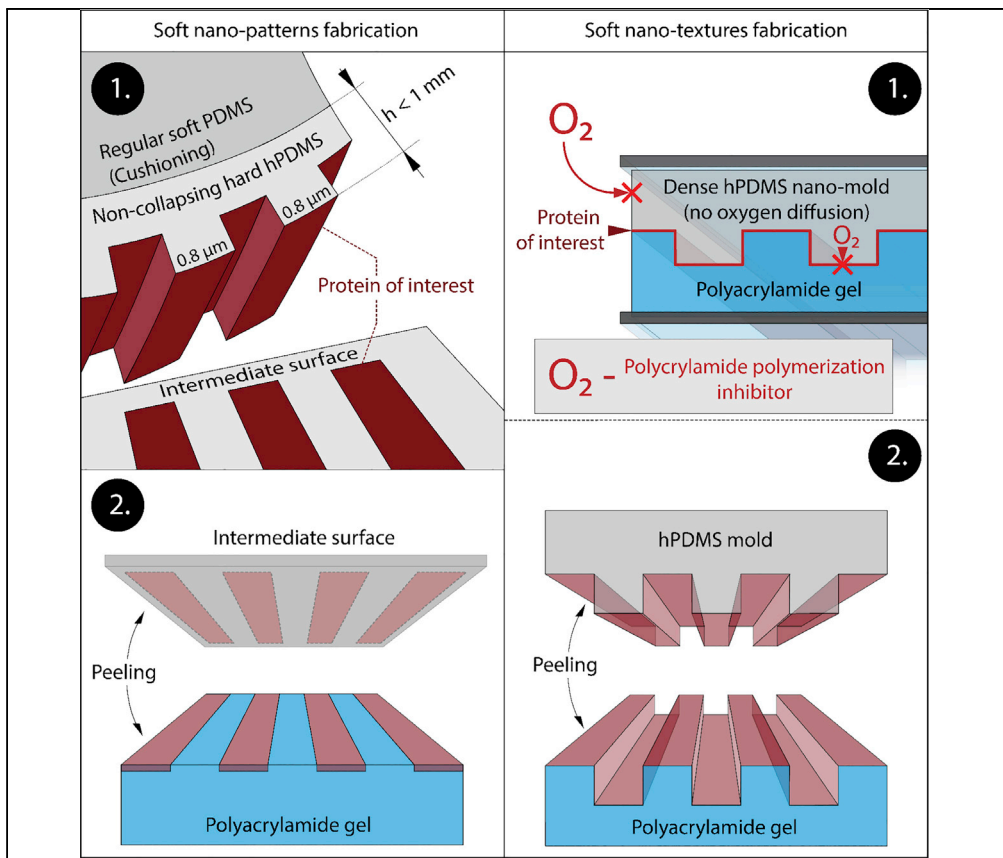


Protocol

Engineering Elastic Nano- and Micro-Patterns and Textures for Directed Cell Motility



We present a reproducible protocol for fabrication of polyacrylamide (PAA) hydrogel-based nano-patterns and nano-textures with a wide range of elastic rigidities to study fundamental cell behaviors, such as mechanosensitivity and motility. We explore the benefits of this protocol by successfully testing the compatibility of the PAA platforms with super-resolution microscopy, which is largely unavailable with platforms of nano-scale textures made from different polymers. We also utilized soft and rigid nano-textures to study the mechanosensing basis of T cell behavior and phenotype.

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HIGHLIGHTS

Contact guidance
cues are critical for
directed cell
migration in health
and disease

Manufacturing nano-
and micro- scale
patterns and textures
with a wide range of
rigidities

The engineered
platforms can be used
to study motility in a
wide range of cell
types

Deformable nano-
textures are
compatible with
super-resolution
imaging

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Protocol

Engineering Elastic Nano- and Micro-Patterns and Textures for Directed Cell Motility

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SUMMARY

We present a reproducible protocol for fabrication of polyacrylamide (PAA) hydrogel-based nano-patterns and nano-textures with a wide range of elastic rigidities to study fundamental cell behaviors, such as mechanosensitivity and motility. We explore the benefits of this protocol by successfully testing the compatibility of the PAA platforms with super-resolution microscopy, which is largely unavailable with platforms of nano-scale textures made from different polymers. We also utilized soft and rigid nano-textures to study the mechanosensing basis of T cell behavior and phenotype.

For complete information on the generation and use of this protocol, please refer to Tabdanov et al. (2018b).

BEFORE YOU BEGIN

Prior to beginning the nano-texture or nano-pattern manufacturing session, the basic consumables for the protocols need to be prepared and inventorized. Each of the consumables is described and its formulation listed in this section.

Polyacrylamide Elastic Gels (PAA Gels) Formulations for Projected Shear Modulus G'

⌚ TIMING: 1–2 h

The final mechanical rigidity of the cured polyacrylamide elastic gels (PAA gels) is controlled via modulation of concentration for both 40% acrylamide (40% AA) base (BioRad) and its cross-linking molecular chain, 2% bis-AA (BioRad) in the PAA premixes, as described elsewhere (Fischer et al., 2012; Plotnikov et al., 2014), see Table 1.



Table 1. Formulation of PAA Gels for G' =2.3, 16, and 50 kPa

G'	40% AA	2% bis-AA	Streptavidin-AA	10X PBS	Q-water	TEMED	10% APS
2.3 kPa	9.33 μ L	1.88 μ L	3.33 μ L	5 μ L	30 μ L	0.1 μ L	1 μ L
16 kPa	15 μ L	3.6 μ L	3.33 μ L	5 μ L	21.97 μ L	0.1 μ L	1 μ L
50 kPa	15 μ L	14.4 μ L	3.33 μ L	5 μ L	11.17 μ L	0.1 μ L	1 μ L

1. In addition, streptavidin-acrylamide (Thermo Fisher) is added to a final concentration of 0.133 mg/mL to enable PAA gels cross-linking with biotinylated proteins of interest.
2. Table 1 describes the components required for preparation of 50 μ L of G' at varying rigidities.
3. The premix solutions can be stored for several hours at +4°C before use.

⚠ **CRITICAL:** Ammonium persulfate solution should be added immediately before PAA polymerization step.

⚠ **CRITICAL:** To ensure a proper PAA polymerization into the textured surfaces, the PAA premixes must be properly degassed in a vacuum chamber (vacuum bell connected to the centralized laboratory vacuum line) for 1 hour and/or in an ultrasonication degassing water bath (Cole-Parmer ultrasonic cleaner with timer and temperature control, Model 8891, Cole-Parmer, USA) for 5-6 minutes at 37°C prior to the nano-texture casting procedure. Since degassing and/or ultrasonication may affect TEMED stability and concentration, it is recommended to add TEMED to the PAA premixes after the degassing session.

Preparation of Protein of Interest

⌚ **TIMING:** 9–13 h

Many proteins of interest cannot be directly printed using soft lithography methods, either because of the protein denaturation susceptibility (e.g., ICAM1) or the protein supramolecular assembly and/or polymerization (e.g., collagen type-1). To resolve this problem during the lithographic nano-printing phase, we substitute the protein of interest with non-blocking antibody “substitutes”, specific against the desired proteins of interest.

4. Here, we substituted collagen with α -collagen-1 rabbit pAb (AbCam, Cambridge, UK), conjugated with biotin to ensure cross-linking of the antibody to PAA gels and with Alexa Fluor™ fluorescent tag for fluorescence visibility.
5. For ICAM1 or E-cadherin nano-pattern functionalization, we first nano-patterned fluorescently-labeled and biotinylated Fab fragments of anti-Fc γ antibody.
6. The prepared nano-patterns were then incubated for 8-24 hours with rat monomeric collagen type-I (Corning, NY) or with commercially developed recombinant human chimeric ICAM1-Fc or E-cadherin protein (Sino Biological, China).
7. Incubate 20 μ L of 1 mg/mL antibody for 1 hour with 5 μ L of ((+)-biotin N-hydroxysuccinimide ester (Sigma-Aldrich; as per the commercial protocol) and 5 μ L of fluorescent tag kit (Alexa Fluor® succinimidyl esters of desired excitation/emission spectrum, Invitrogen™, Molecular Probes®; as per the commercial protocol).
8. Dialyze labeled antibody in Slide-A-Lyzer™ MINI Dialysis Device, 7K MWCO (Thermo Fisher) for 8-12 hours at +4°C in cold PBS, then stored at +4°C in the dark.

⏸ **PAUSE POINT:** At this point, the protein of interest can be stored at +4°C in the dark for several months.

