Supporting Information:

Nanoparticle Tension Probes Patterned at the Nanoscale: Impact of integrin clustering on force transmission

*Yang Liu†, Rebecca Medda‡, Zheng Liu†, Kornelia Galior†, Kevin Yehl†, Joachim P. Spatz‡, Elisabetta Ada Cavalcanti-Adam‡, Khalid Salaita†**

† Department of Chemistry, Emory University, 1515 Dickey Drive, Atlanta GA, 30322 USA; ‡ Department of Biophysical Chemistry, Ruprecht-Karls-University, INF 253, 69120 Heidelberg, Germany, and Department of New Materials and Biosystems, Max Planck Institute for Intelligent Systems, Stuttgart, Germany

Corresponding Author: k.salaita@emory.edu

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Materials

(3-Aminopropyl) trimethoxysilane (97%, APTMS), O-(2-carboxyethyl)-O′-(2-mercaptoethyl) heptaethylene glycol (95%, COOH-EG_8-SH), 2,5-dihydroxybenzoic acid (99%, DHB), triethylamine (99%, TEA), dithiothreitol (99.0%, DTT), triethylammonium acetate (TEAA), Oleoyl-L-α-lysophosphatidic acid (LPA) sodium salt (>98%), Y-27632 Dihydrochloride (>98%), cytochalasin D (≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. The heterobifunctional linker azide-NHS (product #: 88902) was purchased from Thermo Fisher Scientific (Rockford, IL). The fluorescent dye Cy3B-maleimide was purchased from GE healthcare Bio-Science (Pittsburgh, PA). Lipofectamine® LTX with Plus™ Reagent for cell transfection and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Life Technologies (Carlsbad, CA). Number two glass coverslips, ascorbic acid (>99.0%), and 96-well plates were purchased from Fisher Chemical & Scientific (Pittsburgh, PA). DMF (>99.5%), DMSO (99.5%) and sodium bicarbonate (99.0%) were purchased from EMD chemicals (Philadelphia, PA). Cyclic(RGDfK)C (Fig. S5) was custom synthesized and HPLC purified by Peptides International (Louisville, KY). All DNA strands used were custom synthesized and desalted by Integrated DNA Technologies (Coralville, Iowa). Alkyne-PEG-SH (MW 3400) was purchased from Nanocs (New York, NY). $CuSO_4.5H_4O$ was purchased from Mallinckrodt (St. Louis, MO) and P4 gel size exclusion beads were acquired from Biorad (Hercules, CA). DI water was obtained from a Nanopure water purification system with a UV sterilization unit and showed a resistivity of 18.2 MΩ.

The AuNPs that were used to generate the randomly immobilized gold particle arrays, and to calibrate the NSET distance were custom synthesized and characterized by TEM by Nanocomposix (San Diego, CA). Based on TEM analysis that was provided by the manufacturer, the mean diameter of these particles was 8.6 ± 0.6 nm. This diameter was intentionally selected to match the average size of BCMN-generated AuNP that had a 8.4 ±1.0 nm diameter (based on SEM and AFM, Fig. S1-2).

Methods

Fabrication of BCMN patterned gold nanoparticle (AuNP) arrays.

The protocol for deposition of 9 nm gold nanoparticles (AuNPs) on surfaces by block copolymer micelle nanolithography (BCMN) using polystyrene-*block*-poly (2-vinylpyridine) (PS-*b*-P2VP) is based on the work by Glass et al.[1] Prior to BCMN, glass surfaces were activated in oxygen plasma (0.4 mbar, 150 W) for 10 min. The micelle solution used to fabricate the 49 nm interparticle distance substrate was prepared by dissolving polymer '1056' (consisting of 1056 polystyrene units and 671 vinyl pyridine units) at a concentration of 9 mg/ml in *o*-xylene and adding $HAuCl_4$ to the polymer solution to obtain a gold loading of 0.3 ($HAuCl_4$*$3H_2O$ from Sigma, Germany). The micelle solution used to fabricate the 99 nm distance substrate was prepared similarly using a polymer concentration of 5 mg/ml and a gold loading of 0.25. In this work, we used dip-coating to obtain the 99 nm distance by using a constant retraction speed of approximately 25 mm/min, whereas in the case of the 49 nm distance samples we employed spin coating with a speed of 7000 rpm. It should be noted that either method could be used for the generation of small or large interparticle distances by only adjusting the retraction, or the spinning velocity. The organic compounds were removed by hydrogen plasma treatment (0.4 mbar, 150 W) for 45 min, which also results in the coalescence of the AuNPs.

Scanning Electron Microscopy.

The quasi-hexagonal AuNP pattern was analyzed with scanning electron microscopy SEM (LEO 1530 Gemini, Carl Zeiss, Germany). To enhance electron scattering, thus enhancing contrast, carbon was sputtered on the surfaces prior to SEM imaging.

Ensemble fluorescence measurements. The fluorescence intensity of Cy3B was recorded using a Biotek Synergy HT plate reader operated using a filter set with excitation/emission λ = 565 nm/610 nm. Each well in the 96-well plate (Fisher scientific) was filled to a volume of 100 µl. All measurements were performed in triplicate, and the reported error bars represent the standard deviation of these measurements.

HPLC. Reaction products were purified using a C18 column (diameter: 4.6 mm; length: 250 mm) in a reverse phase binary pump HPLC that was coupled to a diode array detector (Agilent 1100).

MALDI-Mass Spectrometry. A 10 mM solution of 2,5-dihydroxybenzoic acid (DHB) was prepared in tetrahydrofuran (THF) as the MALDI matrix and 0.1 M sodium chloride was added as cationization agent.[2] All products were also pre-dissolved in THF and then mixed with an equal volume of the DHB matrix. 2 µl of this mixture was then added to each well on the MALDI plate. After allowing the solution to dry for 20 min, the sample was analyzed by a high performance MALDI time-of-flight mass spectrometer (Voyager STR).

Optical Microscopy. Live cells were imaged in phenol red free Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C. During imaging, physiological temperature was maintained with a warming apparatus consisting of a sample warmer and an objective warmer (Warner Instruments 641674D and 640375). The microscope was a Nikon Eclipse Ti driven by the Elements software package. The microscope features an Evolve electron multiplying charge coupled device (EMCCD; Photometrics), an Intensilight epifluorescence source (Nikon), a CFI Apo 100× (numerical aperture (NA) 1.49) objective (Nikon) and a TIRF launcher with two laser lines: 488 nm (10 mW) and 647 nm (20 mW). This microscope also includes the Nikon Perfect Focus System, an interferometry-based focus lock that allowed the capture of multipoint and time-lapse images without loss of focus. In all the reported experiments, we used the following Chroma filter cubes: TIRF 488, TRITC, and reflection interference contrast microscopy (RICM).

AFM imaging. The density of AuNP tension sensor on the functionalized glass coverslip was measured by using an atomic force microscope mounted on an anti-vibration stage (MFP-3D, Asylum Research, CA). Silicon AFM tips (MikroMasch) with a force constant (5.4-16 N/m) were used to image the sample in tapping mode at a scan rate of 1 Hz. All images were processed and rendered using IgorPro.

