Interplay of Matrix Stiffness and Protein Tethering in Stem Cell Differentiation

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SUPPLEMENTAL METHODS FOR WEN ET AL

METHODS

Polyacrylamide Gels

To covalently attach hydrogel substrates to glass, glass coverslips (Fisher) were cleaned of organics and oxidized by exposing both sides for 3 minutes to UV/ozone (BioForce). Samples were immediately functionalized with 20 mM 3-(trimethoxysilyl)propyl methacrylate (Sigma) in ethanol, washed with ethanol, and dried. A polymer solution containing acrylamide monomers, crosslinker N,N methylene-bis-acrylamide, 1/100 volume of 10% Ammunium Persulfate (APS), and 1/1000 volume of N,N,N',N'-Tetramethylethylenediamine (TEMED) was prepared. 25 μL of polymerizing hydrogel solution was sandwiched between a functionalized coverslip and a dichlorodimethylsilane (DCDMS)-treated glass slide to ensure easy detachment of hydrogels subsequent to polymerization. Hydrogels were allowed to polymerize for 15 minutes then soaked in water prior to use as a cell culture substrate. The ratio of acrylamide%/bis-acrylamide% was varied in order to control hydrogel stiffness and porosity. For 4 kPa gels, ratios of 4/0.4, 6/0.06, and 10/0.02 were used. For 13 kPa gels, ratios of 6/0.45, 10/0.1, and 20/0.03 were used. For 30 kPa gels, ratios of 8/0.55, 10/0.3, and 20/0.15 were used.

To allow for cell adhesion and fibrous protein tethering, collagen type I (BD Biosciences) was coupled to the surface using the heterobifunctional linker N-sulphosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH) as a protein-substrate linker. Substrates were incubated in 0.02, 0.1, 0.2, 0.5, or 1 mg/ml sulfo-SANPAH (Pierce) in sterile 50 mM HEPES pH 8.5, activated with UV light (wavelength 350 nm, intensity 4 mW/cm^2) for 10 minutes, washed three times in HEPES, then incubated in 50 μg/ml rat tail collagen type I in DI H_2O (BD Biosciences) overnight at room temperature. For AFM experiments, 0.5 mg/ml amine-PEG3400-biotin (Laysan Bio) was used in place of collagen type I. Coated hydrogels were stored in PBS at 4°C and UV sterilized prior to use in cell culture.

PA-PEG-RGD Gels

PA-PEG-RGD hydrogels were fabricated by incorporating acrylated-PEG3400-GRGD-amide (aPEG-RGD; 21st Century Biochemicals) into the polymerizing hydrogel solution with acrylamide monomers, N,N'-methylene-bis-acrylamide (Fisher Scientific), APS, and TEMED prior to polymerization. Acrylamide/bis-acrylamide ratios were as described in the previous section. Hydrogels were allowed to polymerize for 15 minutes then soaked in water prior to use as a cell culture substrate. Concentrations of 0.1 mM, 0.5 mM, or 2.5 mM of aPEG-RGD were incorporated into the hydrogels. In order to visualize RGD concentration differences, a fluorescent hydroxycoumarin dye (excitation 350 nm) was conjugated to the peptide to yield acrylated-PEG3400-[K-7-hydroxycoumarin-3-OH]GGGRGD-amide (21st Century Biochemicals).

PDMS Substrates

PDMS (Sylgard 184, Down Corning) was mixed at various elastomer base:curing agent ratios (50:1, 75:1, 90:1, 100:1), thoroughly mixed, and degased under vacuum for 1 hour before pouring directly into multi-well plates or onto coverslips and baked at 60°C overnight.

In certain instances, substrates were functionalized with sulfo-SANPAH and ligand (see Polyacrylamide Gels) (Supplementary Fig. S13a). For covalent attachment of moieties to the surface, PDMS substrates were treated with UV/Ozone for 5 minutes (BioForce) following a 1 hour incubation under vacuum in the presence of (3-aminopropyl)triethoxysilane (APTES). Surfaces were then incubated in a 1 mg/mL solution of sulfo-NHS-biotin in water (G-Biosciences) for one hour at room temperature (Supplementary Fig. S13b).

Scanning Electron Microscopy

PA and PA-PEG-RGD solutions were polymerized in 1.7 mL microtubes or on glass coverslips. Hydrogels were swelled in water overnight, flashed frozen in liquid nitrogen, then lyophilized over night. Lyophilized samples were sputter coated for 6 seconds with iridium. SEM (FEI XL30 and Zeiss Sigma VP) images were taken at 200x, 1000x, or 5000x at 5 kV.

DNA Gel Electrophoresis

GeneRuler 1 kb (250-10,000 bp) and 1kb Plus (75-20,000 bp) DNA ladders (Thermo Scientific) and HyperLadder V (50 to 500 bp; Bioline) were run through polyacrylamide electrophoresis gels in TAE buffer with ethidium bromide at 30V for 14 hours. Images were taken using a ChemiDoc MP system (Bio Rad). DNA fragment lengths were converted to radius of gyration described elsewhere.[22]

Stem Cell Culture

Human ASCs were isolated from freshly aspirated human subcutaneous adipose tissue (donor age between 26 and 31 years) according to the method described elsewhere[33]. To reduce the effects of donor-to-donor variation, cells from three different donors were pooled. Commercially available MSCs were purchased from Lonza. MSCs and ASCs (P4-P7) were cultured in low glucose Dulbecco's modified eagle medium (DMEM) (Gibco) with 10% fetal bovine serum (Gemini) and 1% antibiotics (Corning). For differentiation experiments, MSCs and ASCs were seeded on PA and PDMS substrates at a density of 1,000 cells/cm^2 and on PA-PEG-RGD gels at a density of 2,000 cells/cm^2 and cultured for 7 or 14 days; the medium was changed every 2-3 days. For studies conducted with osteogenic inductive media, growth media was supplemented with 100 nM dexamethasone (Sigma), 200 μm ascorbic acid (Sigma) and 10 mM glycerol 2-phosphate (Sigma). For studies conducted with adipogenic inductive media, growth media supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 100 nM dexamethasone, 10 μg/ml insulin (Sigma), and 10 μM indomethacin (Sigma).

Immunofluorescence

Cells were fixed in 10% formalin for 15 minutes at room temperature, rinsed in PBS, and permeabilized with 1% triton-X in PBS for 10 minutes at room temperature. Cells were stained with 1:500 rhodamine phalloidin (Life Technologies) and 1:5000 DAPI in 2% bovine serum albumin in PBS for 30 minutes. Samples were washed with DI water and mounted with fluoromount-G (SouthernBiotech). Imaging was performed using a Nikon Eclipse TI microscope equipped with a CARV II confocal system (BD Biosciences), motorized stage and MS-2000 controller (Applied Scientific Instrumentation), and a Cool-Snap HQ camera (Photometrics) and controlled by Metamorph (Molecuar Devices). For osteogenic differentiation studies, cells were stained with 1:100 RUNX2 (ab-23981; Abcam). To quantify RUNX2 expression, CellProfiler[34] (Broad Institute) was used to measure cytoplasmic and nuclear fluorescent intensities using the nuclei and cell outlines as masks to define these regions of interest in the RUNX2 fluorescent channel.

Differentiation Assays

ASCs and MSCs were stained for alkaline phosphatase (ALP), an osteogenic marker, using a Millipore ALP detection kit (SCR004; Millipore) according to the manufacturer's instructions. Briefly, cells were fixed in 10% formalin for 2 minutes at room temperature, rinsed in PBS, then incubated in staining solution composed of a 2:1:1 ratio of Fast Red Violet, naphthol AS-BI phosphate, and DI H_2O at room temperature in the dark for 15 minutes. Subsequent to staining, samples were rinsed in PBS then imaged.

ASCs were stained for Oil Red O (ORO), an adipogenic marker. Briefly, cells were fixed in 10% formalin for 15 minutes at room temperature, rinsed in PBS, incubated in 60% isopropanol for 5 minutes, then incubated in a filtered staining solution composed of 1.8 mg/ml ORO in 60% isopropanol in DI H_2O for 20 minutes. Subsequent to staining, samples were rinsed in DI H_2O then imaged.