Table 2. Hard PDMS Formulation

VDT-731	Catalyst	Modulator	HMS-301
3.4 g	18 μ L	5 drops	1 g

Formulation and Preparation of Regular and Hard PDMS

⌚ TIMING: 1 h

- To prepare regular PDMS (rPDMS), mix 1:5 curing agent/base ratio mixture formulation (Sylgard-184, Dow Corning). Remove the air bubble inclusions in the viscous rPDMS premix by centrifugation at 400 g for 3 minutes at +4°C.
- To prepare hard PDMS (hPDMS), mix VDT-731 (Gelest, Inc.), Pt catalyst (Platinum(0)-2,4,6,8-tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane complex solution) (Sigma-Aldrich), cross-linking modulator (2,4,6,8-Tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane) (Sigma-Aldrich), and HMS-301 (Gelest, Inc.) in the minimum amounts as described in Table 2. Details on the formulation for hPDMS is described elsewhere (Odom et al., 2002).

⚠ CRITICAL: HMS-301 must be added last, immediately before use.

⚠ CRITICAL: To avoid hPDMS polymerization before use, keep it on ice. Vortex thoroughly for at least 15 seconds before use.

Alternatives: VDT-731, HMS-301 and modulator can be premixed first, and Pt catalyst added last, immediately before use. Mix the hPDMS premix thoroughly on a Vortex mixer for 30 seconds.

Preparation of Cover-Glasses and Glass-Bottom Dishes

⌚ TIMING: 10 h

- Place all cover glass (round and rectangular) in a 50 mL beaker and cover with aluminum foil.
- Bake at 450°C for 10 hours in a furnace. This step removes the organic residues and surface impurities from the cover glass surfaces.
- After baking, the cover glass can be stored in the sealed beaker to keep away from dust.

Optional: For the best result we recommend activating glass-bottom dishes with methacrylate immediately before use. This step facilitates the covalent bonding between curing polyacrylamide and glass surface.

Note: Glass-bottom 35 mm Petri dishes (MatTek Corp., Ashland, MA) are activated with 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich) as per commercial protocol in ethyl alcohol (Pharmco-Aaper) and acetic acid (Fisher Chemical):

- Dilute 100 μ L of 3-(trimethoxysilyl)propyl methacrylate in 20 mL of absolute ethanol, and just before use add 600 μ L of 10% acetic acid (1:10 glacial acetic acid/water).
- Pour resulting solution onto clean dishes to cover glass bottom completely and allow to react for ~3 minutes.
- Aspirate off excess, and then rinse dishes with ethanol to remove the residual reagent.
- Dry thoroughly before use.

⏸ PAUSE POINT: At this point the functionalized petri dishes can be stored for 1-2 hours in dry air.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Collagen I polyclonal antibody, Rabbit	AbCam	Cat#ab34710; RRID:AB_731684
AffiniPure Fab Fragment Goat Anti-Human IgG, Fc _γ fragment specific	Jackson ImmunoResearch Laboratories, Inc	Cat#109-007-008; RRID:AB_2632440
Chemicals, Peptides, and Recombinant Proteins		
HMS-31, (25-35% Methylhydrosiloxane)-Dimethylsiloxane Copolymer, Trimethylsiloxane Terminated	Gelest, Inc	Cat#HMS-301; CAS#68037-59-2
VDT-731, (7.0-8.0% Vinylmethylsiloxane) - Dimethylsiloxane Copolymer, Trimethylsiloxy Terminated	Gelest, Inc	Cat#VDT-731; CAS#67762-94-1
2,4,6,8-Tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane	Sigma-Aldrich	Cat#396281; CAS#2554-06-5
Platinum(0)-2,4,6,8-tetramethyl-2,4,6,8-tetravinylcyclotetrasiloxane complex solution	Sigma-Aldrich	Cat#479543; CAS#68585-32-0
SYLGARD™ 184 Silicone Elastomer Kit, 0.5 kg KIT	Dow Corning, Sigma-Aldrich	Cat#4019862; CAS#68988-89-6
40% Acrylamide Solution, Electrophoresis purity reagent, 500mL	BioRad	Cat#161-0140
2% Bis Solution, 500mL	BioRad	Cat#161-0142
Streptavidin Acrylamide, 1 mg	Thermo Fisher, Life Technologies	Cat#S21379
TEMED	Thermo Scientific	Cat#17919; CAS#110-18-9
Ammonium Persulfate, BioUltra, for molecular biology	Fluka Analytical	Cat#09913-100G; CAS#7727-54-0
3-(Trimethoxysilyl)propyl methacrylate	Sigma-Aldrich	Cat#6514; CAS#2530-85-0
Ethyl Alcohol 200 Proof, Absolute, Anhydrous ACS/USP Grade	Pharmco-Aaper	Cat#111000200; CAS#64-17-5
Collagen Type I, Rat Tail High Concentration, 100 mg, 8.95 mg/mL	Corning	Cat#354249
E-Cadherin Protein, Human, Recombinant (Fc Tag)	Sino Biological, China	Cat#10204-H02H
ICAM1, Human Protein, Recombinant, hlgG1-Fc.His Tag, Active	Sino Biological, China	Cat#10346-H03H-50
Paraformaldehyde, reagent grade, crystalline	Sigma-Aldrich	Cat#P6148-500G; CAS#30525-89-4
Hoechst 33342, Fluorescent Dye for labeling DNA	Tocris	Cat#5117; CAS#23491-52-3
Bovine Serum Albumin (BSA), fatty acid-free powder	Fisher Bioreagents	Cat#BP9704-100; CAS#9048-46-8
PBS pH7.4 (1X), Phosphate Buffer Saline	Gibco	Cat#10010-023

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
DMEM, 1X (Dulbecco's Modification of Eagle's Medium) with 4.5 g/L glucose, L-glutamine & sodium pyruvate	Corning Cellgro [®]	Cat#10-013-CV
ImmunoCult™-XF T Cell Expansion Medium	STEMCELL™ Technologies Inc., USA	Cat#10981
Human Recombinant IL-2	STEMCELL™ Technologies Inc., USA	Cat#78036.1
0.25% Trypsin, 2.21 mM EDTA, 1X [-] sodium bicarbonate	Corning	Cat#25-053-CI
Penicillin Streptomycin Solution, 100X	Corning	Cat#30-002-CI
Fetal Bovine Serum	HyClone [®]	Cat#SH30910.03
Acetic Acid, Glacial	Fisher Chemical	Cat#BP2401-500; CAS#64-19-7
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich	Cat#472301-100ML; CAS#67-68-5
Silanization solution-I	Sigma-Aldrich	Cat#85126; CAS#75-78-5
Cover Glasses, Circles, 15 mm, Thickness 0.13-0.17 mm	Carolina Biological Supply Company	Cat#633031
Cover Glasses, FisherFinest [®] Rectangles, 25X60-1, Thickness 0.13-0.17 mm	Fisher Scientific	Cat#24X60-1
Critical Commercial Assays		
EasySep™ Human CD4+ T Cell Isolation Kit	STEMCELL™ Technologies Inc., USA	Cat#17952
ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator	STEMCELL™ Technologies Inc., USA	Cat#10970
Slide-A-Lyzer™ MINI Dialysis Device, 7K MWCO, 0.1 mL	Thermo Fisher	Cat#69560
(+)-Biotin N-hydroxysuccinimide ester	Sigma-Aldrich	Cat#H1759; CAS#35013-72-0
Alexa-Fluor™ 488 carboxylic acid, succinimidyl ester	Molecular Probes	Cat#A20000
Alexa Fluor™ 568 carboxylic acid, succinimidyl ester	Molecular Probes	Cat#A20003
Phalloidin-iFluor 647 Reagent - CytoPainter	AbCam	Cat#ab176759
SIR-Actin Kit	Cytoskeleton, Inc.	Cat#CY-SC001
Experimental Models: Cell Lines		
Human Breast Adenocarcinoma Cell Line MDA-MB-468 (ATCC [®] HTB-132™), Female	ATCC [®]	Cat#HTB-132™; RRID:CVCL_0419
Human CD4 ⁺ T cells (derived from human whole peripheral blood).	STEMCELL™ Technologies, Inc., USA	Cat#70507.1
Software and Algorithms		
NIS-Elements Advanced Research 3.0	Nikon Instruments	RRID:SCR_014329
NIS-Elements Confocal software 3.0	Nikon Instruments	RRID:SCR_002776
Adobe Photoshop CC, 20161012.r.53x64	Adobe Systems, Inc.	RRID:SCR_014199
Adobe Illustrator CC, 21.0.0.	Adobe Systems, Inc.	RRID:SCR_010279
Leica SP8 STED 3X system	Leica Microsystems https://www.leica-microsystems.com/	n/a

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Huygens Professional software version 18.10.0	SVI, Hilversum, NL https://svi.nl/Huygens-Professional	n/a
ImageJ 1.52p (Fiji)	National Institutes of Health https://imagej.net/Fiji	n/a
ClearVolume plugin (Fiji)	Max Planck Institute of Molecular Cell Biology and Genetics https://clearvolume.github.io/	n/a

MATERIALS AND EQUIPMENT

To test the high precision 2D nano-patterns, we utilized the human breast adenocarcinoma cell line MDA-MB-468 (ATCC® HTB-132™). To test the elastic nano-textured patterns we utilized the human CD4+ T cells were generated from the human whole peripheral blood (STEMCELL™ Technologies Inc., USA).