Cell culture and transfection. NIH/3T3 fibroblast cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Cosmic Calf Serum (Mediatech), HEPES (9.9 mM, Sigma), sodium pyruvate (1 mM, Sigma), L-glutamine (2.1 mM, Mediatech), penicillin G (100 IU ml^{-1}, Mediatech) and streptomycin (100 µg ml^{-1}, Mediatech) and were incubated at 37 °C with 5% CO_2. Cells were passaged at 60-80% confluency and plated at a density of 10% using standard cell culture procedures. All cell transfection was performed in a 24-well plate. All procedures are based on a standard protocol provided by Life Technologies (Carlsbad, CA). Briefly, 4×10^4 cells were plated in each well one day before transfection. During transfection, 0.5-1 µg DNA was mixed with Lipofectamine® LTX with Plus™ Reagent for each well and incubated for 24-48 hr before imaging.

Supporting Movie Caption

SI Movie1:
NIH/3T3 fibroblasts transiently transfected with GFP-LifeAct were plated on a substrate presenting randomly arranged AuNP tension sensors with ~100 nm average spacing. The timelapse movie was captured from *t* = 5 min to *t* = 43 min. Top row shows the brightfield and RICM channel, while the bottom row displays the F-actin dynamics (red channel) and tension (green channel).

SI Movie2:
NIH/3T3 fibroblasts were plated on a substrate presenting randomly arranged AuNP tension sensors with ~100 nm average spacing for 2.5 hr, followed by treatment with 40 μM Y-27632. The timelapse movie was captured from *t* = 10 min to *t* = 28 min following drug treatment. Brightfield (left), RICM (middle), and tension signal (right) channels were acquired for the movie.

SI Movie3:
NIH/3T3 fibroblasts were plated on a substrate presenting randomly arranged AuNP tension sensors with ~100 nm average spacing for 5.5 hr, followed by direct treatment with 10 μM cytoD for 6 min. Timelapse movie was captured from *t* = 0 min to *t* = 6 min. Brightfield (left), RICM (middle), and tension signal (right) channels are displayed side by side in the movie.

Characterization of BCMN patterned AuNP arrays

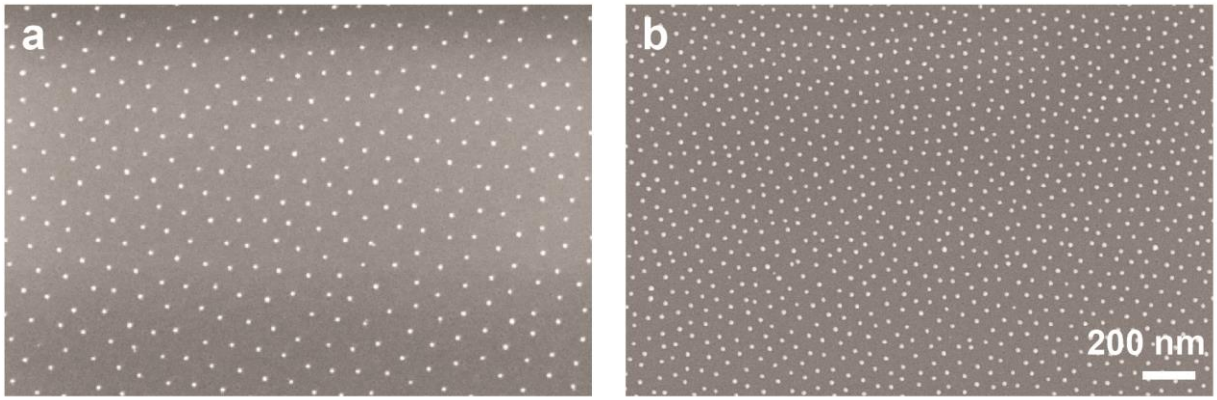

Figure S1. Representative SEM images of BCMN-patterned 9 nm AuNP arrays with a mean spacing of (a) 99 ± 12 nm and (b) 49 ± 9 nm.

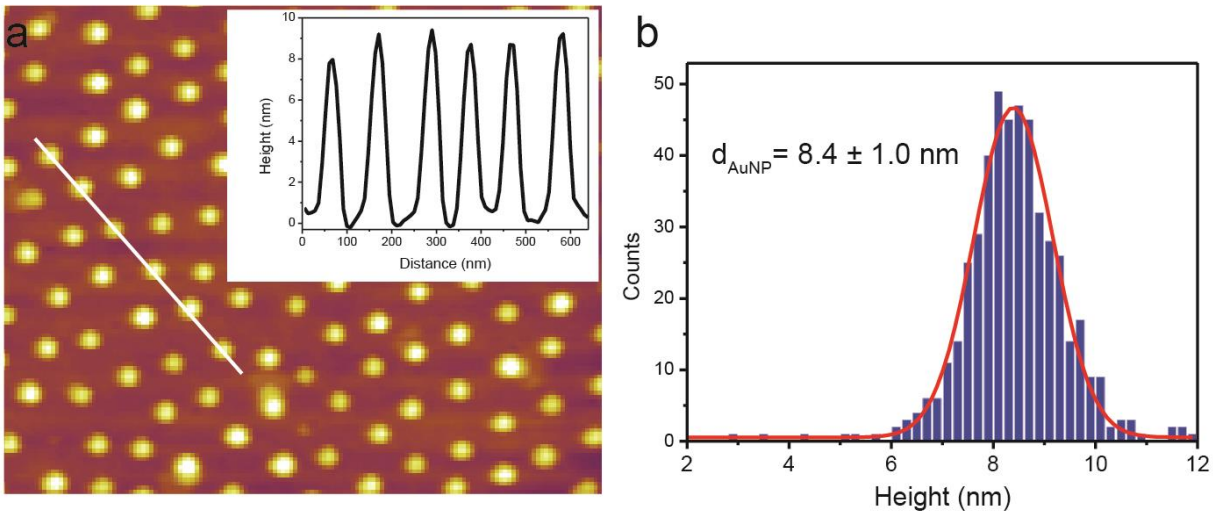

Figure S2. Analysis of BCMN-generated AuNP heights using AFM. (a) Representative AFM image of the 99 nm-spaced AuNP array. The white line indicates the region of the image where a linescan analysis was performed on six particles (inset). (b) Histogram showing the height distribution of ~ 400 AuNPs. The histogram was generated by using the Igorpro software package provided with the Asylum MFP-3D AFM system.

Quantification of the value of d_0 in NSET model

bp	DNA Duplex Length (nm)	DNA sequences (5' to 3')
16	6.9	HS-$(CH_2)_6$-GCC TAG AGC ATC AGT G
21	8.6	HS-$(CH_2)_6$-GCC TAT GAA TGA GCT TCA GTG
33	12.7	HS-$(CH_2)_6$-GCC TAT ATA GTC ATC AGC CGT ATA GCA TCA GTG
45	16.8	HS-$(CH_2)_6$-GCC TAT GAA CCG AAT TCG ATA GTC ATC AGC CGT ATA GCA TCA GTG
60	21.9	HS-$(CH_2)_6$-GCC TAT GAA GGA GCT CGC CTA CTA CCG AAT TCG ATA GTC ATC AGC CGT ATA GCA TCA GTG

Table S1. DNA strands used to generate the NSET calibration plot to determine d_0. The table displays the number of bases, the calculated end-to-end distance of the duplex, and the composition (shown in 5'-3' orientation) of the thiolated DNA sequence. These strands were hybridized with their fluorescently tagged complements prior to surface immobilization. The calculated length of each DNA duplex assumed a 0.34 nm rise per bp as well as a 1.5 nm length for the two C_6 linkers.