Atomic Force Microscopy

To determine the mechanical properties of the various PA hydrogels by indentation and to quantify protein tethering by force spectroscopy, a MFP-3D-Bio (Asylum Research) atomic force microscope (AFM) was used. Chromium/gold-coated, silicone nitride (SiN) cantilevers with pyramid-shaped tips (PNP-TR; NanoWorld) with ~50 pN/nm nominal spring constants as determined from the MFP-3Ds built-in calibration function were used for both methods. All AFM data was analyzed using custom written code in Igor Pro (Wavemetrics). For indentation, samples were mounted on glass slides using vacuum grease and then placed on the AFM stage. Samples were placed in PBS and indented at a velocity of 2 μm/s until a trigger of 2 nN was detected using. Indentation curves were analyzed to determine the Young's modulus as previously described[35].

PDMS substrates were indented with the same cantilevers mentioned above. Additionally a cantilever with a 45μm diameter polystyrene bead tip (Novascan) with 0.03 N/m nominal spring constant was used. Prior to indentation, all PDMS substrates were pretreated with a solution of 1% bovine serum albumin in PBS to limit non-specific adhesive interactions between the tip and the PDMS surface. Indentation curves were obtained and analyzed as described above. For retraction experiments, samples were indented with approach and retraction velocities ranging from 1 nm/s to 10 μm/s. The substrate spring constants were determined by fitting the linear portion of the retraction curve starting at the undeformed surface.

For force spectroscopy, cantilevers were functionalized (Fig. 3a) with the antibody C2456, a mouse monoclonoal anti-collagen type I antibody (Sigma), or avidin (PROSPEC) using a previously established method.[36,37] Briefly, the cantilevers were cleaned with chloroform for 30 seconds and incubated overnight immersed in 5 M ethanolamine-HCl in dimethylsulfoxide at room temperature resulting in amine group functionalization on the cantilever tips. After rinsing with phosphate-buffered saline (PBS), tips were incubated in 25 mM bis[sulfosuccinimidyl] suberate (BS3; Pierce) for 30 minutes. After rinsing again in PBS, tips were immersed in 200 μg/ml C2456 or 1 mg/mL avidin for 30 minutes to crosslink the protein to the tip. Functionalized cantilevers were rinsed, dried, and kept in 4°C until use. Samples were placed in PBS and force curves were taken in a regular 10x10 array of points spaced ~10 μm apart using a functionalized SiN cantilever at 2 μm/s. To promote binding of the antibody to collagen or avidin to biotin, a dwell time of 1 second was added between approach and retraction cycles. Force curves were converted to force vs. tip Z-position curves (Supplementary Fig. S6a) and then analyzed for rupture events using a previously described algorithm;[38] rupture lengths and forces were determined.

Traction Force Microscopy

Traction force microscopy was performed as previously described.[23] Briefly, fluorescent 580/605 0.2 μm microspheres (Invitrogen) were added to the pre-polymer solution to a final concentration of 1% v/v. Substrates were functionalized and treated as described above. The microspheres underneath selected live cells were imaged with a 40x dry confocal objective (Zeiss) using the CV1000 high-speed laser confocal imaging system equipped with an EM-CCD camera and a stage-top incubation system (Yokogawa). Cells were released with 2.5% trypsin and the same confocal stacks were acquired. Bead displacements were determined using a particle image velocimetry script in Matlab (MathWorks).

Förster Resonance Energy Transfer

Fibronectin was isolated from human plasma using gelatin-sepharose binding and eluted with 6M urea. Isolated fibronectin was concentrated to ~3 mg/ml using an Amicon Ultra Centrifugal Filter (10 kDa NMWL) (Millipore), according to manufacturer's instructions, and denatured for 15 minutes in 4 M guanidine hydrochloride (GdnHCl). Denatured fibronectin was dual-labeled

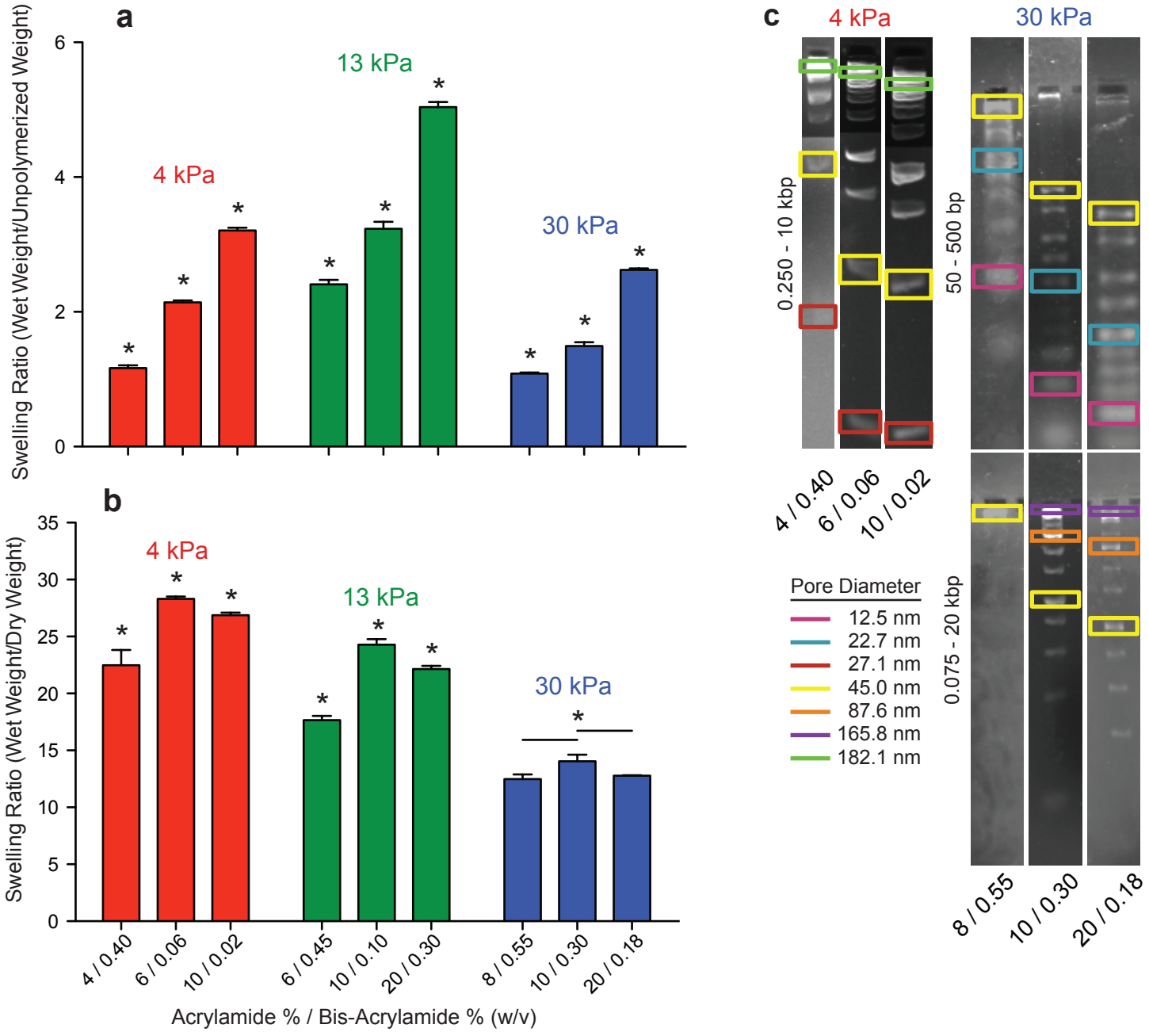
with Alexa Fluor 488 (donor) and Alexa Fluor 546 (acceptor) fluorophores, as previously described.[13] Briefly, denatured fibronectin was incubated with a 30-fold molar excess of Alexa Fluor 546 C5 Maleimide (Life Technologies) for 2 hours to label cysteine residues within the III_7 and III_{15} domains of fibronectin. The single-labeled fibronectin was buffer exchanged into 0.1 M sodium bicarbonate pH 8.3 and separated from unreacted acceptor fluorophores using a spin desalting column (Thermo Scientific), according to manufacturer's instructions. The single-labeled fibronectin was then incubated with a 40-fold molar excess of Alexa Fluor 488 succinimidyl ester (Life Technologies) for 1 hour to label amine residues throughout fibronectin. Unreacted donor fluorophores were removed using a spin desalting column and dual-labeled fibronectin was stored with 10% glycerol at -20 °C. The average number of acceptor and donor fluorophores per fibronectin dimer was 3.6 and 8.8, respectively, and was determined using published extinction coefficients and the absorbances of the dual-labeled fibronectin at 280, 498, and 556 nm.