For microprinting, we used Alexa Fluor™ 568 fluorophore- and biotin-prelabeled anti-collagen rabbit pAb, and Alexa Fluor™ 488 fluorophore- and biotin-prelabeled Fab fragments of anti-Fc_γ antibody (Jackson ImmunoResearch), ICAM1-Fc (Sino Biological, China), human E-cadherin-Fc chimeric protein (Sino Biological, China), collagen type-I (Corning, NY).

For imaging, F-actin was stained with either fluorescent phalloidin (Alexa Fluor™ phalloidin conjugates, Thermo Fisher Scientific) or SiR-Actin (Cytoskeleton, Inc.), chromatin was labeled with Hoechst solution (Tocris, USA).

STEP-BY-STEP METHOD DETAILS

Part A. Preparation of Elastic Nano-textured Patterns

The soft elastically deformable PAA-based nano-textures are manufactured by confining the polymerizing PAA premixes (of chosen shear modulus G' formulation) with molding negative replica of the desired nano-texture design. The cross-linking between biotin and streptavidin is used to functionalize the PAA surfaces with a protein of interest. Streptavidin is introduced directly into PAA premix (see [Before You Begin](#) for more details) as a streptavidin-acrylamide (streptavidin-AA) conjugate (Thermo Fisher) whereas biotin ester tags (Sigma-Aldrich) the protein of interest (See [Before You Begin](#) for more details). The negative molding replica used for molding confinement is precoated with biotin-tagged protein of interest for better efficiency of protein-PAA cross-linking. Optionally, protein of interest is also pre-labeled with a fluorescent tag for further nano-texture visualization (See [Before You Begin](#) for more details).

Manufacturing PAA Molding Casts

⌚ TIMING: 1 h

This section describes the generation of molding casts - negative PDMS replicas of the substrate topography prototype (positive master) used for casting the PAA nano-textured surfaces.

Note: As a positive master, we use either the pre-passivated silicon-crystal matrix (UMN NanoCenter, MN, USA), fabricated via photoresist development method ([Figure 1A, step 1](#)) or textured polyurethane nano-surfaces (NanoSurface Biomedical, Seattle, WA), glued onto the glass slide with SuperGlue® (Loctite, USA) and passivated with silanization solution-I (Sigma-Aldrich) as per [commercial protocol](#).

Note: Use hard PDMS (hPDMS) ([Odom et al., 2002](#)) instead of the regular PDMS (rPDMS) air-containing xerogels (See [Before You Begin](#) for more details). hPDMS xerogel features a higher

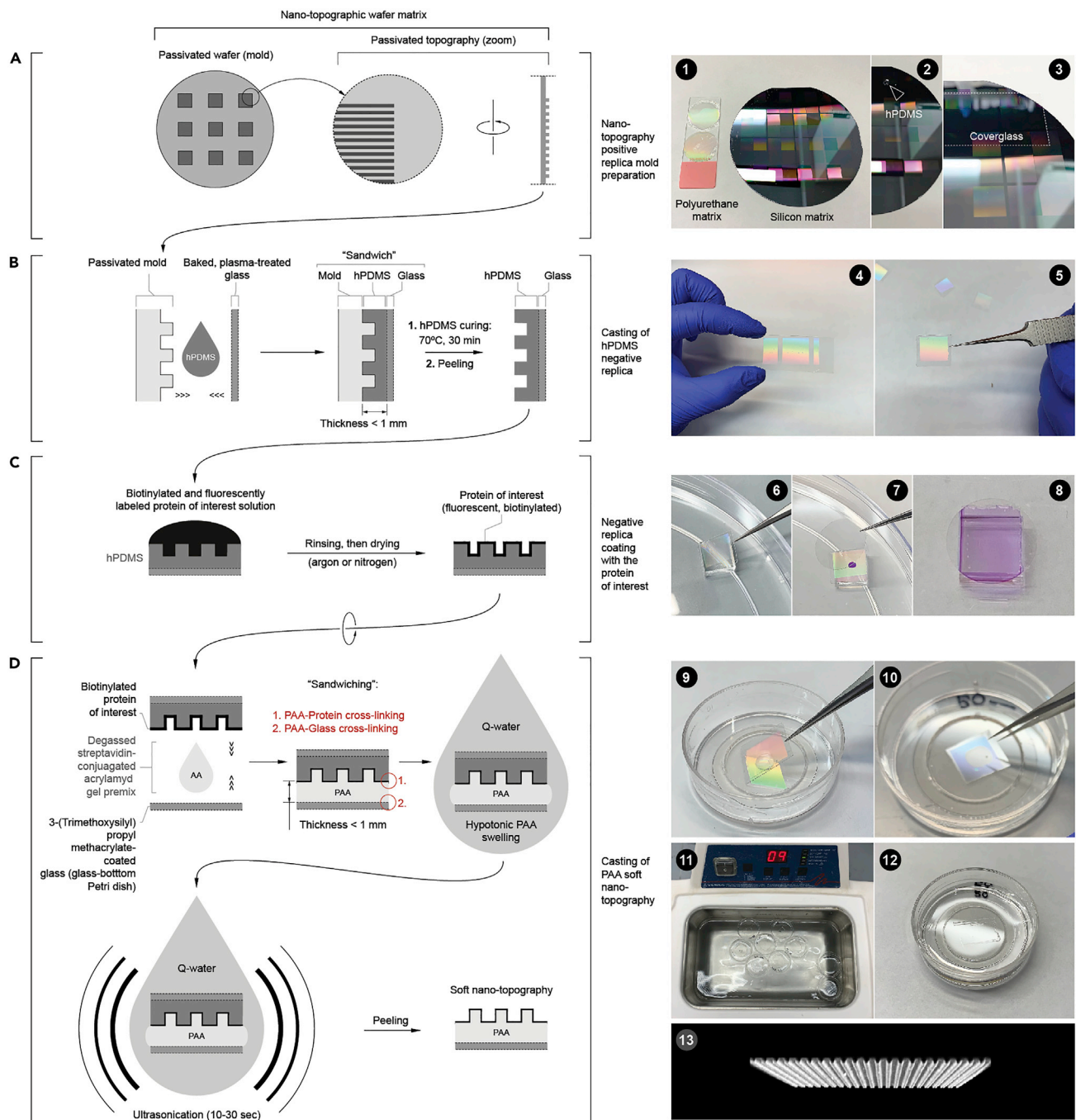


Figure 1. Fabrication of the Elastic Polyacrylamide Protein-Functionalized Nano-Topographies

(A) Passivated mold (matrix) with nano-features is either prepared commercially via conventional photoresist silicone crystal development or assembled from the polyurethane nano-surfaces (Nanosurface Biomedical, Seattle) by gluing the circular nano-features surfaces to the glass-slide with the following silanization in Silanization Solution-I (Sigma-Aldrich) (1).

(B) Casting hPDMS-based mold: hPDMS premix 5 μ L droplet (2 - arrowhead) is “sandwiched” between baked (450°C) plasma-treated cover-glass and the molding matrix (3 - dashed contour) to a submillimeter thickness. hPDMS in the “sandwich” is cured at \sim 70°C, 30 minutes, then peeled from the casting surface together with cover-glass (4), and cut in \sim 1 cm² square pieces (5).

(C) hPDMS molds placed “face-up” atop of the regular PDMS blocks (rPDMS pedestal) (6) to stick and immobilize the pieces. 5-7 μ L droplet of the fluorescently labeled biotinylated 200 mg/mL protein of interest solution is “sandwiched” between the hPDMS molding surface and clean pre-baked coverglass (7-8).