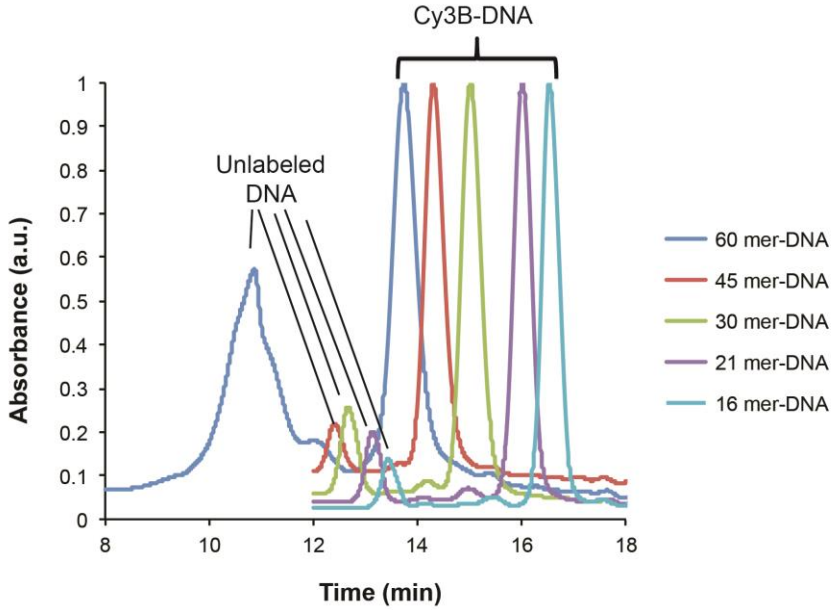

Figure S3. A series of overlaid RP-HPLC chromatograms of the products of the reaction between Cy3B-succinimidyl ester and 5 different amine-modified DNA strands in PBS with 0.1 M sodium bicarbonate. The reaction was allowed to proceed overnight, and then the product was initially purified through a size-exclusion column loaded with P-4 gel (Biorad, 4000 M_w cutoff). The reaction product was then injected into the HPLC using the protocol described below.

5' Disulfide-modified oligonucleotides (modification code: /5ThioMC6-D/) were custom synthesized by Integrated DNA Technologies (IDT). The disulfide group was reduced by incubation with 5 nmol of lyophilized oligonucleotide with 100 µL of disulfide cleavage buffer (0.1 M dithiothreitol (DTT), 170 mM phosphate buffer at pH 8.0) for 3 hr at room temperature. The reduced oligonucleotides were then purified using a NAP-5 column (GE Healthcare, Piscataway, NJ) using DI water as the eluent.

5’ Amine-modified oligonucleotides (modification code: /5AmMC6/) were custom synthesized by IDT and used without further purification. 10 nmol of the oligonucleotide was reacted with 100 nmol of Cy3B-NHS ester dye in 1X PBS (pH 7.4) for 12 hr at room temperature. The product was subsequently purified by reverse phase HPLC (flow rate 1 ml/min, solvent A: 0.1M DI TEAA, solvent B: 100% acetonitrile, initial condition was 10% B with a gradient of 1% per min). The final concentration was determined by using a Nanodrop spectrophotometer.

Equal amounts of 5’SH-DNA and 5’ amine-modified complementary DNA (10% Cy3B labeled, 90% unlabeled) were mixed together at ~25 μM concentrations in 1XPBS and hybridized by heating to 95^{o}C for 5 min, then cooling at a rate of 3.3 °C/min over a period of 20 min. Afterwards, 8.7 μM of dsDNA was then added to 3.0 mL of 9 nm diameter gold nanoparticles (8.4 nM), bringing the concentration of oligonucleotide and gold nanoparticles to ~2.7 μM, and ~5.8 nM, respectively. The pH of the solution was adjusted to pH 7.4 by adding 296.3 μL of 100 mM phosphate buffer, thus bringing the phosphate buffer concentration to 9 mM. The particles were then stabilized by adding sodium dodecyl sulfate (SDS) to the solution and bringing its final concentration to 0.1% (g/mL) by using a stock solution of 10% SDS. The particles were successively salted with four NaCl additions that were spaced 20 min apart using a stock solution of 2.0 M NaCl. The final NaCl concentration of the DNA-AuNP solution was increased to 0.3 M. The first NaCl addition increased the concentration to 0.05 M, while the remaining three NaCl additions increased the NaCl concentration by 0.1 M increments. The fully salted particles were then incubated overnight, in the dark and at room temperature. The following day, the particles were centrifuged five times and reconstituted in PBS for each wash.