The emission spectrum of the dual-labeled fibronectin was characterized in varying concentrations of denaturant by fluorescence spectroscopy using a Synergy 4 Microplate Reader (BioTek). Briefly, 100 μg/ml dual-labeled fibronectin in PBS was denatured in 0 to 4 M GdnHcl and excited at 484 nm. The resulting emission spectrum was measured from 510 to 700 nm (Supplementary Fig. 9b) and the ratio of the maximum acceptor emission (~570 nm) to the maximum donor emission (~520 nm) was determined at each concentration of GdnHCl (Supplementary Fig. 9c).

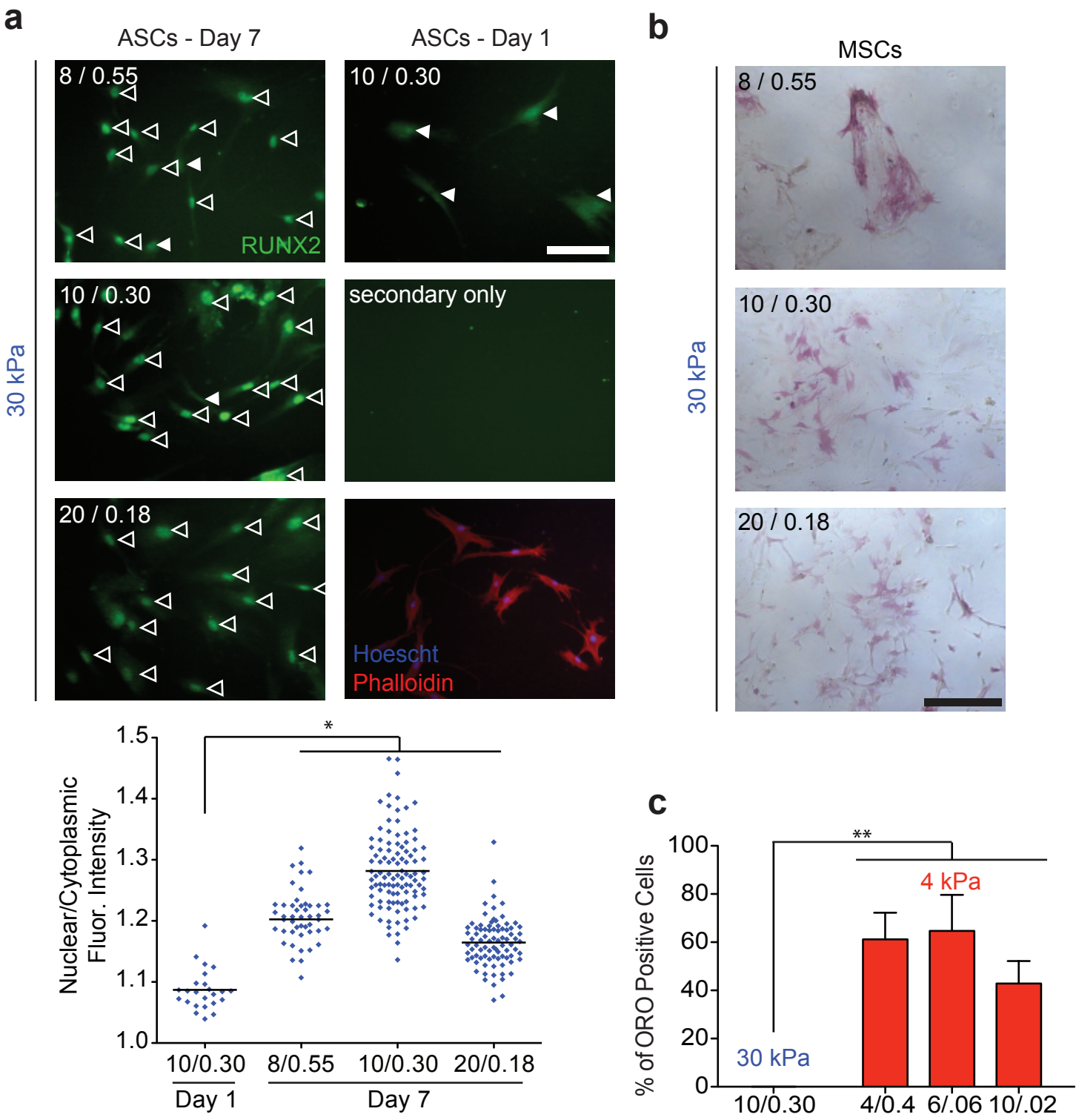
Images of the dual-labeled fibronectin were acquired on a Zeiss LSM 780 Confocal Microscope and analyzed using a custom MATLAB script, as previously described.[14] Briefly, images were averaged with a 3 x 3 pixel sliding block and perinuclear regions of DAPI stained cells were manually selected for analysis. The FRET ratio for each pixel within a selected region was calculated by dividing that pixel's intensity in the acceptor image by its corresponding intensity in the donor image. FRET ratios less than 0.05 and greater than 1.0 were excluded from analysis. The mean FRET ratio within the selected regions was calculated for each cell and then averaged over all the cells in each condition (n = 16 cells per condition) (Fig. 2g).

Statistics

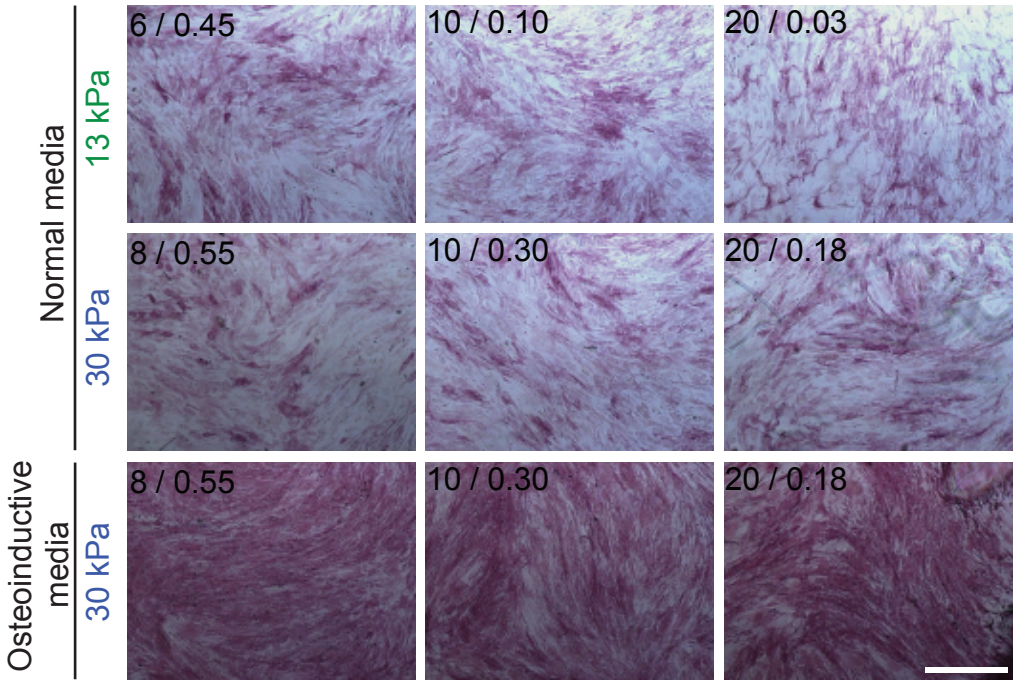
Data are expressed as mean ± standard deviation or standard error of the mean as indicated. For statistical analyses, student's t-test were performed on the data presented in Supplementary Fig. S9d, Fig. 2c, Fig. 3d, and Supplementary Fig. S12b. 2-way ANOVAs were performed on the data in Fig. 2f and Fig. 2g. 1-way ANOVAs were performed on all other data where significance is shown. Multiple comparisons Tukey post tests were performed where appropriate. Differences were considered significant when $*p<0.05$ and $**p<0.0001$. All experiments were performed in triplicate unless otherwise noted, and in such cases, the number of cells used in the measurement has been stated.



