 100-280 nm in 3T3 Fibroblasts and MCF-7 cells. The combined measure of nanoscale architecture and force exerted shows a high level of stress accumulation at a single site of adhesion.
2.1 Introduction

It has come as a surprise recently that cells respond not only to biochemical cues but also to the mechanical properties of their local environment \[1–4\]. Studies showed the stiffness-dependent differentiation of stem cells \[5\], stiffness-directed cell motility (durotaxis) \[6\], and the importance of the environmental mechanical properties in disease like cancer \[7, 8\]. While these phenomena may have different biological relevance, all start at a common origin: the measurement of the mechanical response of the microenvironment performed by the cell, followed by some unexplored mechano-chemical coupling that finally leads to a cellular phenotype.

The cellular structures at which mechanical signals could be measured and analyzed are the focal adhesions (FA). At these sites, the physical connection between the internal contractile cytoskeleton and the extracellular matrix is made through integrin-dimers spanning the cell membrane. On the cytosolic side of the integrins a huge multi-protein complex is formed which attaches to the actin cytoskeleton. The latter forms an active cellular mechanical network contracted by myosin-motor activity. More than 100 proteins have been identified in FAs, that define a biological network with a multitude of interactions \[9\]. Several FA proteins have been suggested to potentially serve as mechano-chemical transducers that alter their biochemical function according to the amount of mechanical force exerted. These mechanosensors include talin \[10\], vinculin \[11\], p130Cas \[12\], zyxin \[13\] and paxillin \[14\]. It has been proposed that upon force exertion on those mechanosensors, specific binding sites become available that promote further biochemical interaction. However, it remains unclear whether the FA complex undergoes enough deformation and force exertion to physically stretch such proteins to perform their mechanosensing activity. We should thus examine on what length scale deformations occur and how much force is carried by FA proteins to enable a comparison to \textit{in vitro} studies. Knowledge about the nanostructured organization of a FA relative to a local site of force exertion has the potential to address these open issues and to provide novel insights about the physical interpretation of local mechanosensory mechanisms.

The lack of knowledge is in part due to a lack of experimental methodology that permits direct measurement of the locally exerted force and simultaneously quantify the local molecular stoichiometry inside a FA complex. Here, we present methodology that combines two high-resolution
optical imaging techniques that enabled us to directly correlate molecular arrangements and cellular forces. In our approach, micropillar array technology was used to quantify sub-nN local cellular forces \[15\] \[17\]. We show the ability to combine this technique with fixed- and live-cell fluorescence microscopy, giving diffraction limited results comparable to previous work \[18\] \[20\]. Simultaneously, we employed super-resolution optical microscopy \[21\] \[23\] with a localization precision of 30 nm to quantify the nanostructure of paxillin and phosphorylated paxillin in focal adhesions.

Prior studies have demonstrated that super-resolution microscopy yields insights into the dynamics and composition of focal adhesion complexes \[24\] \[25\]. To those super-resolution studies we here add the simultaneous readout of cellular forces at high resolution in an inverted micropillar arrangement. First, we show that inversion of the micropillar array to an upside-down configuration on an optical microscope with high-sensitivity multi-color fluorescence imaging capability allows us to accurately measure whole-cell mechanics in both fixed- and live cells. Second, we demonstrate that simultaneous super-resolution microscopy allows us to zoom in onto FAs to generate a molecular density map of phosphorylated paxillin stained by antibodies as well as two-color super-resolution on actin and paxillin. Taken together, our approach provides a sub-nN force precision map of cellular force exertion together with a super-resolved paxillin density map which directly measures the nanoscale architecture of force-bearing focal adhesions. Finally, our measurements demonstrate that multiple small, elongated FAs with dimensions of 100-280 nm carry forces of 10-20 nN, which leads to local stress accumulation up to 300 nN/\(\mu m^2\). Force is thus carried through smaller structures than could be quantified using diffraction-limited microscopy and the diffraction limited stress measured on the same micropillar arrays was an order of magnitude smaller. Quantification of the stress accumulation at a focal adhesion site indeed provides the potential to check whether specific FA proteins can act as mechanosensors.

