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Patterning on Topography for generation of cell culture substrates with independent nanoscale control of chemical and topographical extracellular matrix cues

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Abstract

The cell microenvironment plays an important role in many biological processes, including development and disease progression. Key to this is the extracellular matrix (ECM), a complex biopolymer network serving as the primary insoluble signaling network for physical, chemical, and mechanical cues. *In vitro*, the ability to engineer the ECM at the micro- and nanoscales is a critical tool to systematically interrogate the influence of ECM properties on cellular responses. Specifically, both topographical and chemical surface patterning has been shown to direct cell alignment and tissue architecture on biomaterial surfaces, however, it has proven challenging to independently control these surface properties. This protocol describes a method termed Patterning on Topography (PoT) to engineer 2D nanopatterns of ECM proteins onto topographically complex substrates, which enables independent control of physical and chemical surface properties. Applications include interrogation of fundamental cell-surface interactions and engineering interfaces that can direct cell and/or tissue function.

Keywords

Extracellular matrix; microtopography; fibronectin; biomaterials; microcontact printing; surface initiated assembly

INTRODUCTION

Cells in tissues are surrounded by a complex microenvironment that consists of chemical, mechanical, and physical cues provided by the extracellular matrix (ECM), a dynamic

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2015), shown in Fig. 1F–J. The SIA technique is a key step that enables formation of insoluble ECM protein fibers, sheets and other patterned structures (Feinberg and Parker, 2010). This PoT approach provides independent control of the micropatterned ECM cues that can be conformally overlaid onto surface microtopography. Thus, PoT provides a unique capability to engineer substrates with defined chemical and topographic structure in order to determine possible synergistic, inhibitory, or other effects on cell and tissue behavior.

The PoT process involves two major steps to conformally nanopattern ECM proteins onto nano- or microtopographically structured substrates. In the first step, ECM protein patterns of fibronectin (FN), laminin (LAM), and/or collagen IV (COL IV) are μ CP onto a smooth, sacrificial poly(N-isopropylacrylamide) (PIPAAm) layer (Basic Protocol 1). In the second step (Basic Protocol 2), this ECM-patterned PIPAAm substrate is placed against a topographically complex substrate and submerged in warm water. Cooling of the water causes PIPAAm swelling and dissolution, which releases the ECM via a SIA process and transfers the 2D pattern to the contours of the topographic substrate (Feinberg and Parker, 2010; Sun et al., 2015). In this unit, Support Protocols 1–3 describe (1) fabrication of PDMS stamps, (2) fabrication of PIPAAm-coated coverslips, and (3) fluorescent labeling of proteins for use in Basic Protocol 1. Support Protocols 4 and 5 describe generation of topographically complex substrates with photolithography techniques or from rough materials. Support Protocol 6 describes cell seeding on PoT-printed substrates. Note that we use the term micropattern generically to refer to μ CP from the nano- to millimeter scales in terms of lateral dimensions, and that the PoT patterned protein layer is approximately 5 nm thick (Feinberg and Parker, 2010; Renault et al., 2003; Sun et al., 2015; Szymanski and Feinberg, 2014)

BASIC PROTOCOL 1: MICROCONTACT PRINTING OF ECM PATTERNS ON PIPAAm-COATED COVERSGLIPS

This protocol describes μ CP of ECM patterns onto PIPAAm-coated glass coverslips in order to perform SIA. We previously reported using SIA to generate ECM protein nanofibers (Feinberg and Parker, 2010) and to shrink wrap (encapsulate) cells in a layer of ECM protein to modulate the chemomechanical microenvironment (Palchesko et al., 2014). Here, we μ CP ECM protein micropatterns on PIPAAm in order to transfer them onto complex surface topographies as part of the PoT process, as described in (Sun et al., 2015) and Basic Protocol 2. The main steps of μ CP are first to adsorb protein from solution onto PDMS stamps with microscale features (Fig. 1 A–B, see Support Protocol 1 for PDMS stamp preparation) and second to bring the PDMS stamp into conformal contact with the target substrate (Fig. 1C). This transfers a protein pattern matching the PDMS stamp features to the PIPAAm substrate. The result is a ~5nm thick layer of patterned ECM protein on the PIPAAm (Fig. 1D–E) (Feinberg and Parker, 2010; Szymanski and Feinberg, 2014).