Determination of minimal ligand concentration for cell adhesion

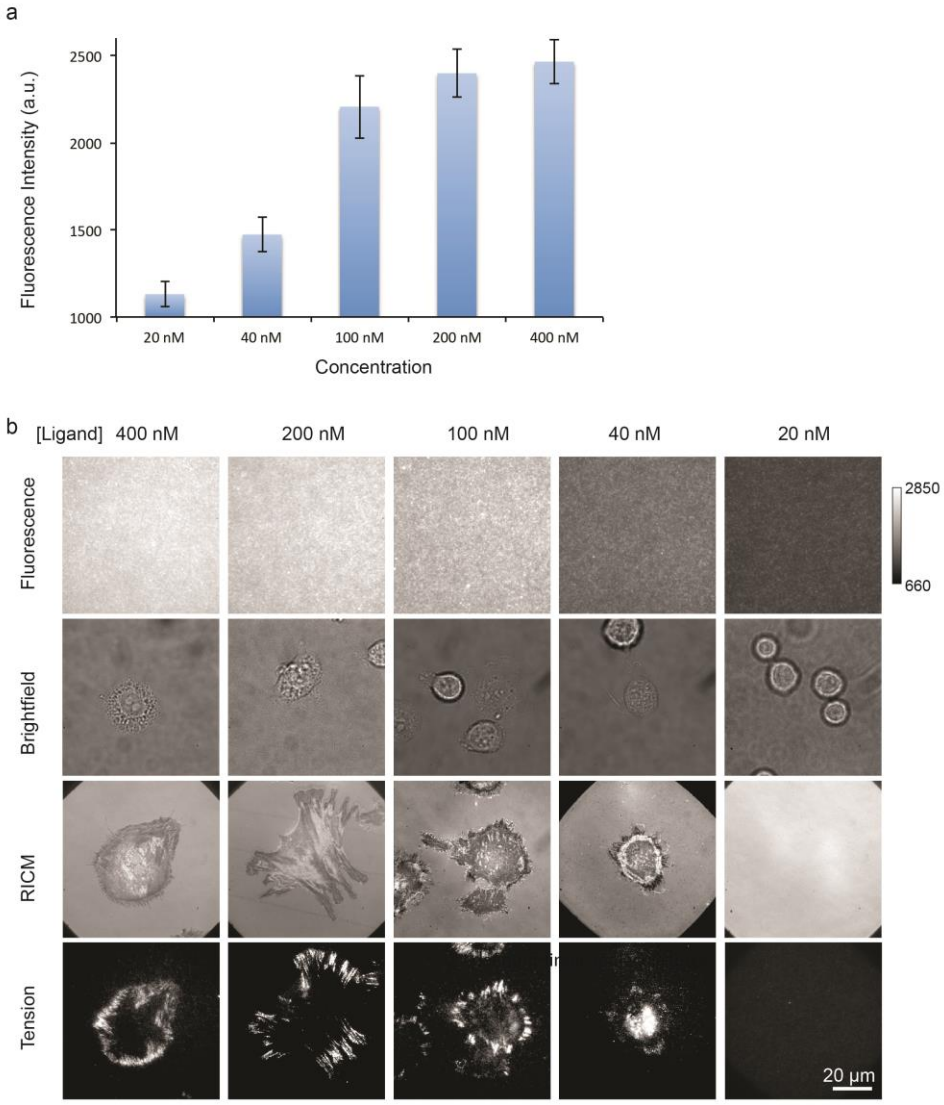

Figure S4. (a) Plot displaying the average substrate fluorescence intensity as a function of incubation concentration of the fluorescent tension ligand. Note that in all incubations, the total thiol concentration was maintained at 40 μM by using a passivating PEG thiol ($SH(CH_2)_2(OCH_2CH_2)_8COOH$). (b) The first row shows representative fluorescence images of the substrate prior to the addition of cells. The brightfield, RICM and tension rows of images show cell adhesion as a function of ligand concentration. This data indicates that incubating the substrate with 200 nM of tension ligand along with 39.8 μM of passivating PEG thiol is sufficient for producing robust cell adhesion with minimal fluorescence background intensity.

Synthesis of Tension Sensor Ligand

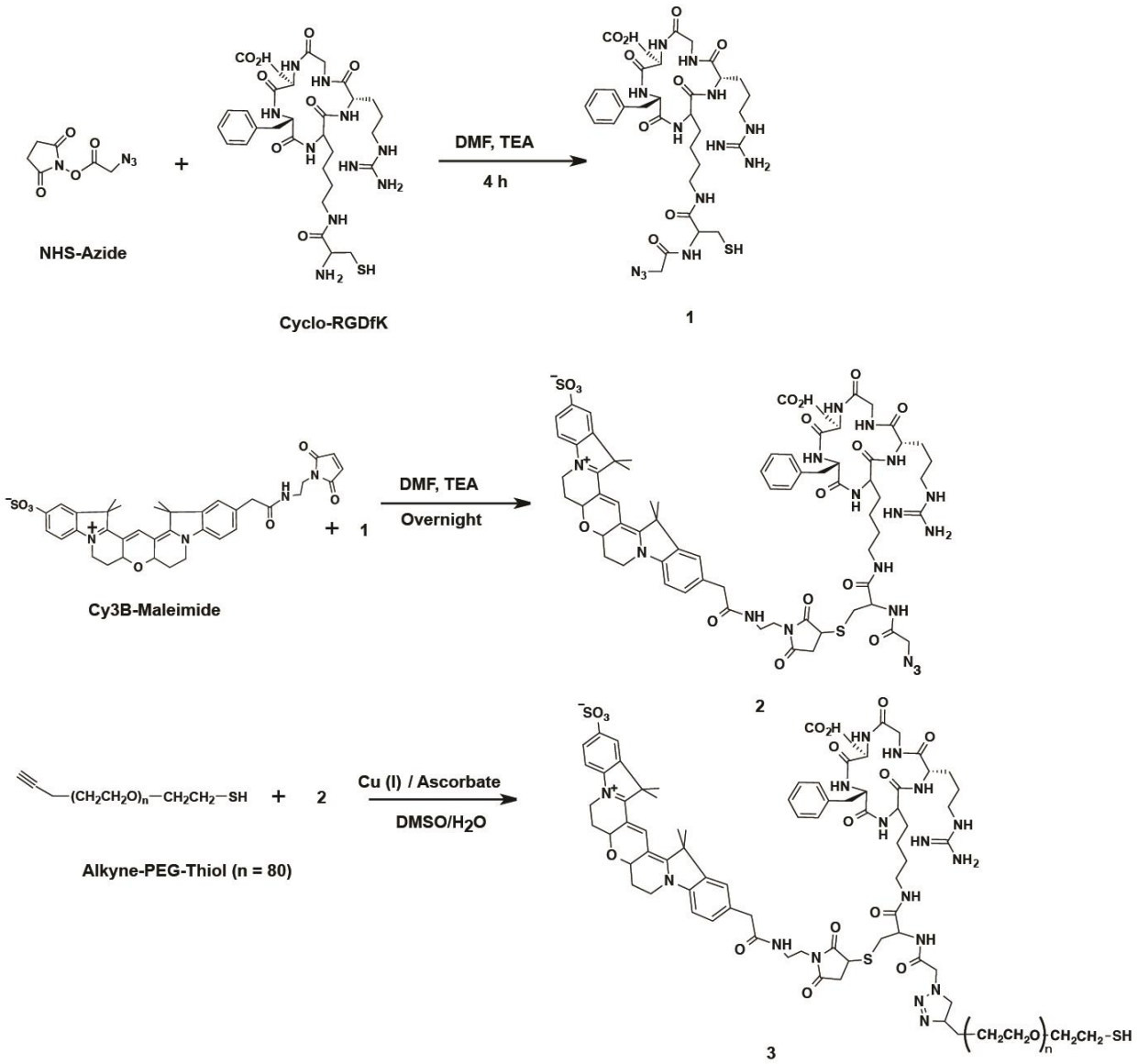

Figure S5. Schematic of chemical synthesis of Cy3B tagged tension sensor ligand.

Synthesis of **1**. 200 µg of cRGDfK(C) peptide (MW: 706.81) was reacted with 0.1 mg azide-NHS linker (MW: 198.14) in 15 µL DMF. To this reaction mixture, 0.1 µL of neat triethylamine was added as an organic base and the reaction was allowed to proceed for 4 hr.

Synthesis of **2**. A 10-fold molar excess of Cy3B-maleimide dye in 10 µL DMF was directly added to eppendorf tube that contained the products of the reaction to generate **1.** 0.1 µL of neat TEA was added and the reaction was allowed to proceed overnight. The product of this reaction, **2**, was subsequently purified by reverse phase HPLC (flow rate 1 ml/min; solvent A: 99.5% DI water, 0.05% TFA; solvent B: 99.5% acetonitrile 0.05% TFA; initial condition was 10% B with a gradient of 1% per min). The sample was then dried in a Speedvac for 6 hr.

Synthesis of **3**. A 0.7 molar ratio of alkyne-PEG-SH (~1 mM) to **2** was dissolved in 10 µL DMSO and 5 µL DI water. 2 µL of 2 M TEAA buffer and 2 µL of 5 mM ascorbic acid was then added to this solution.

Finally, 1 µL of Cu(II)SO_4 was added to the solution and vortexed. The reaction was incubated overnight at room temperature with foil to protect from ambient light. The reaction mixture was then treated with 0.1 M DTT in phosphate buffer for 1 hr to reduce disulfides that formed overnight. Afterwards, size exclusion chromatography was performed with a spin column (cutoff: 4000 MW) to remove all other by-products except the final product **3.** HPLC (method was the same as the one used for **1** and **2** and the chromatogram shown below), and MALDI-TOF (observed mass = 5204.32; calculated mass = 5206.44) were used to confirm the synthesis of **3** with a near 99% purity, which was based on HPLC peak integration.