\[2.2\] Methods

\[2.2.1\] Cell biology

3T3 fibroblasts were cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% calf serum (Thermo Scientific), 2 mM glutamine and 100 \(\mu g/ml\) penicillin/streptomycin. MCF-
7 cells stably expressing a paxillin-GFP fusion construct (a gift from Erik Danen at Leiden University) were cultured in DMEM supplemented with 10% fetal calf serum (Thermo Scientific), 2 mM glutamine and 100 µg/ml penicillin/streptomycin. Cells were seeded at single cell density directly on the micropillar array. Cells were allowed to spread for 8 to 24 hours. Micropillar arrays were subsequently inverted onto #0, 25 mm diameter, round coverslips (Menzel Glaser). The micropillar arrays were kept from floating using a support weight of glass. Live-cell measurements were performed in overnight time-lapse measurements on a confocal spinning-disk setup with a home-built focus-hold system. The temperature was kept at 37 °C with constant 5% CO₂ concentration in a stage-top incubator (Tokai Hit, Japan). Cells were fixed in 4% Paraformaldehyde 16-24 hours after seeding for immunolabeling.

2.2.2 Confocal microscopy

All diffraction-limited fixed and live-cell imaging was performed on a home-built setup based on an Axiovert200 microscope body (Zeiss). An in-house focus-hold system performing feedback on the reflected light from a 850 nm laser-diode at the glass-water interface assured overnight experiments with axial drift <1 µm. Confocal imaging was achieved by means of a spinning disk unit (CSU-X1, Yokogawa). The confocal image was acquired on an emCCD camera (iXon 897, Andor). IQ-software (Andor) was used for basic setup-control and data acquisition. Specifically developed software (Labview, National Instruments) controlled the autofocus and automated XY positioning (Marzhauser XY-stage). Illumination was performed with five different lasers of wavelength 405, 488, 514, 561 and 642 nm (CrystaLaser, Coherent, Cobolt (2x) and Spectra Physics, respectively). Accurately controlled excitation intensity and excitation timing was achieved using an acousto-optic tunable filter (AA Opto-electronics). Light was coupled into the confocal spinning-disk unit by means of a polarization maintaining single-mode fiber.

2.2.3 Force measurement on micropillar arrays

A hexagonal array of poly-di-methyl-siloxane (PDMS, Sylgard 184, Dow Corning) micropillars of 2 µm diameter, 2 µm spacing and with a height of 6.9 µm were produced using replica-molding from a silicon wafer into which the negative of the structure was etched by deep reactive-ion
etching (for details see Supplemental methods). The pillar arrays were flanked by integrated 50 µm high spacers (shown in figure 2.1A and E) such that pillar tops and hence cells attaching to them were within the limited working distance of a high-NA objective (<170 µm) on an inverted microscope. The use of a high-NA objective is a prerequisite for any high-resolution optical imaging. Further, such objectives provide the high collection-efficiency which is essential to super-resolution imaging.

The tops of the micropillars were coated with a mixture of Alexa405-labeled and unlabeled fibronectin (1:5) using micro-contact printing. This approach ensured that cells were solely attached to the tops of the micropillars as confirmed by confocal microscopy (data not shown). Finite element analysis that was fed the exact micropillar dimensions (figure S1) as measured by in-situ scanning electron microscopy (SEM, FEI nanoSEM) allowed us to precisely calibrate the force-deflection relation (for details see Supplemental methods). Pillars on the array had a characteristic spring constant of 16.7 nN/µm. The position of the pillar tops was observed by fluorescence microscopy at 405 nm excitation. From those fluorescence images (figure 2.1B, pillar array without cells) the exact pillar-centroid positions were determined down to 30 nm accuracy using specifically designed software (Matlab, Mathworks). The deflection precision of 30 nm, that is solely limited by the fluorescence signal from an individual pillar, corresponded to a force accuracy of 500 pN (figure 2.1C).

Experimental results are presented with at least 3 independent experiments per graphical representation, performed on at least 4 different cells per experiment. When fitting was performed to results, R² analysis gives the accuracy of the fit. An example of a fixed and dehydrated cell on pillars when imaged in a low-vacuum mode SEM is shown in figure 2.1D. It should be noted that the deflections of the pillars in this image are larger than in live-cell measurements due to the de-hydration procedure needed for electron-microscopy.