All steps of Basic Protocol 1, except for sonication, should be performed in a biosafety cabinet to minimize dust or particles from interfering with transfer of protein from the

PDMS stamp to the PIPAAm substrate. This also maintains sterility if cells will eventually be seeded on the PoT-printed substrates generated from these patterns.

Both for sterility purposes and to protect the microscale PDMS stamp features, tweezers should be used to handle PDMS stamps, by contacting the PDMS stamp edges. It is important to avoid applying pressure to or scratching the feature surface of the PDMS stamp.

Materials

PDMS stamps (Support Protocol 1)
Ethanol (200 proof)
Distilled water
Sonicator (Branson 3510)
Nitrogen gun or compressed sterile air stream
Micropipettes and micropipette tips
Frozen ECM protein aliquot (FN, COL IV, or LAM, see recipes)
Frozen fluorescently labeled ECM protein (optional) (see Support Protocols 3 for succinimidyl ester (SE) labeling of proteins)
100 mm diameter Petri dishes
PIPAAm coated coverslips (see Support Protocol 2)
Tweezers
Scientific marker

Clean and prepare PDMS stamps

- 1 Sonicate PDMS stamps in 50% ethanol in distilled water for 30 minutes.
Fill a beaker with enough 50% ethanol to cover the PDMS stamps and transfer PDMS stamps to the beaker feature side up. Sonicate for 30 minutes. PDMS stamps should be dried within ten minutes after sonication is complete. Otherwise, they will take up ethanol and swell.
- 2 Dry PDMS stamps with nitrogen or sterile compressed air stream.
PDMS stamps must be completely dry so that no ethanol solution mixes with the protein solutions that will be applied.
- 3 Place dry PDMS stamps feature side up in a sterile Petri dish.

Coat PDMS stamps with ECM proteins (FN, COL IV, or LAM)

- 4 Thaw an ECM protein aliquot and dilute to the desired concentration.
Concentrations may be varied. Typically, we use 50 µg/ml for FN, 100 µg/ml for LAM, and 100 µg/ml for COL IV. Work by Toworfe and coworkers (Toworfe et

al., 2004) demonstrates the effect of time and concentration on FN adsorption to PDMS.

- 5 If fluorescent protein will be used, thaw a fluorescently labeled ECM protein aliquot and dilute to desired concentration.
- 6 If fluorescent protein will be used, use the unlabeled ECM solution and fluorescently labeled ECM solution to make a mixture of fluorescently labeled and unlabeled ECM solutions.

For example, to make a 40% fluorescently labeled solution using a 50 µg/ml unlabeled FN solution and a 50 µg/ml labeled FN solution, mix 600 µl unlabeled FN and 400 µl labeled FN for a 40% labeled solution. Using fluorescently labeled protein allows for verification of the results of µCP, and for the verification of the pattern transferred to a topographic substrate in Basic Protocol 2. The percentage of labeled protein that is used can be varied. Ideally, the percentage of fluorescent protein should be as low as possible. Enough labeled protein should be used to generate sufficient contrast to take quality images, but since fluorescently labeling proteins modifies amino acids, too much labeled protein may interfere with ECM protein's native functions (Doyle, 2016).

- 7 Use a micropipette to add a 200 µl drop of the 40% labeled ECM solution to the PDMS stamp surface. More or less solution can be used depending on PDMS stamp size.
- 8 Use a micropipette tip to spread the ECM solution drop over the PDMS stamp surface.