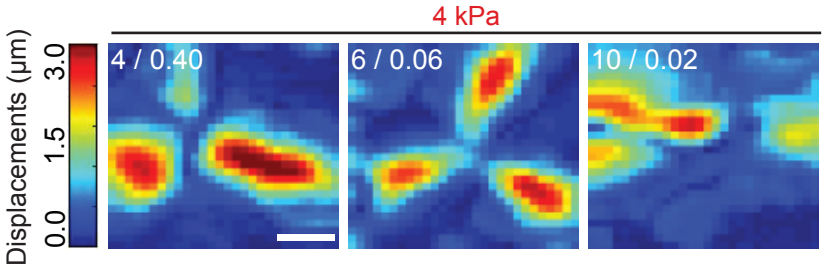
Supplementary Figure 1. *Characterization of porosity on PA gels made with various monomer to crosslinker ratios.* **a**, Volume swelling ratio - polymerized wet weight/unpolymerized wet weight - and **b**, mass swelling ratio - polymerized wet weight/dry weight (n = 3; mean ± S.D.; *p< 0.05). **c**, Ethidium bromide stained DNA fragments that were subjected to electrophoresis through the hydrated hydrogels of indicated compositions. For 4 kPa hydrogels (left), the DNA ladder ranged from 250-10,000 base pairs (bp). For 30 kPa hydrogels (right), the DNA ladders ranged from 50-500 bp (top) and 75-20,000 bp (bottom).



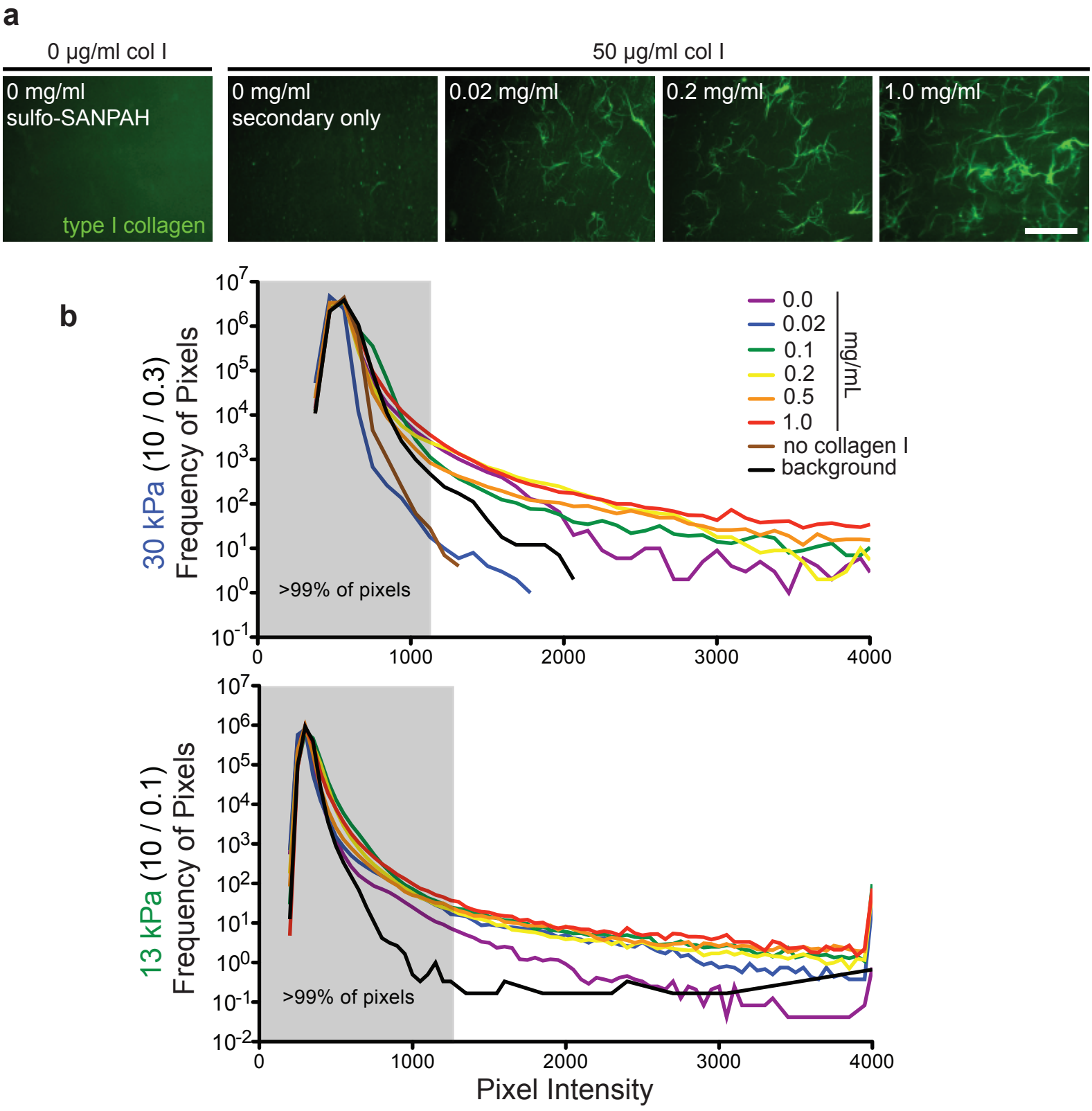
Supplementary Figure 2. Osteogenic and adipogenic differentiation as a function of porosity. **a**, ASCs stained for RUNX2 on different formulations of 30 kPa PA hydrogels after 1 and 7 days. Open arrows indicate positive nuclear RUNX2 staining. Filled arrows indicate the absence of RUNX2 staining (scale bar, 100 μm). RUNX2 nuclear to cytoplasmic mean fluorescent intensity ratio for ASCs was plotted at the bottom of the panel for the indicated conditions. (n > 20 cells; *p< 0.05). **b**, Images of MSCs stained for ALP on different formulations of 30 kPa hydrogels after 14 days (scale bar, 500 μm). **c**, Percent of ASCs expressing ORO on the indicated formulations of 4 and 30 kPa hydrogels after 7 days of culture in adipogenic induction media. Data represents an average and standard deviation per field of view. (n > 50 cells; **p< 0.0001).



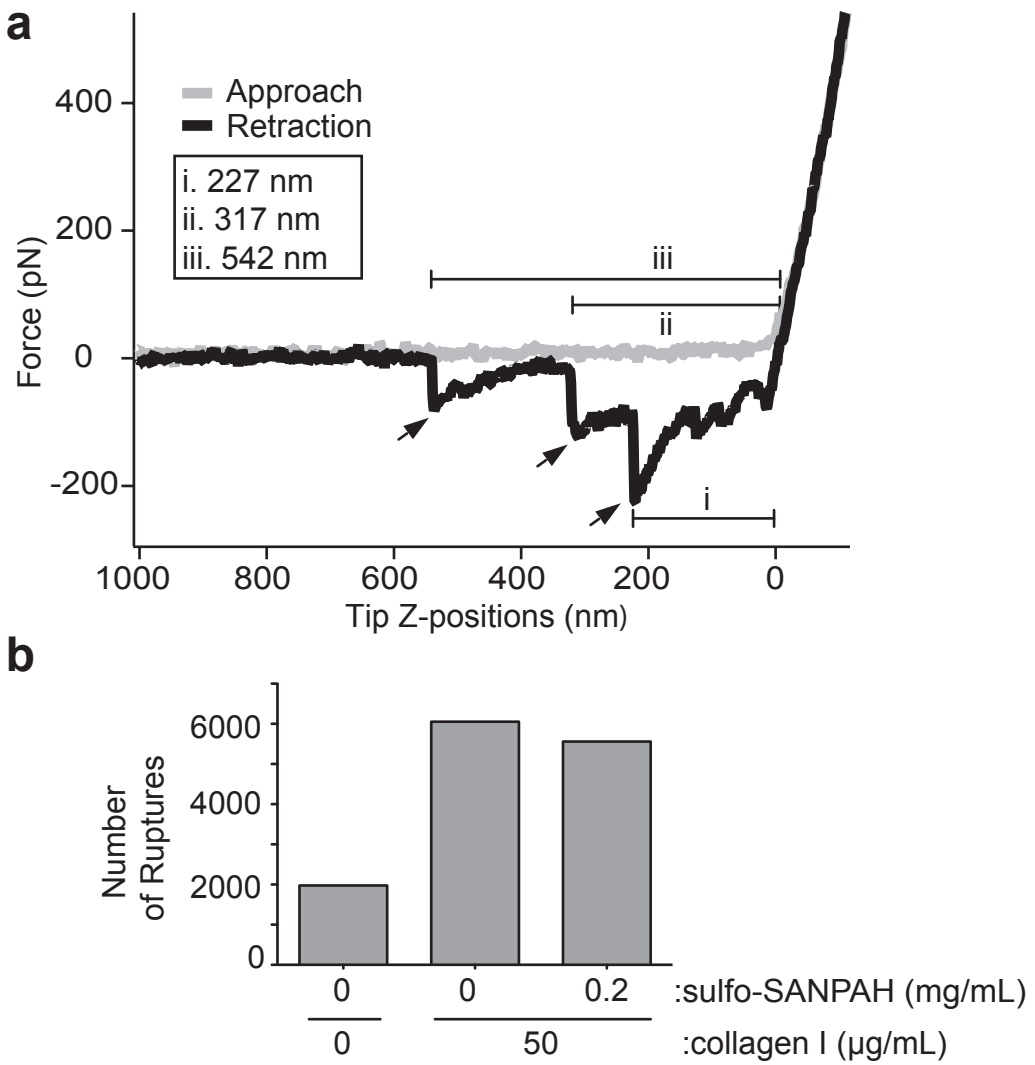
Supplementary Figure 3. Effect of confluence on ASC osteogenesis. Images show representative ALP staining of ASCs grown to confluence on 13 and 30 kPa PA hydrogels of varying compositions in either normal or osteogenic induction media after 14 days (scale bar, 500 μm).