2.2.4 Super-resolution imaging on micropillar arrays

The upside-down micropillar approach with integrated spacers and high-NA fluorescence imaging on an inverted microscope allowed us to combine force measurements with super-resolution imaging (for details see Supplemental methods). In brief, we labeled paxillin-GFP in the MCF-7 cells with Alexa647-labeled GFP-traps (Chromotek) and phosphorylated
Inverted micropillars with well-defined spacers provide simultaneous super-resolution fluorescence- and force measurements. (A) Schematic overview of configuration during microscopy (not to scale). With a 100 μm coverslip and 50 μm spacer next to the array, micropillars and cells are within working distance of a high-NA objective. (B) A fluorescence image of the micropillars is analyzed to show the resolution of the deflection field (scalebar in lower left is for the pillar deflections corresponding to 500 nm, lower right is for the fluorescence image corresponding to 10 μm). (C) Histogram of the absolute deflections in (B) shows a mean deflection of 30 nm, giving the one-dimensional localization precision. (D) Scanning electron microscopy (SEM) of a 3T3 fibroblast spread on a micropillar array shows deflected pillars. (E) One 50 μm high spacer - as seen with SEM - flanking the micropillar array. (Scalebars in D and E correspond to 10 μm)
paxillin with Alexa647-labeled anti-rabbit antibody (donkey-anti-rabbit, Jackson Immunoresearch) to recognize the primary paxillin pY118 antibody (Invitrogen). Alexa647 was reported to show favorable switching properties to employ direct STochastic Optical Reconstruction Microscopy (dSTORM) [23].

Imaging was performed in 100 mM mercaptoethylamine (MEA, Sigma Aldrich) and a glucose oxygen scavenging system (for details see Supplemental methods). By sequential re-activation with 405 nm light from a diode laser (Crystalasers) at 1-20 W/cm² and imaging with 642 nm light from a diode laser (Spectra Physics) at 1.5 kW/cm², we localized individual molecules to a precision of 30 nm, limited by the signal from an individual molecule of 630 counts per 10 ms of illumination. For simultaneous two-color imaging of the actin structure we stained with Alexa532-phalloidin (Invitrogen) and adapted the buffer conditions by removing the oxygen-scavenging system to facilitate favorable switching conditions for Alexa532. The 405 nm switching light also excited the Alexa405-labeled fibronectin on the pillar tops, such that we simultaneously obtained high-resolution information of local cellular forces with each activation step. The combined single-molecule and micropillar approach yielded both a molecular density map of focal adhesion proteins and the local forces exerted by the cell.
2.3 Results

2.3.1 High-resolution measurement of cellular forces.

First, we assured that cell behavior on inverted pillar arrays did not differ from cell behavior in upright chambers. Phenomenologically we found that cells showed a healthy morphology, migrated and divided as expected. We further performed both live-cell and fixed-cell imaging on 3T3 fibroblasts to confer the phenomenological findings. We found that force exertion evolved from the cell periphery, and was largely aligned with actin stress fibers (figure 2.2A). After careful calibration of the micropillars using finite-element analysis, the total vectorial force per
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The absolute force per pillar in each cell varied over a large range from 0 to 10 nN (figure 2.2B) with a mean of 4±3 nN (mean±std). Typically, most of the large forces were localized on a few (5-10) pillars per cell.

Multi-color imaging of paxillin and actin confirmed that cellular forces emerged from focal adhesions (FAs) with their edges attached to the top of the pillars as shown in figure 2.2A-B (figure S2 shows separate channels). The FAs were detected by an antibody that recognizes phosphorylated paxillin. The focal adhesion area was distributed between 0.5 to 3 μm² with a mean of (1.4±0.7) μm² (figure 2.2C). Larger-sized FAs were able to support larger forces as indicated by the correlation between FA area and force displayed in figure 2.2C. Assuming a linear relationship the local force increased by (3±1) nN/μm² of FA area. A similar range of forces and FA area was previously found for Human Foreskin Fibroblasts [18] and REF52 Fibroblasts [19].