Figure 1. Continued

(D) ~7 μ L of PAA premix droplet is “sandwiched” between 3-(trimethoxysilyl) propyl methacrylate-coated glass-bottom of 35 mm Petri dish and rinsed and dried protein-coated hPDMS mold (9-10 - hPDMS is oriented “face-down” toward the PAA). After 5 minutes the cured PAA “sandwich” is incubated in deionized water or Q-water for 30 minutes at 20-25°C or at +4°C for 8-24 hours. Glass-bottom Petri dishes with Q-water then placed into ultrasonication chamber for 10-30 seconds to release the hPDMS molds from the PAA gel (11). If hPDMS mold is not released on its own, gently peel the mold manually (12). The nano-textured soft PAA surface is examined for the quality and defects via 3D microscopy (13).

structural density than that of rPDMS, significantly reducing the diffusion of oxygen-containing air from hPDMS bulk into the molded PAA premix that otherwise inhibits PAA polymerization at the PAA/hPDMS interface and compromises the PAA nano-texture quality.

Note: We use rectangular cover-glasses (24X60 mm, #1 thickness, Premium™ cover-glass, Fisher Finest®, Fisher Scientific) as the bearing base for the negative hPDMS replica.

1. Plasma-treat the baked cover-glasses (See [Before You Begin](#) for more details) for 2 minutes in the low pressure, thin rarefied air, generated by vacuum pump (Platinum DV-142N 5CFM, USA), and ionized into plasma at 18W (maximum power setting) in the basic plasma cleaner/sterilizer (Harrick, PDC-32 G, USA), immediately before casting of the hPDMS.
2. Put a 5-7 μ L droplet of hPDMS between passivated master mold and plasma-treated cover-glass ([Figure 1B](#), steps 2 and 3) and then bake at 70°C for 30 min.

▮▮ PAUSE POINT: Fully cured casts are stable for several weeks at 20-25°C.

3. Gently peel the cured molding cast off the master mold together with its bearing base ([Figure 1B](#), step 4), and cut into the 1X1 cm squares ([Figure 1B](#), step 5) by diamond pencil scribbling (on the reverse side of nano-surface).

▮▮ PAUSE POINT: Fully cured casts are stable for several weeks at 20-25°C.

Precoating of Molding Casts with Protein of Interest

⌚ TIMING: 1 or 12 h

This section describes the precoating of molding casts with protein of interest for better efficiency of protein-PAA cross-linking.

Note: We precoat molding casts with biotinylated and fluorescent tag-labeled protein of interest (in our case - Fab fragments of anti-Fc γ antibody (Jackson ImmunoResearch) in 0.2 mg/mL PBS solution (See [Before You Begin](#) for more details).

4. Put a 5-7 μ L droplet of protein of interest solution atop of the hPDMS mold and cover it by the round 15 mm pre-baked cover-glass (Carolina Biological Supply Company) (See [Before You Begin](#) for more details) in the “sandwich” fashion ([Figure 1C](#), steps 6-7) to economize the use of protein solution and to ensure its even distribution. Incubate at 20-25°C for 40 minutes or at +4°C for 8-12 hours in a wet chamber (20X160 mm Petri dish with Q-water-wetted tissue or cotton balls).
5. After incubation, gently rinse the casting mold in deionized Q-water and gently dry it under the jet of filtered air, nitrogen or argon ([Figure 1C](#)), use immediately.

Casting PAA Nano-textures

⌚ TIMING: 24 h

This section describes the casting of PAA nano-textures using molding casts precoated with protein of interest.

Note: We degas streptavidin-conjugated polyacrylamide premix (See [Before You Begin](#) for PAA premix formulations) of volume not greater than 0.5 mL in a vacuum chamber or in an ultrasonication water bath for 1 hour (See [Before You Begin](#) for more details) before casting the PAA nano-topographic surfaces. To avoid TEMED degradation and evaporation during degassing procedure, we recommend to add TEMED after the degassing session.

Note: We activate glass-bottom 35 mm Petri dishes (MatTek Corp., Ashland, MA) using 3-(trimethoxysilyl)propyl methacrylate (See [Before You Begin](#) for more details). Add 100 μ L of 3-(trimethoxysilyl)propyl methacrylate to 20 mL of absolute ethyl alcohol, followed by the addition of the 600 μ L of 10% acetic acid (water solution) and mix well prior to covering the glass-bottom Petri dishes. After 3 minutes of incubation, Petri dishes are rinsed with ethanol, dried and immediately used for PAA casting.

6. Immediately before PAA nano-texture casting, mix the 7-10 μ L volume of PAA premix of desired projected shear modulus G' (see [Table 1](#)) with ammonium persulfate solution (Fluka Analytical), and "sandwich" it as a single droplet between protein-coated casting mold and activated with 3-(trimethoxysilyl)propyl glass-bottom 35 mm Petri dishes (MatTek Corp., Ashland, MA) ([Figure 1D](#), [steps 9 and 10](#)).
7. After PAA curing (1-5 minutes), place the resultant textured PAA nano-chip in cold deionized Q-water for 8-10 hours for reversible hypotonic "swelling" of PAA ([Figure 1D](#)) to loosen the steric interaction between the molding cast and PAA.

Note: The PAA gel "swelling" is reversible and does not cause any structural damage to the PAA nano-surface.

8. Gently peel molding casts from the polymerized PAA surface with sharp tweezers. For the better molding nano-surface release from the cured PAA gel, gently apply pressure in the middle of the glass-bottom of the Petri dish from outside to generate the dome-like glass-bottom surface deformation, directed inward of Petri dish. The dome-like (i.e. spherical or bi-axial) deformation allows for an effective, yet gentle peeling of the molding nano-surface off the molded nano-textured PAA gel surfaces.

Optional: For a better release of the sterically interactive nano-mold, hypotonically treated PAA "sandwiches" were optionally ultrasonicated in the water bath (Cole-Parmer ultrasonic cleaner with timer and temperature control, Model 8891, Cole-Parmer, USA) at 37°C for 10 seconds ([Figure 1D](#), [step 11](#)). This step is particularly relevant to the nano-textures of low shear moduli, as soft PAA gel is susceptible to the structural damage during the casting mold peeling process.

△ CRITICAL: The incorrect peeling may cause structural damage to the PAA nano-texture surface. There is no possibility of reversing the damage caused to the PAA surface if the peeling of the molding nano-surface off the PAA gel fails. The percentage of the successfully peeled off substrates is usually ~50%.

△ CRITICAL: The poor PAA polymerization or poor definition of the PAA nano-texture features are usually caused by the presence of oxygen in the PAA premixes. To avoid this issue, repeat the PAA premix degassing procedure before casting the PAA nano-topographic surfaces (See [Before You Begin](#) for more details).

- Place released PAA nano-textures into isotonic PBS solution for 5 minutes to reverse PAA hypotonic swelling and check for the surface quality under the microscope (Figure 1D, steps 12 and 13). PAA nano-textures are not sterile and can be stored in PBS at +4°C only for several days.

▮▮ **PAUSE POINT:** PAA nano-textured can be stored at +4°C in the dark for several weeks. Optionally, add 0.01% sodium azide as a protein preservative to prevent bacterial contamination of the protein-coated PAA surfaces.

Note: We incubate successfully released PAA nano-textures with 200 µg/mL ICAM1-Fc (Sino Biological, China) solution in cold PBS (4°C, 8-12 hours), rinse with cold PBS, and immediately use for the T cell adhesion and contact guidance assays.

▮▮ **PAUSE POINT:** ICAM1-coated PAA surfaces can be stored for up to a week at +4°C in the dark.

Part B. Preparation of High Precision 2D Nano-patterns

Nano-patterns provide a powerful tool to study directed migration (Ray et al., 2017; Taddanov et al, 2018b). However, fabrication of flat elastic nano-patterns by the traditional soft lithography methods is a challenging task due to the suboptimal size of the printing bas-relief features of the nano-stamp. To address these issues and achieve high precision and definition for the resulting nano-patterns on elastic platforms we substituted regular soft PDMS (rPDMS) nano-stamps with the composite stamps. We combined soft cushioning rPDMS block with a veneering submillimeter-thick layer of hard PDMS (hPDMS, See [Before You Begin](#) for more details) that features rigid non-collapsing printing bas-relief nano-features (Schmid and Michel, 2000; Shen et al., 2008; Tabdanov et al., 2015). Thus, in this design, a soft rPDMS cushioning layer provides an even pressure distribution onto the hPDMS printing veneer for the even lithographic contact with the printed surface, while the submillimeter thickness of the non-collapsing printing layer prevents the effects of hPDMS fragility.

Casting of Nano-stamps

⌚ **TIMING:** 2 h

This section describes the generation of nano-stamps casts - negative PDMS replicas of the substrate topography prototype (positive master) used for nano-contact printing.