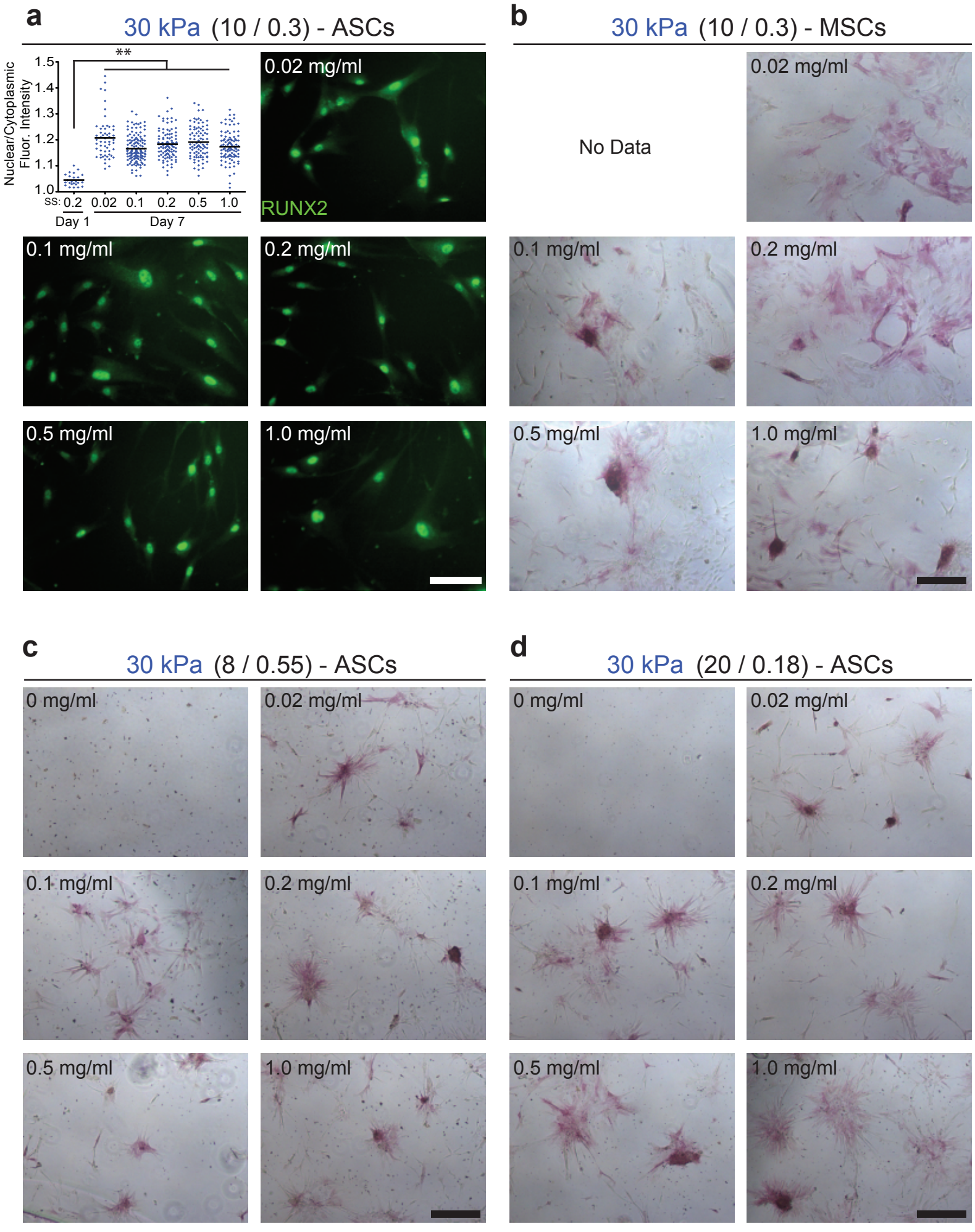
Supplementary Figure 4. Displacement maps computed from traction force microscopy. Representative displacement maps of embedded fluorescent particles resulting from ASC traction forces on 4 kPa hydrogels of varying porosity. Color bar spans particle displacements ranging from 0-3 µm. (scale bar, 50 µm).



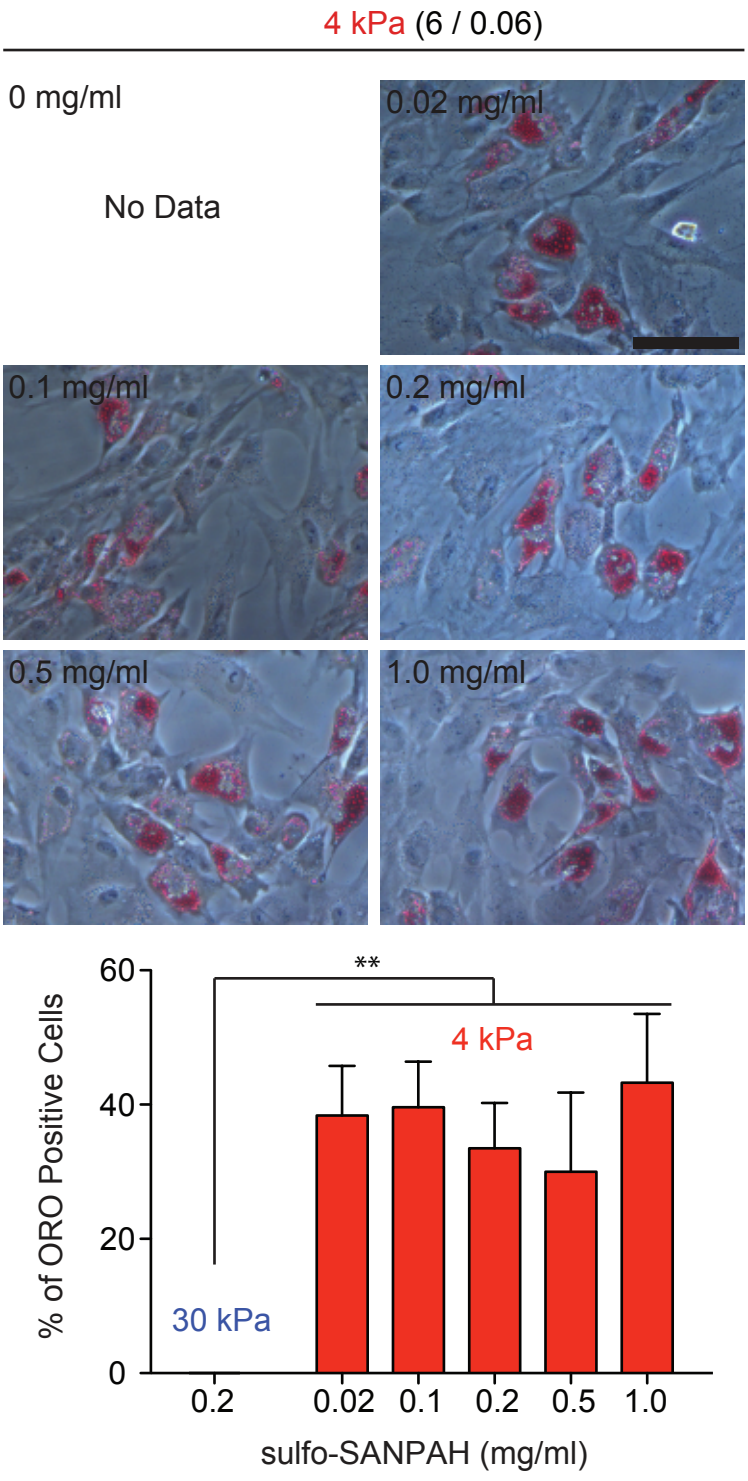
Supplementary Figure 5. *Visualization of surface protein coating on PA substrates*. **a**, Collagen staining of 30 kPa hydrogels activated with 0, 0.02, 0.2, and 1 mg/ml sulfo-SANPAH and subsequently coupled with collage type I (scale bar, 500 µm). **b**, Histograms of image pixel intensities for 13 kPa (bottom, n = 8) and 30 kPa hydrogels (top, n = 5) for a range of sulfo-SANPAH concentrations.