Shapes and sizes of FAs were quantified using custom software to detect the edges of focal adhesions (figure S3). Those quantities were correlated to local cellular traction forces. Actin stress fibers (mostly cortical fibers) were formed throughout the cell and emerged from force-bearing FAs (figures 2.2A-B). Throughout all cells, we observed a homogeneous distribution of orientation for both forces and focal adhesion elongation. This was predicted, since there is no preferential orientation for cells on hexagonal pillar arrays. However, we found a clear correlation between the direction of force and the direction of FA elongation. FAs were oriented within 10° (standard deviation) with respect to the direction of force (figure 2.2D).

Live-cell imaging of FAs in MCF-7 cells stably expressing a paxillin-GFP fusion construct in the inverted configuration further confirmed the ability to perform live-cell experiments in the inverted configuration and local force-FA area correlations. In figure 2.3A snapshots of a migrating cell on micropillars (blue) expressing paxillin-GFP (green) are shown. FA dynamics were directly quantified. The local FA area followed the force again in an apparent linear relationship. For two individual pillars (figure 2.3B) force and FA area correlated over time as one decreased in force and FA area and the other increased in force at an approximately constant small FA area. The force in live-cell experiments increased with FA area with (13±2) nN/μm² (figure 2.3C).

Our measurements of FA characteristics, cellular forces and their cor-
Figure 2.3
Live-cell dynamics of focal adhesions and force. (A) Live MCF-7 cell expressing paxillin-GFP on a micropillar array exerts significant forces locally where focal adhesions (FAs) are present. (B) Force and FA area correlate over time, from two individual pillar locations. Color correlates to time, indicating FA and Force decrease for one pillar, while another grows in force at a constant small FA size. (C) Over multiple focal adhesions and sites of force exertion on live MCF-7 cells, a clear correlation is given by a linear fit with $(13 \pm 2) \text{ nN/µm}^2$ ($R^2 = 0.711$).
relations corroborate previous observations of fibroblast-behavior when grown in the upright configuration \cite{18,20}. Using our inverted approach we can furthermore characterize live-cell force and FA area dynamics at the highest possible diffraction-limited resolution. Hence, we conclude that cells that are spread on inverted pillar arrays remain viable and their phenotypic and mechanical behavior is unaltered with respect to other situations.

2.3.2 Super-resolution imaging of focal-adhesion proteins on micropillar arrays.

In order to relate cellular forces to the molecular structure of focal adhesion complexes, we performed super-resolution imaging of the FA protein paxillin in cells that pulled on micropillar arrays\cite{23}. After adhesion, cells were fixed and immunostained for phosphorylated paxillin (pY118) using the primary rabbit-anti-pY118Paxillin antibody. Subsequently, a secondary Alexa647-anti-rabbit antibody was used for fluorescence-labeling. Cells were imaged in phosphate-buffered saline at pH 8 supplemented with 100 mM mercapto-ethylamine and a glucose-oxidase oxygen-scavenging system. After pre-bleach, Alexa647 was re-activated with 405 nm light for 10 ms every 20 frames with increasing intensity (1-20 W/cm$^2$) such that a constant 10 to 20 Alexa647 molecules were observed in each image. Individual Alexa647 molecules were identified and localized when excited at 642 nm for 10 ms with an intensity of 1.5 kW/cm$^2$. In total 15,000 images were recorded within 5 minutes. From those data we reconstructed a super-resolved image of FAs on pillars with a mean one-dimensional localization-precision of 30 nm. Figure 2.4A exemplifies the largely increased resolution of the super-resolved image (right) in comparison to the image taken at diffraction-limited resolution (left, the full diffraction-limited image is depicted in figure S4). Note the scalebar of 2 µm in this image when comparing to the image in figure 2.2B. FAs that appear as elliptical globular structures in the diffraction-limited images (figure 2.2B) appear rather as elongated stretches of 100-500 nm by 1-5 µm in the super-resolved image.