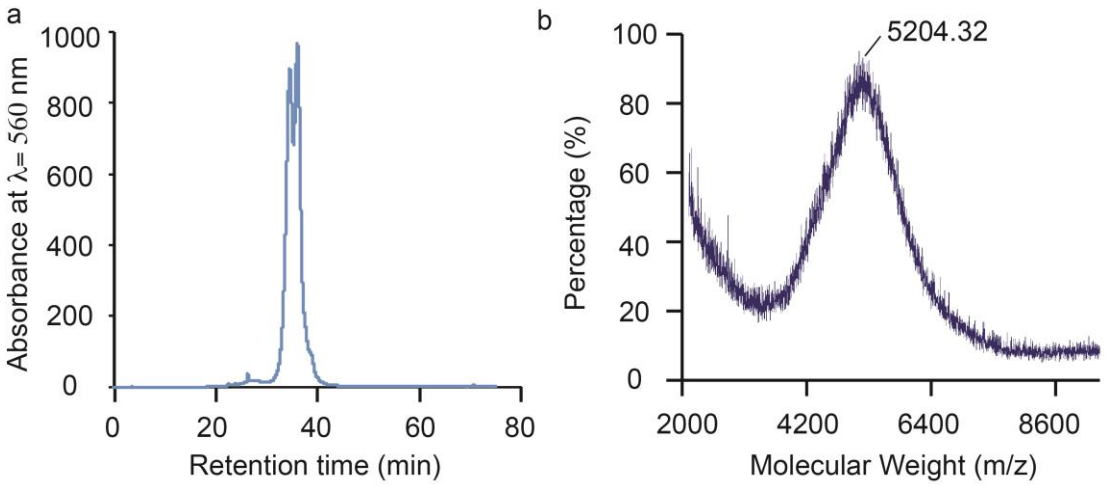

Figure S6. HPLC chromatogram (a) and MALDI-TOF spectrum (b) of the final product **3.**

Quantification of the average number of tension sensor ligands per AuNP and the quenching efficiency of Cy3B

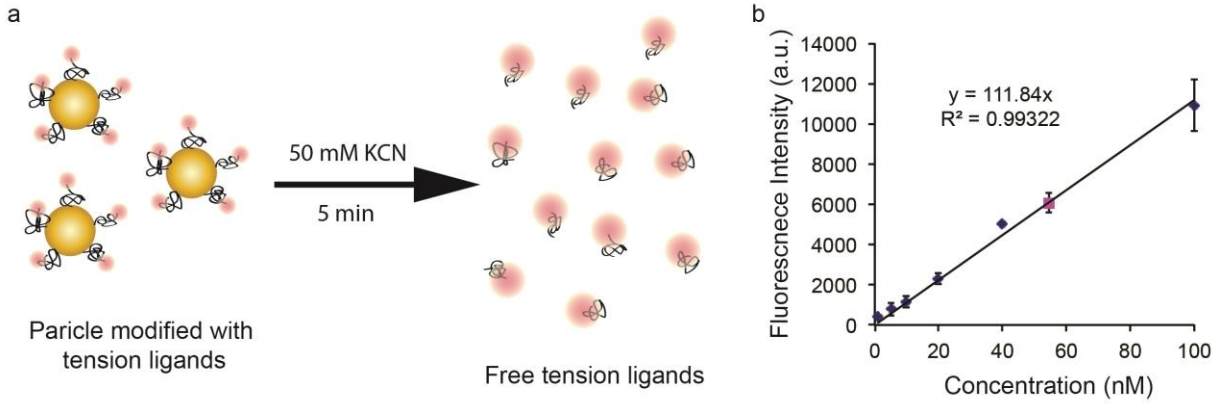

Figure S7. (a) Scheme showing the approach used to determine the average stoichiometry between Cy3B-labeled tension probe ligands and AuNP. Briefly, the concentration of AuNP was determined from the absorbance at λ=520 nm. Subsequently, AuNPs were dissolved by treating the sample with 50 mM KCN, which released Cy3B-labeled tension probe ligands within 5 min. The fluorescence intensity of the released Cy3B dye was then used to determine the number of dye molecules in the sample. Note that the quenching efficiency of Cy3B was determined by measuring the fluorescence intensity of the sample before and after AuNP dissolution and using the following relation: $1 - I_{quenched}/I_{de\text{-}quenched}$, and this was determined to be 0.91 in our experiments, indicating an average distance of 8.1 nm between Cy3B dye and AuNP surface. (b) The calibration curve used to quantify the number of tension sensor ligands per AuNP. Blue diamonds represents the calibration curve, while the purple box represents the unknown sample containing the released Cy3B ligands from AuNP-tension probes. All measurements were performed in triplicate.

Image analysis

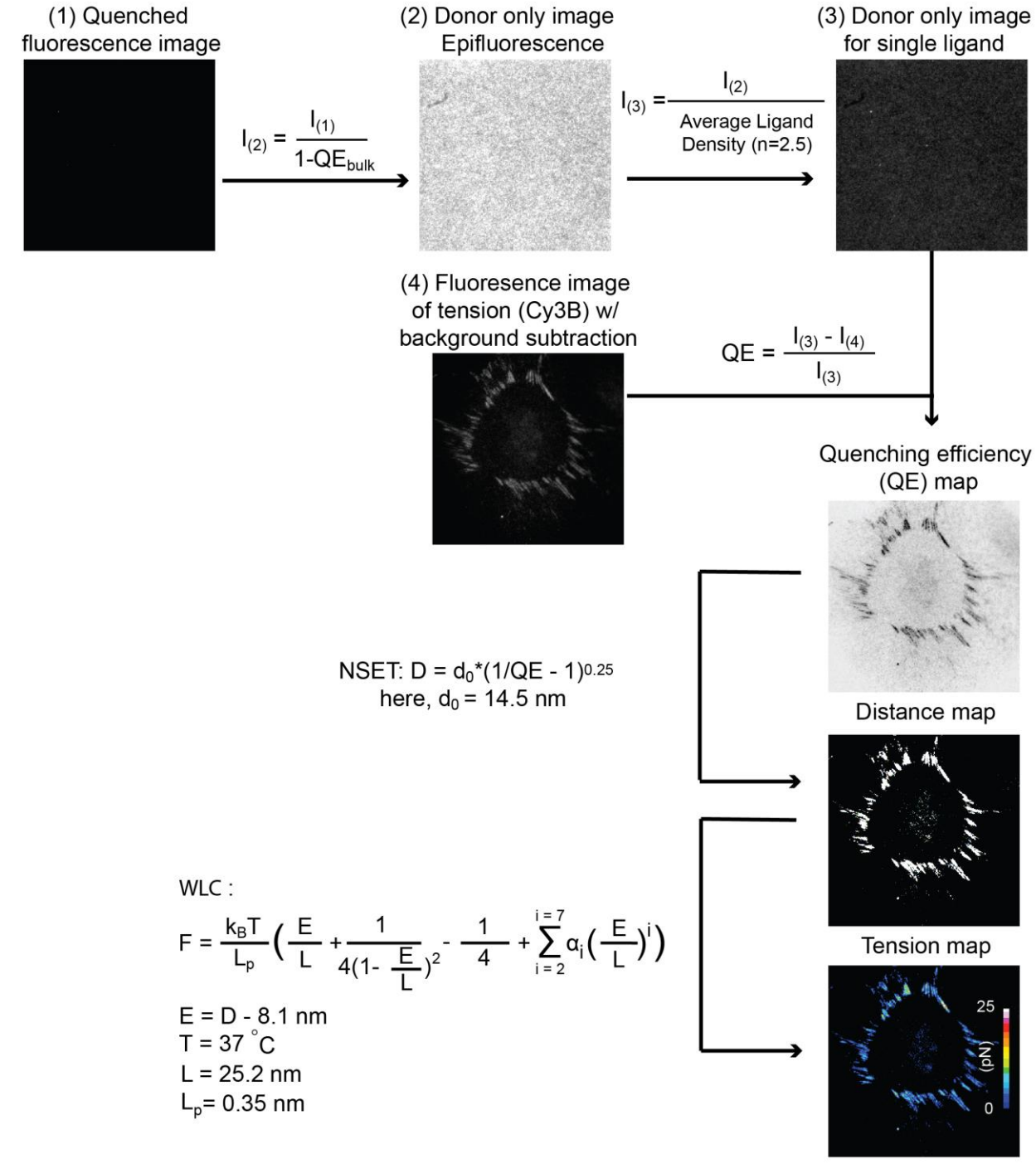

Figure S8. Stepwise image analysis used to relate the raw fluorescence images to a calculated force per integrin. The image analysis employed the NSET and extended WLC models[3].