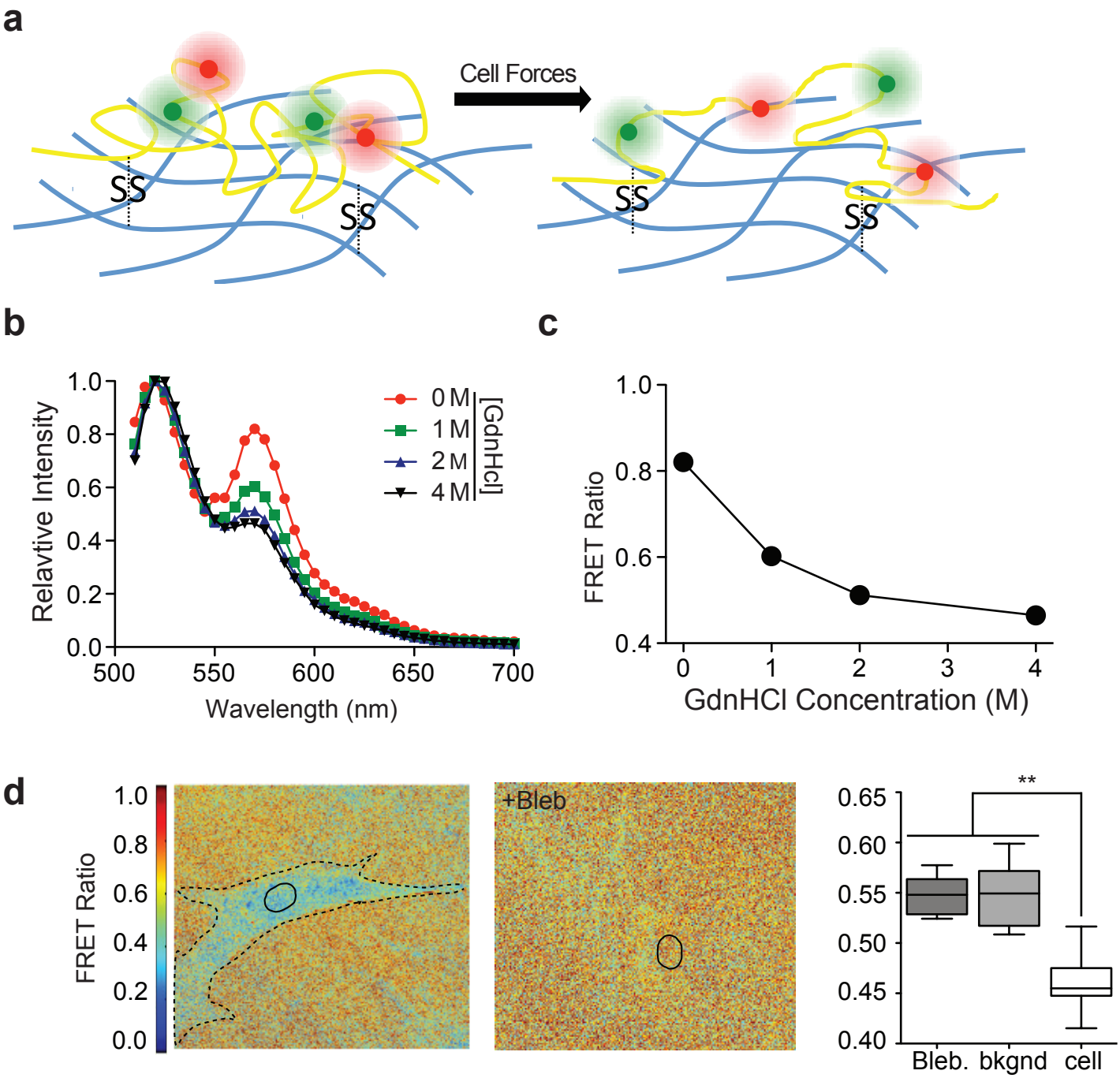
Supplementary Figure 6. *Atomic force spectroscopy validation.* **a**, Representative force vs tip-sample separation spectrogram of the collagen antibody-functionalized tip approaching (grey) and retracting (black) from a collagen I functionalized hydrogel. Rupture events between the antibody functionalized tip and collagen (arrows) are indicated with their distances from the substrate. **b**, The number of rupture events detected for PA hydrogels with and without collagen and sulfo-SANPAH as indicated (n = 500).


a
30 kPa (10 / 0.3) - ASCs
**
Nuclear/Cytoplasmic Fluor. Intensity
1.5
1.4
1.3
1.2
1.1
1.0
SS: 0.2 0.02 0.1 0.2 0.5 1.0
Day 1
Day 7
0.02 mg/ml
RUNX2
0.1 mg/ml
0.2 mg/ml
0.5 mg/ml
1.0 mg/ml
b
30 kPa (10 / 0.3) - MSCs
No Data
0.02 mg/ml
0.1 mg/ml
0.2 mg/ml
0.5 mg/ml
1.0 mg/ml
c
30 kPa (8 / 0.55) - ASCs
0 mg/ml
0.02 mg/ml
0.1 mg/ml
0.2 mg/ml
0.5 mg/ml
1.0 mg/ml
d
30 kPa (20 / 0.18) - ASCs
0 mg/ml
0.02 mg/ml
0.1 mg/ml
0.2 mg/ml
0.5 mg/ml
1.0 mg/ml