Labeling the micropillars with Alexa405-fibronectin allowed us to overlay the super-resolution images of FAs with diffraction-limited images of the micropillar arrays resulting in high-resolution force-maps. Figure 2.4B shows such overlay in which the pillar indicated by an arrow was deflected significantly with a force of 6.5 nN, which is carried by two
Figure 2.4
Nanoscale architecture of force bearing focal adhesions (FAs). (A) Super-resolved structure of phosphorylated paxillin using dSTORM. The diffraction limited image (left side) cannot resolve the small structural features of focal adhesions, but the super-resolved image (right side) reveals smaller features. (B) Micropillar locations are correlated to the super-resolved FAs through direct observation of the Alexa405-labeled fibronectin in blue during dSTORM re-activation. The arrow corresponds to a force of 6.5 nN. (C) A closer look at a single site of force exertion reveals a super-resolved image of a force-bearing FA. (scalebars in A-B correspond to 2 µm and in C to 1 µm) (D) Collapsed histogram of multiple line profiles along the FA base, as denoted by i in C. The base of the elongated FAs is only 125 nm wide and is perpendicular to two elongated FA structures.
individual elongated FAs. From such images it becomes obvious that the cell’s major forces emerge from elongated FAs that end on pillars, and that FA elongation is parallel to force exertion (as we observed in figure 2.2D). However, in the super-resolved image the attachment region of FAs to fibronectin is more clearly resolved. The FA is elongated, but also clearly localized on the very top of the pillars. Furthermore, we observe that the FA cluster extends several micrometers beyond the pillar. Fibroblasts produce fibronectin by which they model a fibrous network left behind when cells move forward (staining for fibronectin is shown in figure S5). The elongated FA in figure 2.4B attached to fibronectin left behind by the cell leading to the elongated growth of FAs towards the cell center.

With super-resolved imaging we further characterized the nanoscale architecture of the focal adhesions bearing the force exerted on a single pillar, as exemplified in figure 2.4C. In this case, two focal adhesions emerged in parallel at a distance of 1 µm and carried a force of 6.5 nN. The two parallel focal adhesions each measured a width of 280 nm at the full-width of half-maximum (FWHM, see figure S6). Strikingly, the base of contact in figure 2.4C on top of the pillar shows an additional structure 125 nm wide (FWHM, figure 2.4D) perpendicular to the two elongated FAs. In all super-resolution images analyzed, we found that force-exertion clearly emerged from the top of the pillars and from localized FA structures that had a width of 125-280 nm FWHM for 3T3 fibroblasts.

We also examined the super-resolved structure of paxillin in the MCF-7 cells used for live-cell microscopy (see figure 2.3). Directly labeled Alexa647-nanobodies recognizing the beta-barrel of GFP on the paxillin and Alexa532-labeled phalloidin were used to reconstruct a two-color super-resolution image (see figure 2.5). The structure of the FAs (figure 2.5A) appeared similar to what we obtained from super-resolved phosphorylated paxillin in figure 2.4C. Actin bundles preceded the elongated FAs carrying the exerted force (figure 2.5B). Again, multiple elongated stretches emanated from the top of the micropillars bearing forces in the order of 0-20 nN (zoom in on a single pillar in figure 2.5C) with a width of 100-150 nm.

With the super-resolved structure of FAs carrying a local force, we measured the FA area more accurately as compared to diffraction-limited microscopy. For both cell types it was apparent that the local force
2.3 Results

Figure 2.5
Super-resolved focal adhesions and actin correlated to force. Fixed MCF-7 cells show super-resolved paxillin using Alexa647-nanobody staining directly to paxillin-GFP and Alexa532-phalloidin to actin. (A) Paxillin molecular density and (B) actin molecular density through dSTORM imaging relative to force exertion. (C) A closer look at the location of one pillar shows multiple elongated FAs that emanate into actin bundles with a width of 100-150 nm. (Scalebars in A, also valid for B: lower-left corresponds to 20 nN and lower-right to 2 μm. Scalebar in C corresponds to 1 μm)

exerted was distributed over small FAs, consisting of multiple elongated 100-280 nm wide stretches. From these smaller FA areas, the actual force per area amounted to 10-300 nN/μm². The stress accumulated is thus approximately one order of magnitude higher as compared to the values we and others [18–20] obtained using diffraction-limited experiments. The individual sites of attachment as in the enlargement in figure 2.5C thus carried an even larger force per FA area, making it a locally highly stress-bearing state.
2.4 Discussion and conclusion