Additional analysis of FA size, integrin tension, and traction force

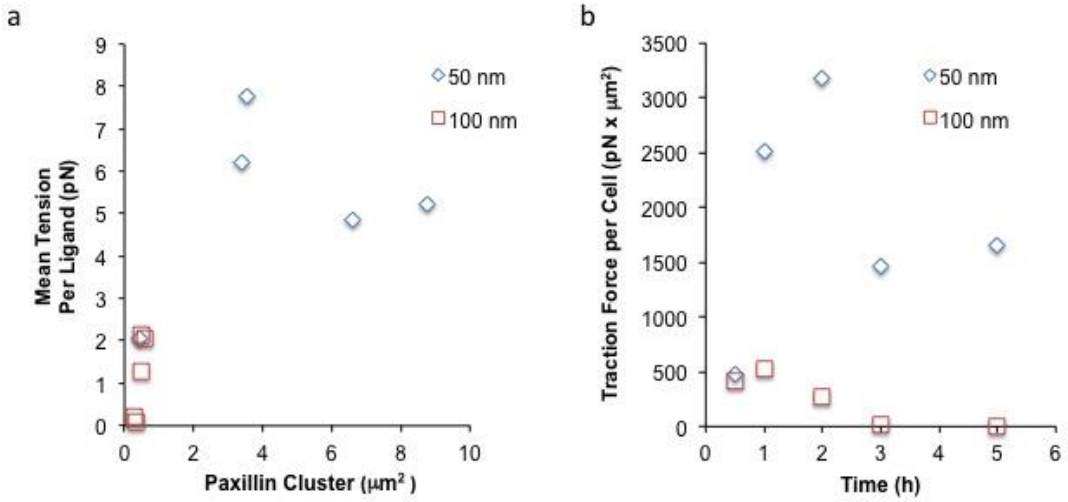

Figure S9. (a) Plot showing mean tension per integrin ligand as a function of FA size, as indicated by GFP-paxillin. The data is analyzed from the experiments shown in Fig. 3b-c (*n* = 10 cells). The blue diamonds indicate data analyzed from the 50 nm spaced AuNP array, while the red square shows the data collected from the 100 nm AuNP arrays. Each data point represents the average FA size and the corresponding average integrin ligand tension at a specific time point. (b) Plot showing total traction force per cell as a function of time. The data was obtained from 10 cells, and the adhesion area was determined by GFP-paxillin fluorescence.

Stimulating myosin contractility with LPA treatment

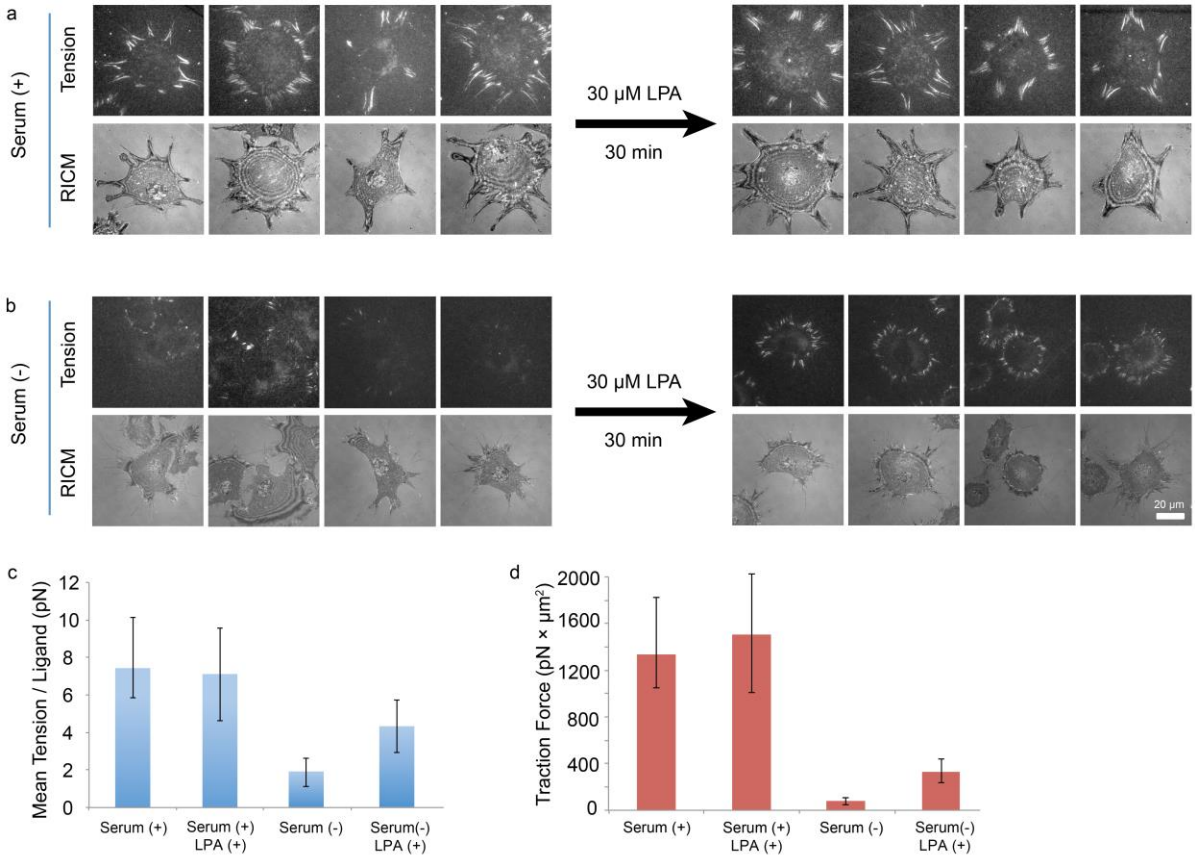

Figure S10. (a-b) Representative tension and RICM images of cells prior to and following 30 μM Oleoyl-L-α-lysophosphatidic acid (LPA) simulation. For cells that are cultured in serum free media, LPA addition led to an increase in myosin-driven integrin tension as well as an increase in FA size for NIH/3T3 fibroblasts. (c-d) Plots showing the mean integrin ligand tension and total traction force for cells (cultured for 5.5 hrs) stimulated with LPA for 30 min under serum-free and serum-enriched conditions. Total traction force is calculated as the product of the mean ligand tension by the area under the cell that displayed tension above background levels. Cells were cultured on the randomly arranged AuNP-MTFM substrates.