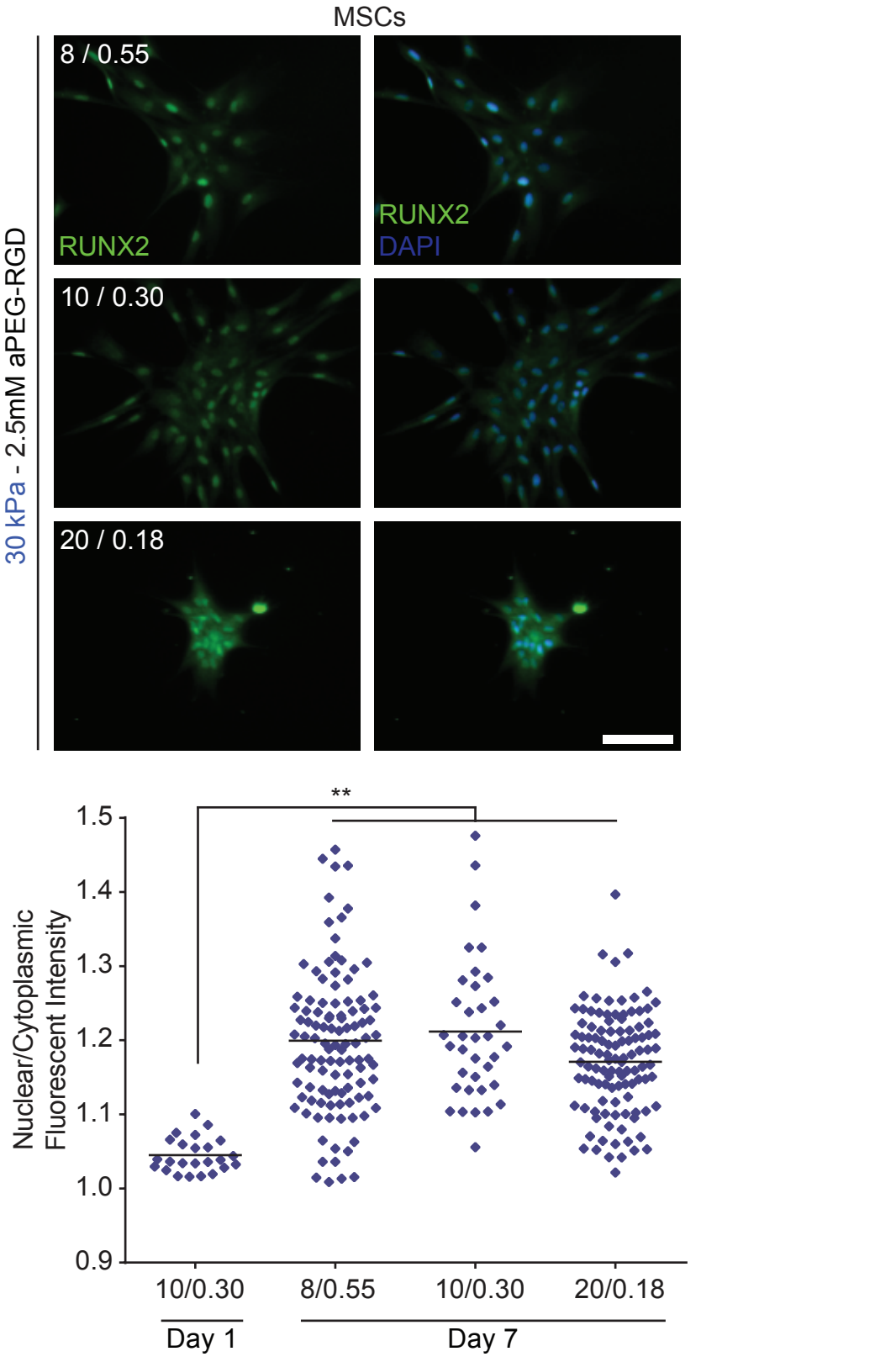
Supplementary Figure 7. *Osteogenic differentiation as a function of sulfo-SANPAH concentration.* **a**, ASCs stained for RUNX2 on 10/0.3 30 kPa hydrogels as a function of sulfo-SANPAH concentration after 7 days (scale bar, 100 μm). RUNX2 nuclear to cytoplasmic mean fluorescent intensity of ASCs was plotted for the indicated time points and sulfo-SANPAH concentrations ($n > 20$ cells; $^{**}p < 0.0001$). **b**, MSCs stained for ALP on 10/0.3 hydrogels as a function of sulfo-SANPAH concentration after 14 days (scale bar, 500 μm). **c**, ASCs stained for ALP on 8/0.55 30 kPa hydrogels and **d**, 20/0.18 30 kPa hydrogels as functions of sulfo-SANPAH concentration after 14 days (scale bar, 250 μm). Sulfo-SANPAH concentrations range from 0 to 1 mg/ml as indicated.



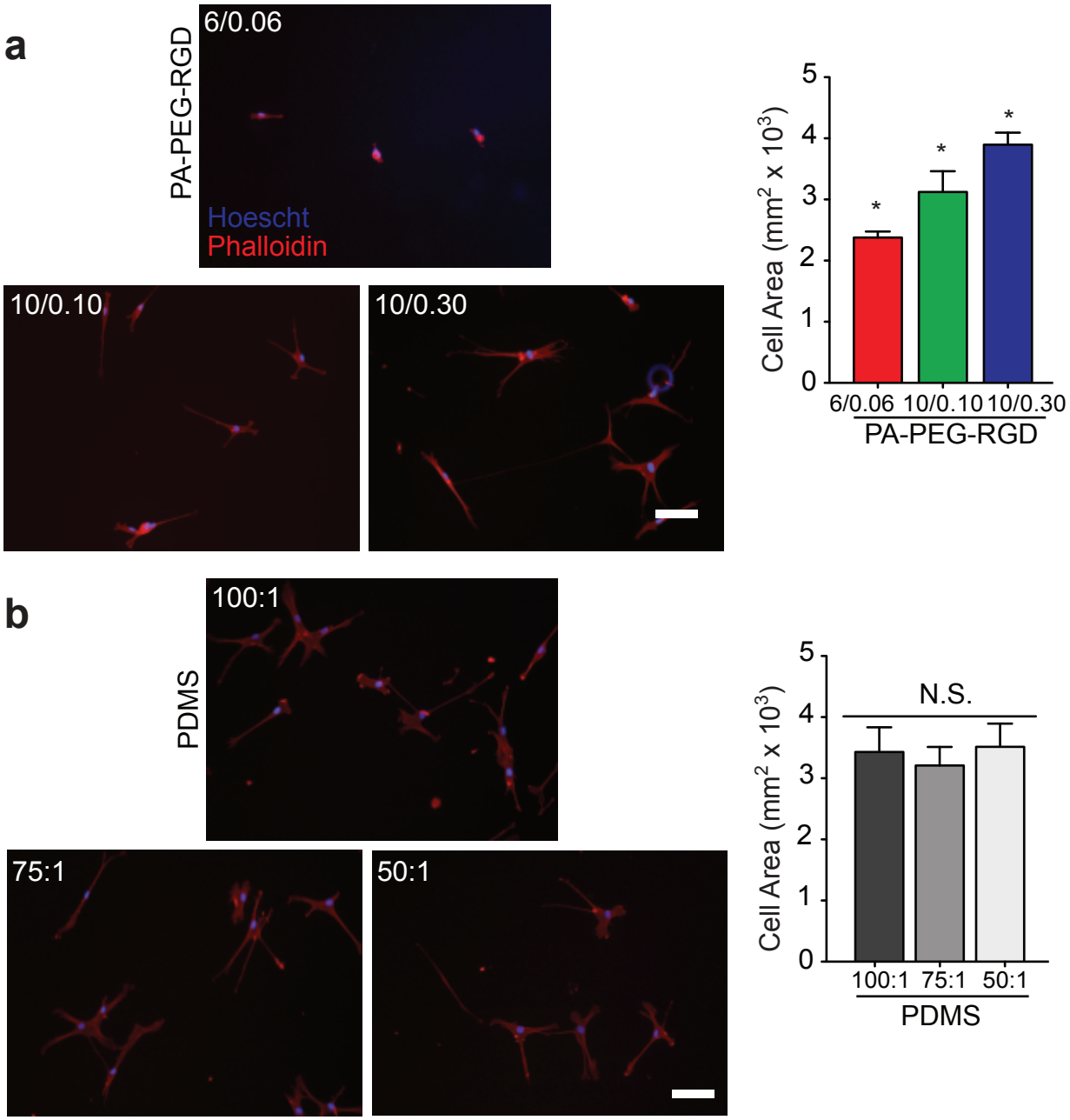
Supplementary Figure 8. *ASC adipogenic differentiation as a function of sulfo-SANPAH concentration.* ORO staining of ASCs on 4 kPa 6/0.06 hydrogels for sulfo-SANPAH concentrations ranging from 0 to 1 mg/ml (scale bar, 250 μm) (top). Percent of ASCs expressing ORO on the indicated formulations of 4 and 30 kPa hydrogels after 7 days of culture in adipogenic induction media (bottom). Data represents an average and standard deviation per field of view. (n > 50 cells; **p< 0.0001).