Note: We use commercially manufactured polyurethane nano-surface disks (NanoSurface Biomedical, Seattle, WA) and the pre-passivated silicon-crystal matrix, commercially manufactured via photoresist development method (UMN NanoCenter, MN, USA) (Figures 1A and 2A) as the casting matrices. Textured nano-surface disks (NanoSurface Biomedical, Seattle, WA) were glued onto the glass platform with SuperGlue® (Loctite, USA) and passivated with silanizing solution-I as per the commercial protocol (Figure 1A, step 1) (Sigma Aldrich).

- Coat silanized plastic nano-surface or silicone crystal matrix with ≤0.5 mm hPDMS gently spreading it with soft Parafilm-made spatula (Hach, USA), and then cure matrix at 70°C for 30 minutes (Figure 2A).
- After hPDMS layer rigidification, pour the rPDMS atop of the hPDMS to the layer's final thickness of 8 mm. Cure at 70°C for ~1 hour.

▮▮ **PAUSE POINT:** Fully cured casts are stable for several weeks at 20-25°C.

- Gently peel composite nano-stamps from the master matrix, and cut into 5X5 mm or 1X1 cm ready-to-use nano-stamp pieces (Figure 2A, step 1).

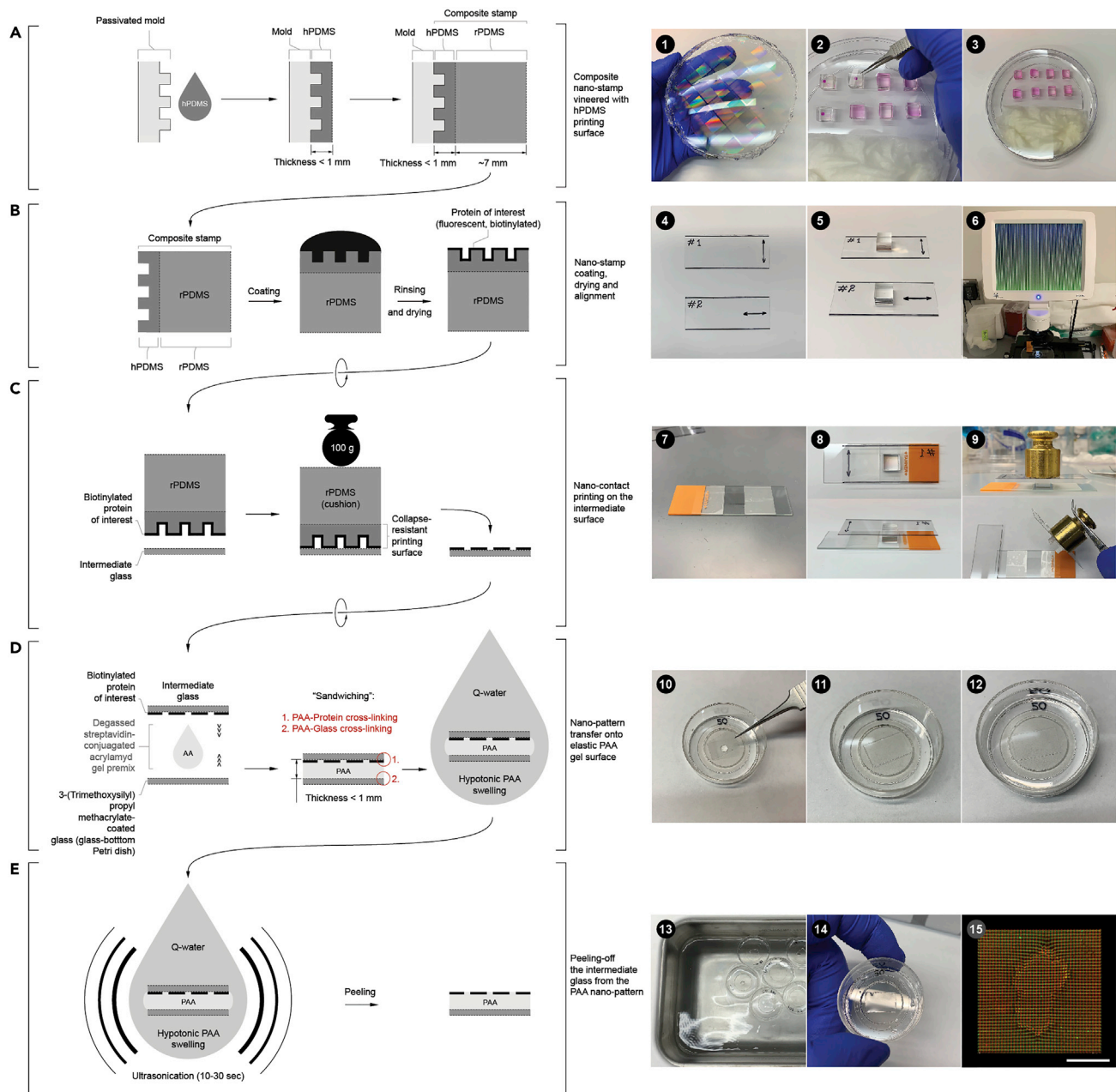


Figure 2. Fabrication of the Elastic Polyacrylamide Protein-Functionalized Nano-Patterns

(A) Preparation of the composite nano-stamp: thin submillimeter layer of hPDMS premix is spread atop of the molding matrix passivated surface (manually or by spin-coating), cured at $\sim 70^{\circ}\text{C}$ for 30 minutes. Regular PDMS (rPDMS) premix layer is poured at the top of the hPDMS layer to the final stamp thickness of ~ 7 mm and consequently cured at $\sim 7^{\circ}\text{C}$ for 1 hour. Cured composite PDMS is gently peeled off the matrix (1) and cut in $\sim 0.5\text{-}1$ cm² pieces (2, 3). For the composite double protein nano-grids printing, prepare 2 rectangular cover-glasses and label them as shown on (4).

(B) Composite nano-stamps are coated with 5-7 μL of protein of interest (200 mg/mL, fluorescent, biotinylated) by "sandwiching" the protein solution between the stamp's surface and clean coverglass in the wet chamber (3) for at least 40 minutes at $20\text{-}25^{\circ}\text{C}$ or at $+4^{\circ}\text{C}$ for 8-12 hours. Protein-coated, rinsed and dried nano-stamps are placed on the rectangular cover-glass (5) and aligned to the cover-glass' edges either vertically or horizontally (6).

(C) Clean dust-free cover-glass ("intermediate glass") pieces are immobilized on the glass-slides with the Scotch-Tape™ (7). The protein-coated nano-stamp is gently placed with its printing surface facing the "intermediate" surface cover-glass, in one-touch movement with holding coverglass edges aligned to the glass-slide edges (8). A weight (~ 100 g) is placed atop of the nano-stamp to ensure a proper nano-contact stamping.

Note: For the convenience the weight's bottom must be wet to ensure easy removal of the weigh together with the nano-stamp in one movement (9). For the dual protein grid printing, repeat steps 4-9 with the second protein of interest and nano-lines orientation orthogonal to the orientation of the previously printed nanolines.

Figure 2. Continued

(D) 5–7 μL of PAA premix (10) of desired projected rigidity is “sandwiched” between 3-(trimethoxysilyl) propyl methacrylate-coated glass-bottom of 35 mm Petri dish and nano-patterned “intermediate” surface, facing the PAA solution (11), to ensure submillimeter thickness of PAA gel. After gel polymerization (~ 5 minutes) the Petri dish is filled with Q-water for 30 minutes at 20–25°C or at +4°C for 8–24 hours (12).

(E) After hypotonic incubation the Petri dishes are briefly ultrasonicated for 10–30 seconds (13) and “intermediate” cover glasses are peeled off the PAA gels (14). The microscopy-inspected for quality of the nano-patterns substrates are used for experiments (15).

▮▮ **PAUSE POINT:** Fully cured casts are stable for several weeks at 20–25°C.

Coating of Nano-stamps with Protein of Interest

⌚ **TIMING:** 1 or 12 h

This section describes the precoating of nano-stamps with protein of interest for printing on “intermediate” cover-glass.

- Place 5 μL droplets of 0.2 mg/mL solution of biotin- and fluorophore-labelled (see [section 2. for more detail](#)) protein of interest (anti-collagen type-1 (AbCam) antibody or Fab fragments of anti-Fc γ antibody (Jackson ImmunoResearch)) atop of the 5X5 mm or 1X1 cm square nano-stamps ([Figure 2B, step 2](#)).

Note: To ensure an effective stamp surface coating with protein of interest, “sandwich” protein solution droplet between the stamp’s printing surface and pre-baked 15 mm round glass coverslip (See [Before You Begin](#) for more details) (Carolina, USA) as shown on [Figure 2 \(step 2\)](#).