Do not contact the patterned features with the micropipette tip, as this can damage the PDMS stamp. Contact the top edges of the PDMS stamp with the side of the micropipette tip in order to spread the droplet of protein solution over the PDMS stamp.
- 9 Cover the Petri dish and incubate for one hour. Protect from light to prevent photobleaching.

This step allows time for the ECM proteins in solution to adsorb to the PDMS stamp surface.

Removal of excess ECM protein

- 10 Fill two 100 mm Petri dishes with sterile distilled water.
- 11 Rinse excess protein from PDMS stamps.

With tweezers, hold the PDMS stamp feature side up and submerge it in the first water dish. Move the PDMS stamp through the water for 3–5 seconds to rinse excess ECM solution from the PDMS stamp. Repeat this process with the second Petri dish. This step removes excess ECM protein that is not adsorbed to the PDMS stamp features.

- 12** Dry the PDMS stamps with nitrogen or sterile compressed air.

PDMS stamps must be completely dry because any water will partially dissolve the PIPAAm.

Transfer of ECM protein from PDMS stamp features to PIPAAm substrate

- 13** Place PDMS stamps on PIPAAm-coated coverslips.

With tweezers, gently bring the PDMS stamp into contact with the PIPAAm coated coverslips, feature side down. Contact one edge of the PDMS stamp to the coverslip, then tilt the PDMS stamp to bring the feature face into contact with the PIPAAm surface.

- 14** Gently tap each corner of the PDMS stamp with tweezers.

This ensures conformal contact between the PIPAAm and PDMS stamp features.

- 15** Mark pattern location and orientation of the PDMS stamp on the PIPAAm with scientific laboratory marker (optional).

This is important if the pattern should be transferred to topography in a particular orientation and is also useful for locating the pattern for imaging.

- 16** Leave the PDMS stamps in contact with the PIPAAm for one hour.

This allows time for protein transfer from the PDMS stamp to the PIPAAm.

- 17** Slowly and gently peel the PDMS stamps off the coverslips.

Use one set of tweezers to hold down the coverslip, and another set to hold two ends of the PDMS stamp. Slowly tilt the tweezers to peel the PDMS stamps off the coverslips.

SUPPORT PROTOCOL 1: Preparation of PDMS stamps for μ CP of ECM protein patterns on PIPAAm

This Support Protocol describes photolithography techniques using SPR 220.3 photoresist to generate micropatterned master molds for making PDMS stamps with 2–10 μ m deep features for μ CP of ECM proteins. PDMS is used to fabricate the stamps because it can reproduce submicron features of the master molds (Zhang et al., 2006) and is elastomeric, so slight deformations allow the stamp features to fully contact the target substrate for pattern transfer in μ CP (Ruiz and Chen, 2007). Parameters such as lamp height and exposure time are optimized based on individual photolithography setup and feature size. For this Support Protocol we use photoresist coated cover glasses, but photolithography can also be done using photoresist coated silicon wafers, and for this reason we will generically refer to the substrates as wafers. Patterned substrates generated with this Support Protocol can be used to make PDMS stamps up to ten times, sometimes more. The PDMS stamps generated from the wafers can be used multiple times.

It should be noted that Support Protocol 1, for generating PDMS stamps (feature depth of 2–10 μ m) for μ CP, and Support Protocol 4, for generating PDMS topographic substrates

(feature depth of 10–200 μ m), both use photolithography techniques to micropattern wafers for making PDMS substrates. Our previous paper (Sun et al., 2015), used PDMS stamps prepared from glass wafers (Support Protocol 1, which may be less expensive and more accessible) for μ CP, and PDMS substrates prepared from silicon wafers (Support Protocol 4) for topographic substrates. In practice, separate substrates prepared from silicon wafers could be used as both μ CP stamps and as topographic substrates, with the caveat that, depending on silicon wafer and resulting PDMS stamp depth, it will be more difficult to ink (coat) ECM protein solutions onto deeper PDMS stamps due to decreased wettability. Alternately, substrates prepared from glass wafers could be used for both applications, but the topographic features will be less deep. PDMS stamps for μ CP can be used multiple times for μ CP, but should only be used for one protein and one fluorophore to avoid cross-contamination and fluorescent artifacts. Topographic substrates should only be used once.