With one additional step in the micropillar fabrication procedure, we achieved simultaneous high-resolution cellular traction force measurements and super-resolution imaging. The precisely fabricated and integrated spacers next to the microfabricated pillar array allowed us to perform microscopy using high-NA objectives that are required for super-resolution microscopy. With our approach it became possible to measure local focal adhesion molecule-densities and the local force exerted by the cell at the same location. The ability to perform super-resolution measurements further makes our approach an attractive technique to address the question of a global versus local mechanosensory mechanism.

Cells that were attached on inverted micropillar arrays remained viable, as observed by proliferation and mechanical characteristics. Cellular force maps with high resolution down to the sub-nN force precision were obtained for fixed 3T3 fibroblasts and live MCF-7 cells. Simultaneously, we were able to image the nanostructural organization of the focal adhesion protein paxillin. Our method further allowed us to characterize exact adhesion size and localization relative to forces. Focal adhesions in 3T3 fibroblasts and in MCF-7 cells were elongated parallel to the direction of force exertion and had a typical width of 100-280 nm. The approximate stress carried by a FA complex was one order of magnitude higher as compared to the diffraction-limited case, since the FA actually consisted of multiple narrow elongated structures. Our measurements thus revealed a higher stress accumulation compared to previous measurements on cellular adhesion sites.

With the ongoing developments to quantify the exact number of molecules in super-resolution imaging [26], our approach can be directly used to quantify the amount of specific force-bearing molecules in a FA. We estimated that the focal adhesion structures localized on the pillar presented in figure 2.4C contained about 780 paxillin molecules. Combined with the measure of the local force we may in the future be able to complete our image of how forces are transmitted through the multifaceted focal adhesion complex. It will be interesting to investigate, using our methodology, the structure-force responses of potential mechanosensory proteins like talin, vinculin, p130Cas and zyxin. Such experiments may shed light on how a cell structurally alters its adhesion organization depending on its mechanical behaviour.
2.5 Supplemental methods

2.5.1 Micropillar fabrication and coating

Micropillar arrays were prepared by replica molding from a micro-fabricated master produced by a two-step Si-technology process following described methods [16, 17, 27, 28]. In the initial step a hexagonal pattern of 2 \( \mu \)m diameter holes and 2 \( \mu \)m spacing was etched into a flat Si-wafer. Roughness of the sidewalls was minimized by an alternating SF6 etching and C4F8 passivation Bosch process in an inductively-coupled deep reactive-ion etcher. In a second step we etched 50 \( \mu \)m deep spacers at two sides of the 1x1 cm micropillar arrays. The etching process resulted in silicon-wafer masters with the negative mold of pillars and spacers.

The masters were cleaned in 100% ethanol, then 100% isopropanol, and finally silanized with trichloro(1H,1H,2H,2H-perfluorooctyl) silane (Sigma Aldrich) for 16 hours. Polydimethyl siloxane (PDMS, Dow Corning) was mixed with 1:10 crosslinker:prepolymer ratio, degassed, poured over the master wafers, and cured for 20 hours at 110 °C. The cured micropillar arrays with spacers were peeled from the wafers.

Subsequently, micro-contact printing with fluorescently labeled fibronectin was performed. A flat PDMS stamp was incubated for 1 hour with a 40 \( \mu \)l drop of 50 \( \mu \)g/ml fibronectin and 10 \( \mu \)g/ml fibronectin-Alexa405 (NHS-labeled with approximately 10 dyes per fibronectin). After two washing steps with ultrapure water, most liquid was adsorbed using a tissue and left to dry in a laminar flow hood. Prior to labeling the micropillars were activated using a UV-ozone cleaner (Jelight) for 10 minutes. The stamp was applied 2-5 minutes. Finally, the PDMS micropillars were blocked for 1 hour with 0.2% pluronic (Sigma) in phosphate buffered saline.