- Incubate the nano-stamps with “sandwiched” droplet of protein of interest solution for 40 minutes at 20–25°C or for ~ 8 –12 hours at +4°C in wet chamber (See [Before You Begin](#) for more details) ([Figure 2B, step 3](#)).

▮▮ **PAUSE POINT:** Incubation in the wet chamber can be utilized as a pause point, 40 min or 8–12 hours.

- After the coating session the nano-stamps were gently rinsed in deionized milli-Q water (Q-water) and gently dried under the jet of filtered air, nitrogen or argon, and then used for nano-printing.

Nano-Contact Printing

⌚ **TIMING:** 1 h

This section describes the printing of nano-patterns on “intermediate” cover-glass surface using nano-stamps pre-coated with protein of interest.

Note: For elastic hydrogel micro- and nano-patterning we adopted an “intermediate substrate” technique in which biotin-conjugated protein micro- and nano-patterns were initially printed on “intermediate” cover-glass, then transferred onto hydrogels by cross-linking patterned proteins’ biotin tags to streptavidin-conjugated polyacrylamide (PAA) ([Tang et al., 2012](#)).

Note: To print nano-grids composed of two sets of orthogonally intersecting parallel nano-lines, constituted of two different proteins of interest, we use Alexa Fluor™ 568 fluorophore- and biotin-prelabeled anti-collagen rabbit pAb, and Alexa Fluor™ 488 fluorophore- and biotin-prelabeled Fab fragments of anti-Fc γ antibody (Jackson ImmunoResearch).

16. To ensure the orthogonality of the grid, align the nano-stamps' nano-lines features directionality to the edges of the rectangular "loading" cover-glass (Figure 2, steps 4 and 5), to which the nano-stamps are attached by their non-printing rPDMS "sticky" side. Align the "loading" cover-glass horizontally or vertically to the microscope object table with the hand-made ruler and/or protractor. Use of the DIC microscope with the external monitor helps to align the nano-stamp to the pre-aligned "loading" cover-glass for the precise horizontal or vertical positioning of the corresponding lines patterns (Figure 2, steps 5 and 6): e.g. vertically for protein of interest #1 and horizontally for the protein of interest #2.
17. Immobilize clean (not pre-baked) cut 1X1 cm square "intermediate" cover-glasses to the glass-slide by a scotch-tape (Figure 2, step 7). The composite double protein nano-grid is printed in two printing sessions, one for each of the lines set.
18. First session, gently place vertically oriented nano-stamp with protein of interest #1 atop of the immobilized cover-glass, with the edges of "loading" cover-glass aligned to the edges of the glass-slide with the immobilized "intermediate" cover-glass with nano-stamp's printing surface facing down towards the "intermediate" cover-glass (Figure 2C, step 8).
19. To ensure a tight lithographic seal between the nano-stamp and "intermediate" cover-glass, gently place 100 gram weight atop of the "loading" cover-glass for 30 seconds (Figure 2C, step 9).

⚠ **CRITICAL:** To ensure an accurate, one-step removal of the nano-stamps, wet the bottom of the weight with a little amount of water, to provide a capillary "stickiness" between the weight and the "loading" cover-glass.

20. Following 30 seconds of lithographic seal between the nano-stamp and "intermediate" cover-glass, gently remove the weight in one step together with the capillary effect-attached "loading" cover-glass and the nano-stamp (Figure 2C, step 9).
21. Second session, repeat the procedure (18-20) with the same "intermediate" cover-glass and with horizontally oriented nano-stamp, coated with the protein of interest #2 (Figure 2C, steps 8-9).

⏸ **PAUSE POINT:** Nano-printed intermediate glass surfaces can be stored in the dark for up to 2 hours at 20-25°C.

Transferring Nano-patterns onto PAA Gels

⌚ **TIMING:** 24 h

This section describes the transfer of proteins printed on the "intermediate" cover-glass surface to PAA nano-textures via cross-linking of nano-patterned proteins to the polymerizing PAA gels.

22. Mix a 7-10 μ L droplet of PAA with projected mechanical rigidity (Table 1) with APS in the center of the freshly prepared thoroughly dried 3-(trimethoxysilyl)propyl methacrylate-functionalized glass-bottom Petri dish (See [Before You Begin](#) for more details) (Figure 2D, steps 10)
23. Gently place "Intermediate" cover-glass with pre-printed nano-pattern face-down atop of the droplet to form a PAA "sandwich" (Figure 2D, steps 11).

⚠ **CRITICAL:** PAA begins polymerization immediately after APS addition, both 22 and 23 steps must be performed within ~10 seconds.

⚠ **CRITICAL:** To ensure proper polymerization, wait at least 20 minutes before going to the release step.

24. Gently release cover-glass from PAA gel by reversible hypotonic “swelling” in deionized Q-water (8-12 hours at +4°C or at 20-25°C for 40 minutes) (Figure 2D, step 12).

Optional: For easy and gentle “intermediate” cover-glass release from the cured PAA, use an optional ultrasonication session of the “sandwiched” PAA gel in Q-water for ~10-30 seconds (Figure 2E, steps 13 and 14). This step is particularly relevant for the PAA gels of low shear moduli ($G' < 16$ kPa), susceptible to the structural damage during mechanical peeling of the “intermediate” cover-glass.

25. Place released PAA nano-textures into isotonic PBS solution for 5 minutes to reverse PAA hypotonic swelling and check for the printing quality under the microscope.

▣ **PAUSE POINT:** Nano-patterned PAA surfaces can be stored for up to a week in the dark in PBS with 0.25% sodium azide.

△ **CRITICAL:** Thoroughly rinse the PAA surfaces before utilization from cytotoxic sodium azide.

Note: The resultant fluorescent PAA nano-patterns of α -collagen-1 (red) and α -Fc γ Fab (green) we incubate in two steps:

- The α -collagen-1 nano-lines sets are functionalized with 1 mg/mL rat monomeric collagen type-I (Corning, NY) in cold PBS (Gibco) at +4°C for 8-24 hours, and then rinsed (cold PBS).
- The α -Fc γ Fab nano-lines sets are functionalized with 200 μ g/mL human E-cadherin-Fc chimeric protein (Sino Biological, China), PBS solution, +4°C, for 8-24 hours, then rinsed and used for experiments (Figure 2, steps 14 and 15).

EXPECTED OUTCOMES

The following proceedings are the final product testing protocols and demonstration of compatible cells morphometric measurements.

Cell Adhesion and Spreading Assay, Samples Preparation

We utilized the human breast adenocarcinoma cell line MDA-MB-468 (ATCC[®] HTB-132[™]) as a model system to test both cell-ECM type adhesion to collagen type-1 nano-patterns, and biomimetic cell-cell type adhesion to E-cadherin nano-lines. For details, please refer to this [Cell Reports paper](#).

Human CD4⁺ T cells were generated from the human whole peripheral blood (STEMCELL[™] Technologies Inc., USA) as a model system for sterically active substrates recognition and interaction via LFA1-ICAM1 adhesion. For cell culture experiments, T cells were maintained in ImmunoCult[™]-XF T Cell Expansion Medium (STEMCELL[™] Technologies Inc., USA) with the addition of Human Recombinant Interleukin 2 (IL-2, STEMCELL[™] Technologies Inc., USA) as per STEMCELL[™] Technologies Inc. as specified by the commercial protocol. After cell adhesion and spreading assay (5% CO₂, 37°C, 2 hours) samples were fixed with cold DMEM with 4% PFA for 15 minutes, followed by rinsing incubation in 1% BSA PBS. F-actin was stained with either fluorescent phalloidin (Alexa Fluor[™] phalloidin conjugates, Thermo Fisher Scientific; 10 U/mL in 1% BSA PBS) or SiR-Actin (Cytoskeleton, Inc.; 1 μ M in 1% BSA PBS for 1 hour). Chromatin was labeled with 1:1000 Hoechst solution (Tocris, USA). All cell work was approved by the University of Minnesota Institutional Biosafety Committee and followed institutional and NIH guidelines.

High-Resolution Imaging

High-resolution 2D and 3D imaging, images reconstruction and linear image parametric adjustments were performed as described previously. Figures were composed using unmodified NIS-

Elements AR-generated TIFF images with Adobe Illustrator CC 2017 (Adobe Systems, Inc.). For details, please refer to this [Cell Reports paper](#).