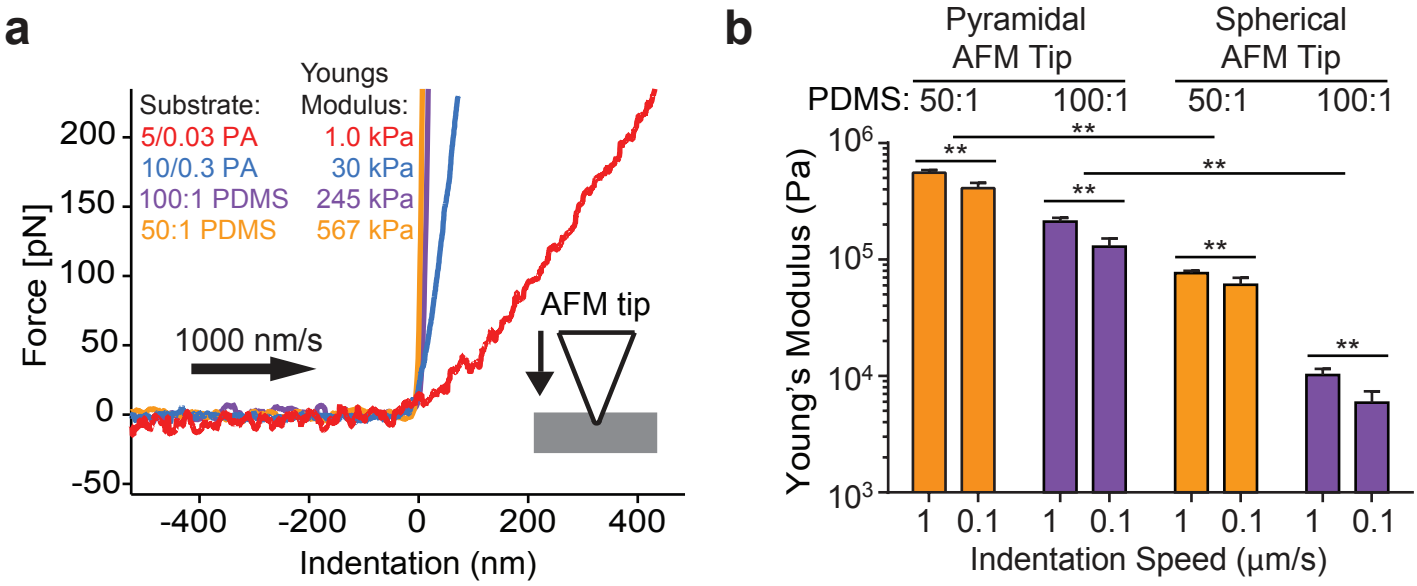
Supplementary Figure 9. *FRET characterization of dual-labeled fibronectin*. **a**, Cell forces are applied to the dual-labeled fibronectin (left to right), causing the fibronectin to unfold, and fluorophores to move apart, resulting in a decrease in FRET intensity ratio. **b**, Emission spectrum of dual-labeled fibronectin denatured in 0-4M GdnHCl and excited at 484 nm **c**, Resultant FRET intensity ratios. **d**, Fibronectin FRET intensity ratio map of an untreated ASC (left) and a blebbistatin treated ASC (center) on a 10/0.3 hydrogels activated with 0.2 mg/ml sulfo-SANPAH (left), and measured FRET intensity ratio for the undeformed protein surrounding a cell, the deformed protein underneath a blebbistatin treated cell and an untreated cell on 10/0.3 hydrogels (right) (n = 8; **p<0.0001).



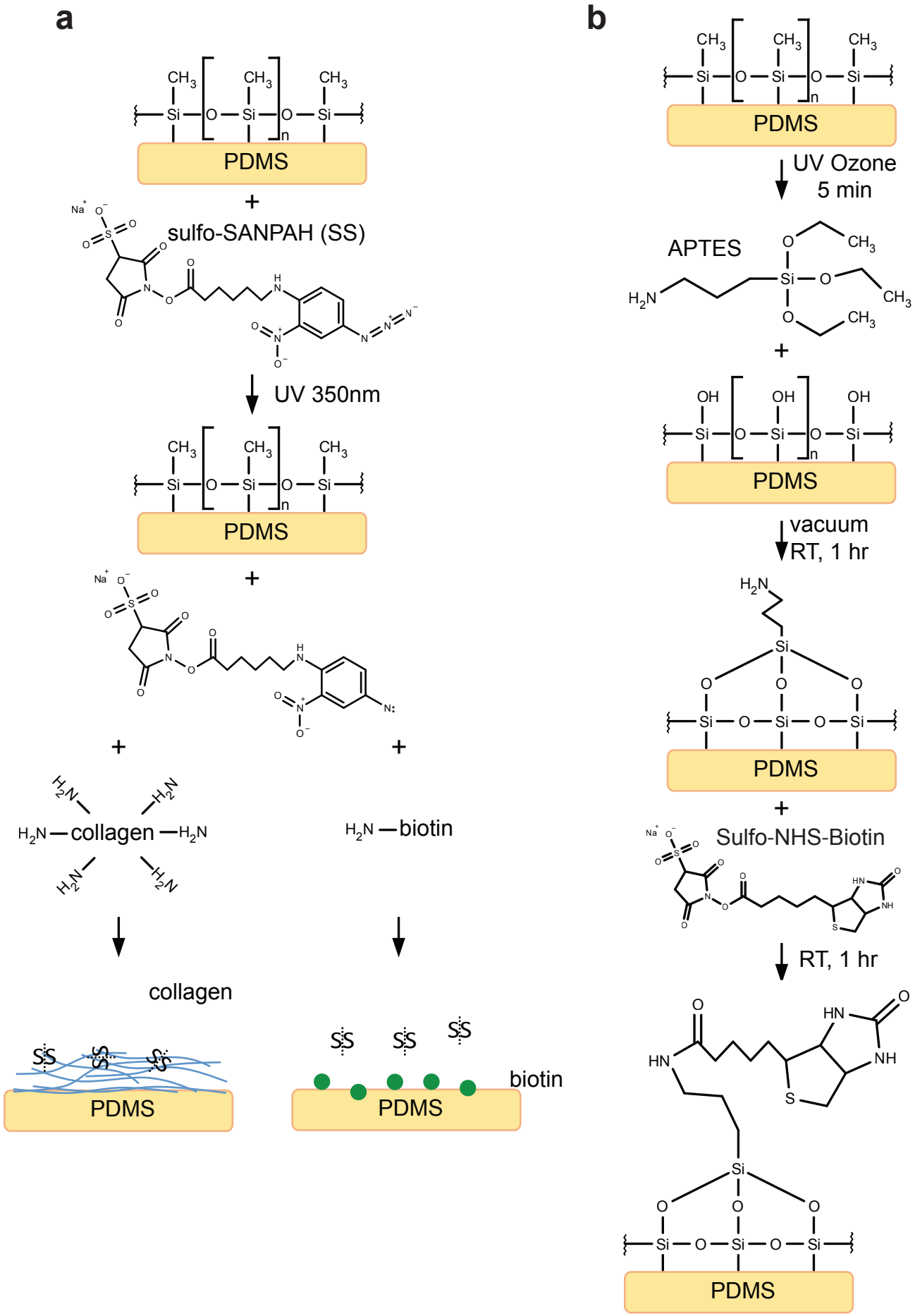
Supplementary Figure 10. *MSC osteogenic differentiation on RGD hydrogels.* RUNX2 staining after 7 days of culture of MSCs in normal media (top) and their corresponding RUNX2 nuclear to cytoplasmic mean fluorescent intensity ratio (bottom) are shown for different formulations of 30 kPa hydrogels and constant 2.5 mM RGD. For the ratio, day 1 cells are shown as a reference (scale bar, 100 μm; n > 35 cells; **p <0.0001).



Supplementary Figure 11. *ASC spread area on tether-less hydrogel systems.* **a**, Images show ASCs on PA-PEG-RGD (2.5 mM aPEG-RGD) 24 hours after seeding (phalloidin staining, red; nuclei staining, blue). At right is the quantification of cell spread area on PA-PEG-RGD substrates 24 hours after seeding (scale bar, 100 μm). **b**, Images show ASCs on PDMS substrates 24 hours after seeding (phalloidin staining, red; nuclei staining, blue). At right is the quantification of cell spread area on PDMS substrates 24 hours after seeding (scale bar, 100 μm). (n > 40; mean ± S.E.M; *p<0.05).



Supplementary Figure 12. *Mechanical characterization of PDMS substrates by atomic force microscopy*. **a**, Force spectrograms from indentations of PDMS substrates fabricated from 50:1 and 100:1 base to curing agent ratio (yellow and purple, respectively) and 1 and 30 kPa PA hydrogels (red and blue, respectively) performed with a pyramidal cantilever. **b**, Calculated Young's modulus from indentations on 50:1 and 100:1 PDMS substrates with either pyramidal (yellow) or spherical tips (purple) at different indentation speeds (**p<0.0001).


a
PDMS
sulfo-SANPAH (SS)
UV 350nm
PDMS
collagen
biotin
collagen
PDMS
biotin
PDMS
b
PDMS
UV Ozone
5 min
APTES
PDMS
vacuum
RT, 1 hr
PDMS
Sulfo-NHS-Biotin
RT, 1 hr
PDMS

Supplementary Figure 13. *Diagram for the covalent attachment of protein to the surface of PDMS substrates.* **a**, Sulfo-SANPAH does not react with the methyl groups of untreated PDMS upon UV activation, but may interact non-specifically with the PDMS surface. Collagen and biotin-NH2 adsorb to the PDMS surface. Collagen amine-containing residues may react with sulfo-SANPAH molecules leading to an accumulation of crosslinked collagen at the surface. Biotin-NH2 one contains a single free amine, and thus cannot aggregate at the PDMS surface. **b**, Activation of the PDMS surface with UV ozone and APTES coats the PDMS surface with primary amines, which react with the sulfo-succinimidyl functional group of sulfo-NHS-biotin, covalently attaching a single biotin molecule.