2.5.2 Force-deflection calibration

We first accurately assessed the pillar dimensions and their bulk material properties. We measured the stress-strain behavior of bulk PDMS in a uni-axial test using a dynamic mechanical analyzer (TA Instruments). From those experiments the Young’s modulus of the pillar bulk material was determined to be 2.5±0.1 MPa with a negligible Loss Modulus. Subsequently, scanning electron microscopy (FEI nanoSEM) was used to directly quantify the dimensions of the non-conductive PDMS micropill-
lars. Images were taken in low vacuum mode at a partial water vapour pressure of 0.7 mbar, such that coating with a conductive material was not necessary. At the base of the micropillars a notch due to undercutting during the etching process is visible. The pillars shown in figure \[57\] had a height of 6.9±0.2 µm and a diameter of 2.0±0.1 µm (see figure \[58\]) and were homogeneously arranged in a hexagonal array of 10x10 mm.

In most previous work, a perfect linear elastic beam is assumed for the force-deflection relationship. Schoen et al. \[29\] showed in their experiments that base-deformation has to be considered. They therefore developed an analytical extension to linear elastic beam theory that includes tilting of the base, shear in the base as well as base displacement. This led to a more accurate relation between force at the pillar top and displacement of the pillar, but still a linear relationship. Because of the more complicated shapes of our pillars we employed finite element modelling (FEM) to give a precise measure of the force-deflection behavior and to account for the effect of the notch at the base of the pillar (see figure \[57\]).

In our FEM model the base of the micropillar was assumed to be an elastic, deformable material of the same stiffness as the pillar (see figure \[51\]), to facilitate base-deformation. FEM analysis confirmed that the small notch on our pillars indeed influences the result from a perfect cylinder modeled with FEM. It further showed that the force-displacement relationship was linear even up to deflections of 2 µm for the relatively high pillars (6.9 µm) used in the experiments presented here, while a non-linear response occurs only for larger deflections. The linear elastic bending, base-tilting, cylindrical FEM and notch FEM bending moduli are given in table \[2.1\]. The force-deflection behaviour over the relevant range of 0 - 2 µm deflection was determined to be 16.7 nN/µm with $R^2=0.999$.

<table>
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<th>Base deformation k (nN/µm)</th>
<th>Analytical Bending</th>
<th>Analytical Model [29]</th>
<th>FEM Cylindrical</th>
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**Table 2.1**
Linear force-deflection response for pure bending, the extended model by Schoen et al. \[29\], the cylindrical- and notched FEM pillar.
2.5 Supplemental methods

2.5.3 Deflection map

Fluorescence imaging of Alexa405-labeled fibronectin shows the homogeneous application of non-fibrous fibronectin to only the top of the micropillars. We determined the micropillar positions with sub-pixel accuracy by fitting a Gaussian or cone-profile to the cross correlation of the measured pillar intensity map and a circle of identical radius. Doing so we were able to obtain a mean deflection precision of 30 nm solely determined by the signal-to-noise ratio of pillar detection. Subsequently we corrected the positions for optical aberrations. In general objectives are perfectly corrected for aberrations only in terms of diffraction limited imaging. As SEM measurements showed with very high precision that pillars were arranged in a perfectly hexagonal array (see figure S8) this knowledge was used to correct positions of an undeflected pillar array that in turn was used to obtain a position correction-field to account for optical aberrations (figure S9). In summary, the high positional accuracy in determination of the pillar positions, including aberration correction and our finite element modeling of the pillar stiffness, allowed us to reliably determine cellular forces at a precision of 500 pN.

2.5.4 Focal adhesion analysis

Automated image analysis of the focal adhesion fluorescence images provided measures for focal adhesion size in the diffraction-limited images. Specifically designed software provided us with the detection and further characterization of discrete focal adhesion patches (see figure S3). Focal adhesion patches were analyzed in terms of area, elongation, angle of elongation towards force, and were assigned to its respective pillar on which force exertion emerged.