Super-Resolution Imaging (Contact Guidance of Human T Cells on Nano-textured Surface)

Super-resolution stimulated emission depletion (STED) microscopy was performed using a commercial Leica SP8 STED 3X system (Leica Microsystems, Mannheim, Germany), equipped with a white light laser with continuous spectral output between the wavelengths of 470 nm to 670 nm, and a 592 nm, 660 nm and a pulsed 775 nm STED depletion lasers, to obtain time-gated STED images on 3 hybrid detectors. We have used initially for imaging the STED white objective 100x/1.4 NA oil immersion objective lens (HCX PL APO STED white, Leica Microsystems). Given the complexity and varying depth of the sample, the STED WHITE Glycerin objective lens (HC PL APO 93X/1.30 GLYC motCORR) (Leica Microsystems) was more advantageous for depth imaging due to the motorized correction collar allowing precise and swift adjustment of optical lenses to specimen inhomogeneity. Labeled samples placed in 35 mm culture dishes with number 1.5 cover glass bottom (MatTek Corporation, Ashland, MA) containing 250 μ l glycerol (90%) in PBS were imaged in sequentially as follows: first sequence STED for SiR-actin (via 647 nm excitation and 660-730 nm emission range) on gated (0.7-6.5 ns time gating) hybrid detector using 775 nm (25% power) as STED depletion laser for best lateral resolution; Second and third sequences were confocal settings for Hoechst and Alexa488 (labeling nucleus and surface of the gel) respectively, via two sequential excitations (405 nm, and 488 nm) and two emission ranges (410-465 nm, and 495-555 nm) respectively, on gated (0.3-6.5 ns) hybrid detectors. Imaging was performed with a scan speed of 600 lines per second, scanning bidirectionally, a pixel size of 30-35 nm (1024 x 1024 pixels), and 6-line averages, pinhole of 0.7 Airy units and Z-stacks were collected at 0.140 μ m-depth intervals throughout the depth of the sample. We deconvoluted images using Huygens Professional software version 18.10.0 (SVI, Hilversum, NL) with the classical maximum likelihood estimation algorithm. We then inspected and reconstructed 3D data using Clear Volume plugin (FIJI). Still frames were saved and montaged using Adobe Photoshop CC.

T Cell On-Ridge Spreading and In-Groove Invasiveness Is Controlled by the Mechanical Rigidity of ICAM1-Coated Nano-topography

Human CD4⁺ T cell behavior on topographical features can be captured on nano-textured PAA gels (Figure 3A) with super-resolution STED microscopy (Figure 3B), which are unavailable commercially compared to manufactured plastic nano-textures, and provide much needed mechanical control of T cell motility analysis. We demonstrate the ability to perform morphometric analysis of T cell motility on soft ICAM1-coated nano-textures, such as T cell height and projected 2D spreading area (Figure 3C and 3D). Along with the basic morphometric analysis, we are able to decipher structurally finer T cell invasiveness features that penetrate into the nano-grooves as a function of the nano-texture mechanical rigidity (Figure 3D). We demonstrate ICAM1 rigidity-controlled T cell spreading-invasion dynamics, finding the mechanically controlled balance between the flat lamellipodium-driven *on-ridge* T cell spreading and *in-groove* steric invasiveness (Figure 3D and 3E) that may help to increase T cell contact guidance in the soft, but not rigid environments.

Adenocarcinoma Cells Spreading on Bi-axially Heterogeneous and Anisotropic Nano-grids

In vivo, cells frequently encounter simultaneous synergistic or competing adhesion cues from cell-cell and cell-ECM interactions that can be studied *in vitro* (Borghi et al., 2010; Tabdanov et al., 2018a). Human breast adenocarcinoma cells (MDA-MB-468) demonstrate overall bi-axially continuous protrusion on the 'collagen type-1 vs. E-cadherin' nano-grids (Figure 4A) on both soft ($G' = 2.3$ kPa) and rigid ($G' = 50$ kPa) mechanical platforms. The morphometric analysis reveals an overall mechanically controlled bi-axial spreading (Figures 4B and 4C). However, we observe no differential preference or spreading competition between either of the ligands nanolines directions on both soft and rigid substrates. Therefore, we hypothesize that at the nanoscale the adenocarcinoma cells are unable to robustly spatially resolve the directionality and spatial organization of the flat

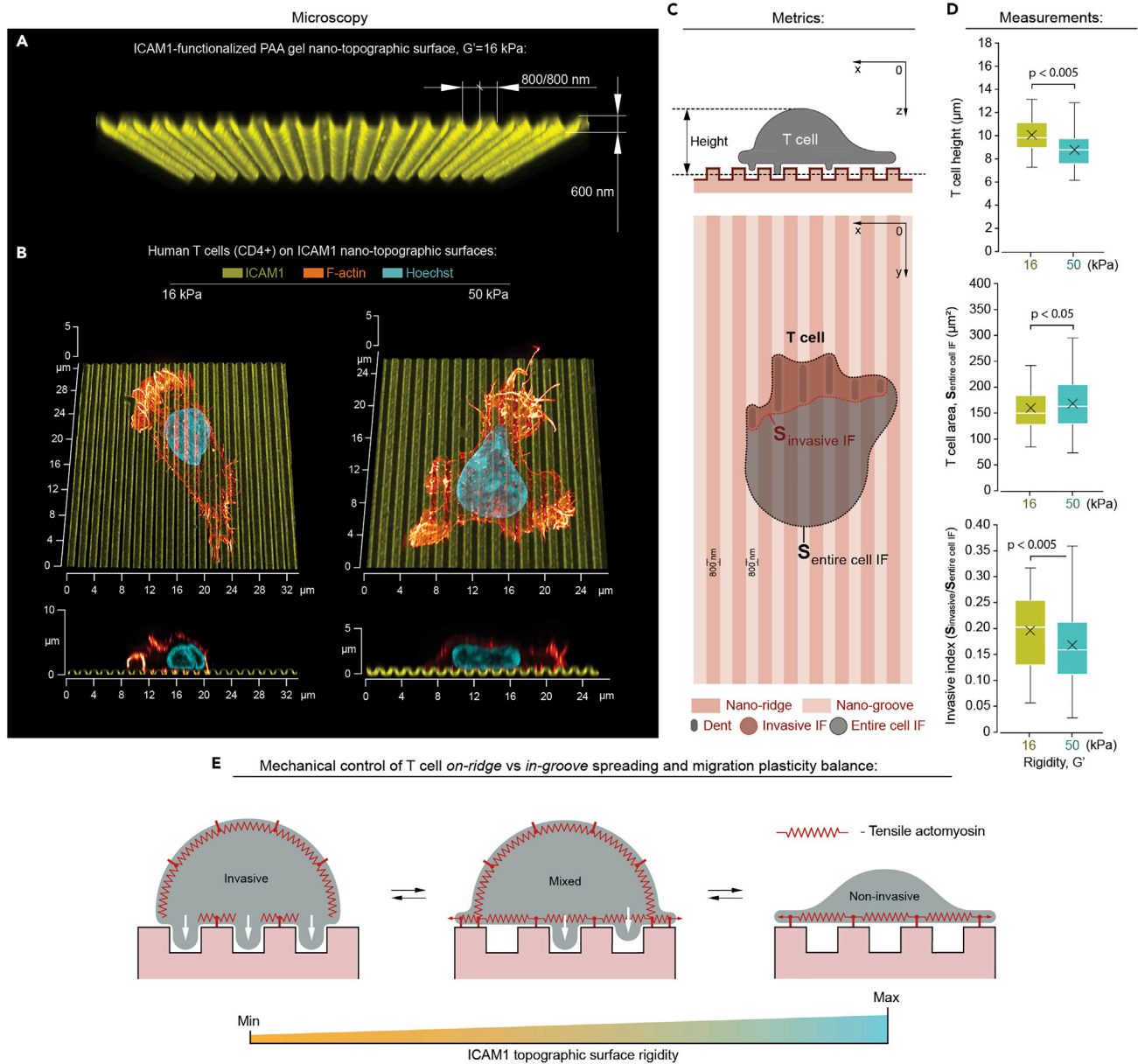


Figure 3. Mechanical Rigidity of ICAM1 Nano-Topographies Controls T Cell On-Ridge Spreading and In-Groove Invasiveness Plasticity Balance

(A) 3D super-resolution reconstruction of the ICAM1-functionalized PAA nano-topographic surface ($G'=16$ kPa).

(B) Test super-resolution imaging (3D reconstruction) of human CD4+ T cells spreading and migrating along the ICAM1-coated nano-topographic surfaces on soft (16 kPa) and rigid (50 kPa) PAA surfaces.

(C) Schematic of the T cell morphometric analysis and metrics: T cell height, projected area of spreading of entire cell interface ($S_{\text{entire cell IF}}$) and projected area of *in-groove* invasive T cell interface ($S_{\text{invasive IF}}$).