Stepwise inhibition of integrin tension

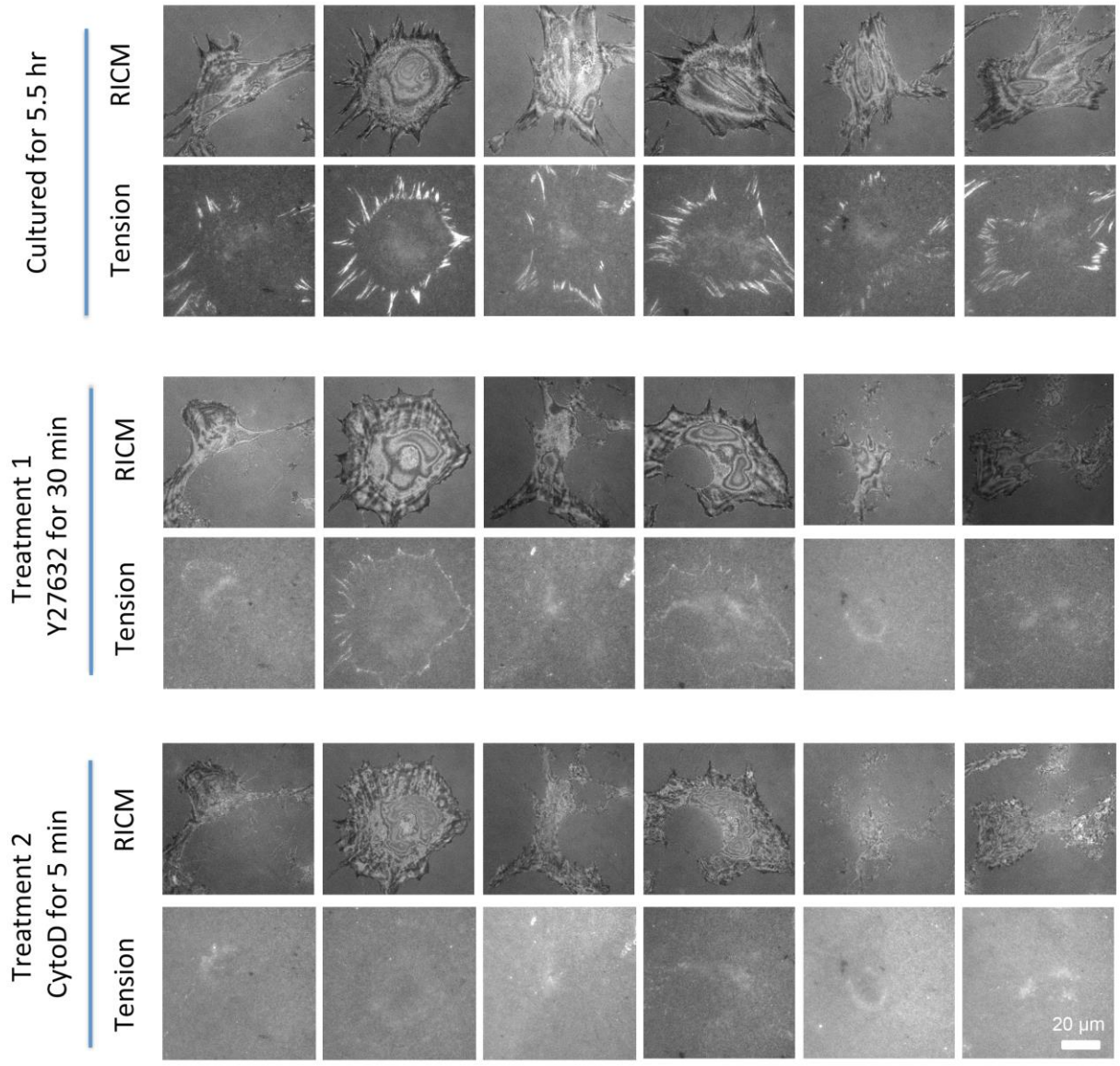

Figure S11. Representative images showing the stepwise decrease in integrin tension for NIH/3T3 cells that had been cultured on the AuNP tension probes for 5.5 hr (top two rows). The six cells were first treated with 40 μM ROCK inhibitor (Y-27632) for 30 min (middle two rows). This led to a significant reduction in integrin tension, and it also altered the localization of tension to a sub-micron region that generally resided around the cell edge. Next, the same six cells were treated with 10 μM CytoD for 5 min (bottom two rows). CytoD led to complete loss of integrin tension signal.