2.5.5 Super resolution imaging

For the dSTORM single-molecule measurements, we took a similar approach as in van de Linde et al. [23]. Primary antibodies recognized paxillin that is phosphorylated at site Y118 (Invitrogen). Secondary antibodies were labeled with the dye Alexa647 (Jackson Immunoresearch). We labeled paxillin-GFP in the MCF-7 cells using Alexa647-labeled GFP-trap (Chromotek) with 1.6 dyes per trap. Actin was directly stained using Alexa532-phalloidin (Invitrogen). Imaging was performed with 100
mM MEA in PBS at pH 8 supplemented with an oxygen scavenging system, containing 10% w/v glucose, 0.5 mg/ml glucose oxidase and 40 µg/ml catalase (all from Sigma Aldrich). The oxygen-scavenging system was not added for the two-color experiments, as it greatly decreased the blinking of Alexa532. The sample was sealed between two coverslips with grease during the measurement, to minimize the influence of oxygen. The sample was imaged on a wide-field single-molecule setup equipped with a 100X, NA 1.4 objective (Zeiss) onto a back-illuminated emCCD camera (Cascade). In the two-color experiments a sCMOS Orca Flash camera (Hamamatsu) was used on the same setup. The illumination intensity of a 642 nm diodelaser (Spectra Physics) was kept at 1.5 kW/cm², while re-activation of molecules was performed every 20 frames with a 405 nm laser (Crystalaser) at an increasing intensity of 1-20 W/cm². The mean localization precision of the single molecules in both x- and y-direction was 30 nm (see figure S10).
2.6 Supplemental figures

**Figure S1**
Finite element analysis of a single micropillar. The exact pillar shape and size were taken into account: including a notch at the base and the base-tilting effect (both pillar and base had the same properties), to provide a precise force-deflection relationship.

**Figure S2**
Diffraction limited imaging of actin, focal adhesions and micro-contact printed fibronectin. Forces exerted by a 3T3 Fibroblast are plotted with staining for actin (left), paxillin (middle) and micro-contact printed fibronectin (right). Arrow corresponds to 20 nN and scalebar to 10 µm in fluorescence images.
Figure S3
Automated analysis gives all focal adhesion properties after spatial frequency filtering of the background (left image, after background subtraction). The resulting focal adhesions (color-coded in the right image) are detected using Canny-edge detection. This yields the cellular outline and pixel-limited geometrical information on the focal adhesion sites (e.g. area, orientation). Scalebar corresponds to 10 µm.

Figure S4
Diffraction limited close-up image of paxillin. Focal adhesions appear as rounded objects and structural features below 200 nm cannot be clearly seen (image size is 10x10 µm).
2.6 Supplemental figures

Figure S5
3T3 Fibroblast spread on micropillars stained for Fibronectin (green) and actin (red). Micro-contact printed fibronectin is present only on the pillar tops, while the fibronectin produced by the cell is visible at the edge of the pillars where the cell attaches (scalebar corresponds to 10 µm).

Figure S6
Sum of collapsed line profiles of a single focal adhesion in figure 4B. Full Width at Half Maximum of elongated focal adhesions is 280 nm.
Figure S7
Scanning electron microscopy image sideview of PDMS micropillars. These micropillars have a height of 6.9 µm and diameter of 2 µm. The force-deflection behaviour is calibrated including the notch at the base using Finite Element Analysis (scalebar bottom right corresponds to 2 µm).

Figure S8
Scanning electron microscopy topview of PDMS micropillars. All SEM was performed using Low-Vacuum Mode, which enabled the imaging of non-conductive samples. Replica-molding from the etched Silicon wafers yields perfectly hexagonal arrays with precision beyond the fluorescence imaging resolution (scalebar corresponds to 10 µm).
Figure S9
High resolution imaging reveals subpixel astigmatism. The micropillar arrays are perfectly hexagonal (see figure S8) below the optical pillar localization precision (30 nm) and an imperfect hexagonal map was observed larger than this precision. This was corrected for in the pillar localization using a two-dimensional mapping as depicted here with deflections corrected up to approximately 1 pixel (corresponding to 138 nm). The correction map is color coded corresponding to the colorbar in pixels.

Figure S10
Localization precision of single molecules. Histogram of localization precision of single molecules from dSTORM as observed on inverted micropillar array in x- and y-direction. Mean localization precision is approximately 30 nm.