(D and E) Mechanoregulation of T cell height, spreading area and invasiveness as indicated by T cell spreading assay on soft ($G'=16$ kPa) and rigid ($G'=50$ kPa) ICAM1 nano-textures. T cell spreading enhances on the rigid ICAM1, accompanied with T cell flattening, i.e. decrease of the T cell height. Results indicate a mechanically controlled dynamic balance between *on-ridge* T cell spreading and *in-groove* invasiveness, as shown on the schematic panel (E). I.e. T cell *in-groove* invasiveness structurally competes with *on-ridge* spreading, indicating that *on-ridge* spreading is mechanically enhanced and out-balances *in-groove* invasiveness on the rigid ($G'=50$ kPa) ICAM1 nano-topography. Alternatively, soft ($G'=16$ kPa) ICAM1 nano-textures are unable to promote the mechanically sensitive *on-ridge* T cell spreading, shifting the balance towards steric *in-groove* T cell invasiveness.

Data on the plots on (D) are as follows: boxes - means, Q1 and Q3; whiskers - max and min, X - medians; p values - one way ANOVA test. Experimental data collected in triplicates, total $n>50$.

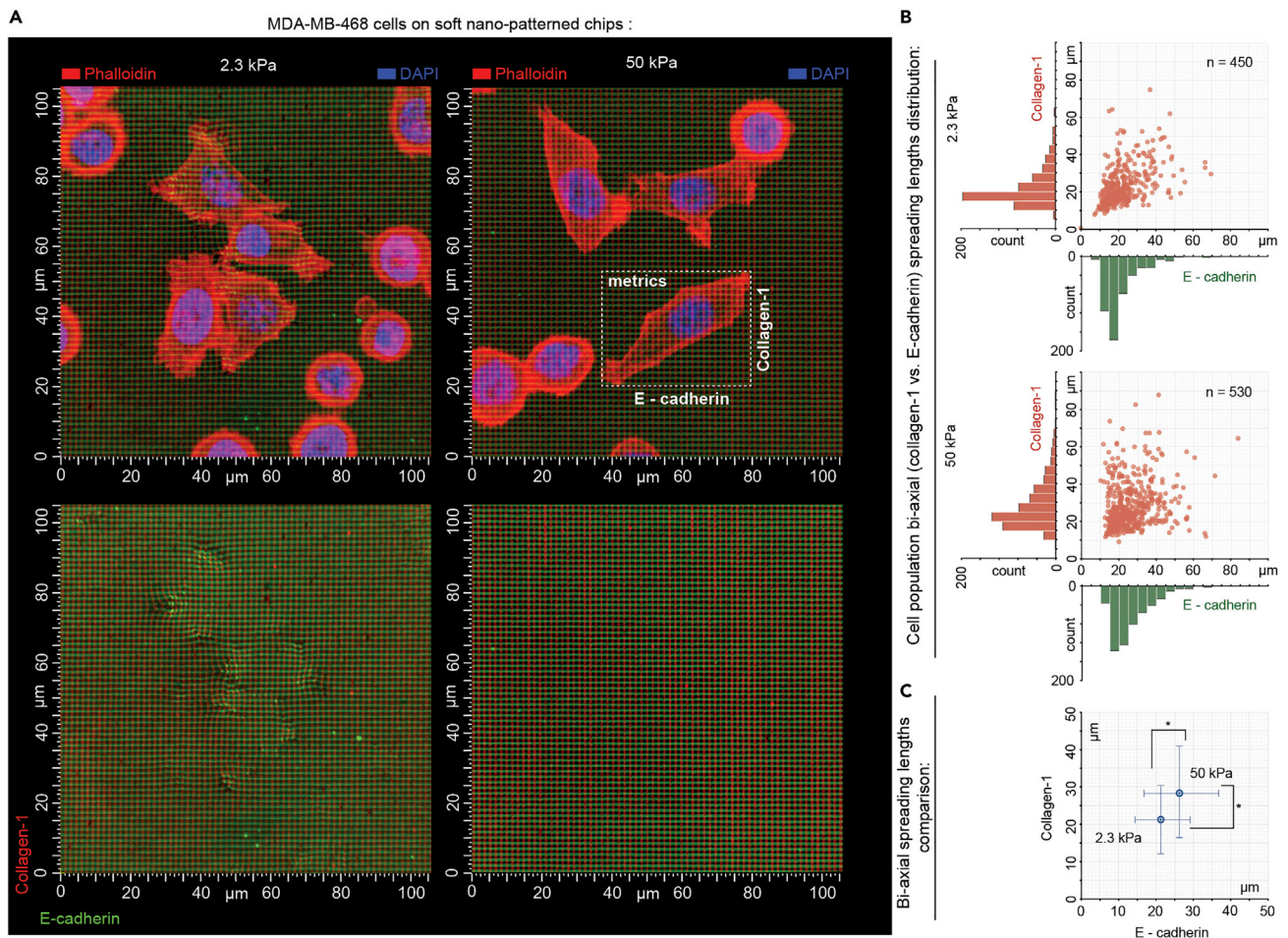


Figure 4. MDA-MB-468 Human Breast Adenocarcinoma Cells on 2.3 (Soft) and 50 (Rigid) kPa PAA Gels with Collagen (Red) and E-cadherin (Green) Orthogonal Grid Nano-Patterns

(A) Note the soft nano-pattern ($G' = 2.3$ kPa, left column) but not the rigid nano-pattern ($G' = 50$ kPa, right column) being deformed by the adhering cells. Metrics for bi-axial cell spreading length measurement along each nano-lines direction is shown with dashed box. (B) Cell population bi-axial spreading data for each mechanical rigidity platform is shown as the combined cloud and distribution diagrams. (C) Statistical analysis of bi-axial cell spreading for each of the mechanical rigidity platforms. Data configurations on the plots on panel B are as follows: bi-axial cell spreading represented as a cloud diagram with individual cell measurement points, and as distribution binned diagrams for each direction (collagen-1 and E-cadherin). Data on panel C represent means \pm SD, p values - one way ANOVA test for each direction of spreading lengths. * $p < 0.05$. Experimental data collected in triplicates, total n values are shown on the corresponding plots.

anisotropic guidance cues, as opposed to the previously reported dynamic and mechanically controlled MDA-MB-468 cell protrusion competition between microscale (1-15 μm) collagen-1 and E-cadherin anisotropic cues directionalities, additionally modulated by the mechanical rigidity of the adhesion-motility platforms (Tabdanov et al., 2018a). Thus, we suggest the utilization of our experimental platforms for the further investigation of cell contact guidance cues sensing on the nanoscale and in correlation with mechanobiological aspects of cell-microenvironment interactions.

LIMITATIONS

Both described methods have limitations at the lithographic nano-printing or nano-molding step, specifically, although hPDMS, due to its mechanical rigidity, allows one to eliminate the problem of nano-textured surface collapse onto the printed surface, such collapse-resistance exists within a limited range of the geometrical dimensions of the printing bas-relief. Although we have not

explored such dimension limitations, we suggest that it may take place at the bas-relief dimensions below 50-100 nm. Similarly, we predict nano-molding limitations at the molding bas-relief dimension below 50-100 nm, as at this size range the oxygen diffusion-related effects may prevent the proper replication of the molding nano-texture by polymerizing PAA gel surface.

TROUBLESHOOTING

Problem

Patterns are inactive due to protein denaturation or the protein supramolecular assembly and/or polymerization during printing.

Potential Solution

Substitute the protein of interest with non-blocking antibody “substitutes”, specific against the desired proteins of interest (see [Before You Begin](#) for more details).

Problem

PAA gel detaches from glass-bottom dishes.

Potential Solution

Activate glass-bottom dishes with methacrylate immediately before use (See [Before You Begin](#) for more details).

Problem

Peeling causes structural damage to the PAA nano-texture surface.

Potential Solution

Use hypotonic swelling, apply pressure in the middle of the glass-bottom of the Petri dish from outside to generate the dome-like glass-bottom surface deformation for better release. Use ultrasonication for peeling. Repeat the PAA premix degassing procedure before casting the PAA.

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AUTHOR CONTRIBUTIONS

E.D.T. participated in the design, execution, and analysis of all nano-patterning and experiments. E.D.T. developed nano-patterning and nano-texture molding methods, and all platforms employed in this study. A.S.Z. participated in the experimental design, conducted experiments, data analysis, and interpretation. V.P. performed experiments and analysis. P.P.P. participated in experimental

design, data analysis, data interpretation, and secured funding. E.D.T., A.S.Z., and P.P.P. wrote the manuscript. All authors read and contributed comments to the final manuscript. E.D.T. and P.P.P. oversaw all aspects of the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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