Quantification of integrin tension for U2OS and REF

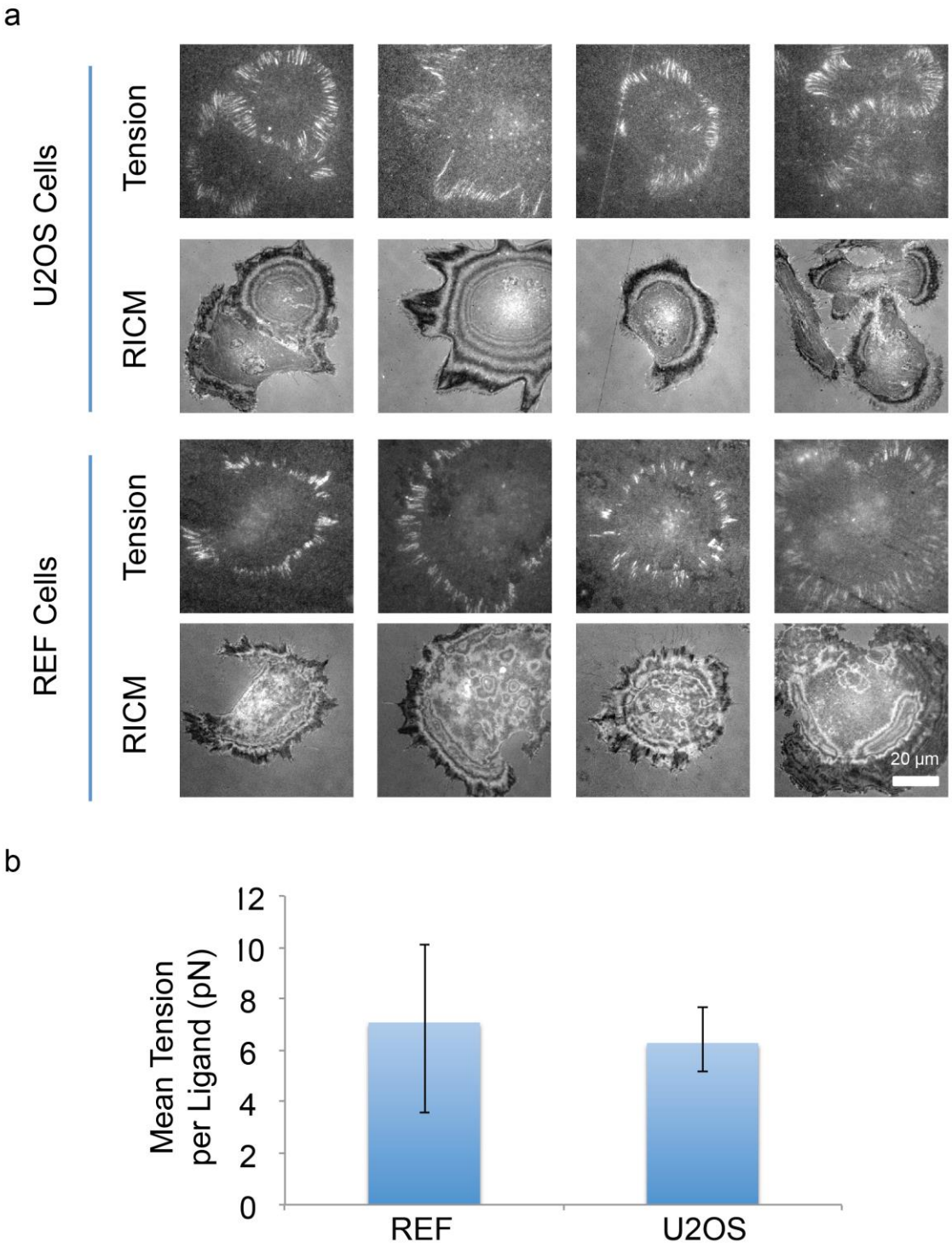

Figure S12. (a) Representative images showing RICM and integrin tension from Rat Embryonic Fibroblasts (REFs) and Human Bone Osteosarcoma Epithelial Cells (U2OS) after cell culture on the randomly arranged AuNP-MTFM substrates. (b) Plot showing the average ligand tension for the REF and U2OS cells (n = 5 cells for each cell type).

Analysis of FA maturation and integrin tension within single FA

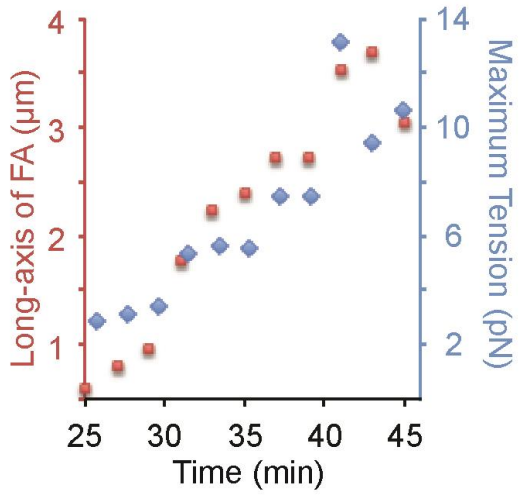

Figure S13. Plot showing the dynamic relationship between the maximum integrin tension within a single FA (right y-axis, blue) and the length of its long-axis (left y-axis, red). The data is analyzed for the highlighted FA shown in Figure 4 of main text.

The data in figure S13 shows a high correlation between integrin tension and FA size. This finding is in agreement with traction force microscopy-derived for mature FA that is typically several microns in length.

Concurrent growth of F-actin template and integrin tension

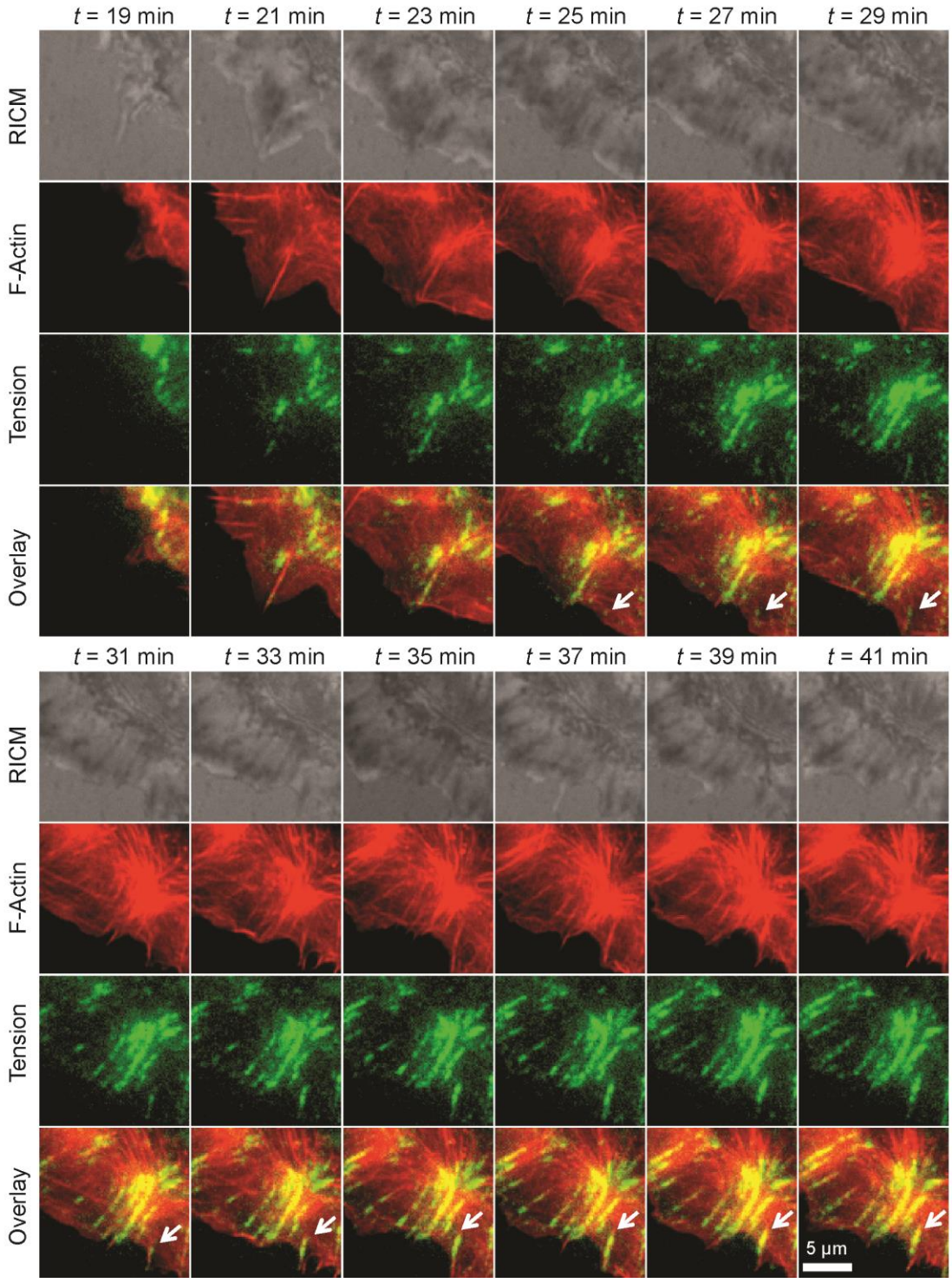

Figure S14. Timelapse RICM, GFP-lifeAct, integrin tension, and overlay actin-tension images recorded for a 3T3 NIH fibroblast during the initial stages of cell spreading and adhesion. The overlay is shown in the main text in Fig. 4, but here we display the individual fluorescence channels for references.

References

1. Glass, R.; Moller, M.; Spatz, J. P., *Nanotechnology* **2003,** *14* , 1153-1160.
2. Pasch, H.; Schrepp, W., *MALDI-TOF mass spectrometry of synthetic polymers*. Springer: 2003.
3. Bouchiat, C.; Wang, M.; Allemand, J. F.; Strick, T.; Block, S.; Croquette, V., *Biophys J* **1999,** *76*, 409-413.