Materials

Chrome mask (Photosciences Inc, acquire in step 2)
Hot plate
Glass rectangular coverslips/wafers (Fisher 12-543F)
Ethanol (200 proof)
Bunsen burner
Transfer pipets
SPR 220.3 photoresist (Microchem)
SPR 220.3 developer (MCF 26A) (Microchem)
Spin-coater
UV lamp
Adjustable stage
Rotator
Glass piece with soft part around edge
Large binder clips
Distilled water
Nitrogen or compressed air for drying wafers
Sylgard PDMS 184 (see recipe)
150 mm diameter Petri dish
Kimwipes

Mask design and acquisition

- 1 First, design a photomask with the desired nanometer to micrometer scale features with Computer Aided Design (CAD) software.

SPR 220.3 is a positive photoresist, meaning UV light will pass through transparent mask regions and solubilize the photoresist, which will dictate resulting PDMS stamp feature geometry.

- 2 Use the CAD file to make/order photomask.

This protocol describes photolithography with chrome on glass photomasks, which need to be used when feature geometries are less than 10 μm .

Transparency-based photomasks are adequate for feature sizes 10 μm or greater and could also be used as described in previous work (Sun et al., 2015).

Prepare workspace

- 3 Set hot plate to 115°C.
- 4 On a rotator, place three dishes large enough to submerge the glass wafer.
- 5 Fill one of the dishes with SPR 220.3 developer (MCF-26A). Fill the other two dishes with distilled water.
- 6 Set up stage for UV exposure.

The main components of this, shown in Fig. 2A, are 1) an orbital rotator 2) an adjustable height lab jack platform on top of the rotator, and 3) A UV lamp mounted above the platform. The platform should be adjusted to about 3 inches from the UV lamp.

Photolithography steps

- 7 Clean glass wafers with ethanol.
Rinse the wafer with ethanol and dry with a Kimwipe. Make sure the wafer is dry and free of debris before applying flame to it in the next step.
- 8 Treat one side of glass wafer with Bunsen burner flame.
This step activates the glass wafer surface so that the photoresist will adhere.
- 9 Place wafer treated side-up on spin-coater chuck.
- 10 Pipet SPR 220.3 photoresist onto the treated cover glass so that about 80% of the glass wafer surface is covered.
- 11 Spin-coat at 5000 rpm for 30 seconds to achieve a 2 μm thick photoresist layer, or at 2000 rpm for 30 seconds to achieve a 10 μm thick photoresist layer.
Spin-coating removes excess photoresist and leaves a thin uniform layer on the wafer.
- 12 Bake coated wafer on hot plate at 115°C for 90 seconds.
This step evaporates solvent from the photoresist layer.
- 13 Move wafer to stage underneath UV lamp. Align the wafer under the center of the lamp.

- 14** Align and apply the chrome mask, with the chrome side down touching the wafer. Make sure the desired micropattern in the mask is aligned with the UV lamp and wafer, so the lamp, micropattern, and wafer are all centered relative to each other. Make sure they are mostly centered when the rotator is turned on.
- 15** To obtain conformal contact between the chrome mask and wafer, we use a large rectangular piece of glass with soft material around its edges (Fig. 2B). The soft side is placed down against the chrome mask and the glass, mask, and stage are secured together with large binder clips (Fig. 2C). The micropattern and wafer should be under the clear part of the glass.
- 16** Turn on rotator.
- 17** Turn on UV lamp. Expose coated wafer to UV light through the photomask for 50 seconds. (Turn the lamp off after 50 seconds, then turn off the rotator).
- SPR 220.3 is a positive photoresist, so it becomes more soluble after exposure to UV light. The actual exposure time required may vary on bulb age, type, and other factors unique to experimental setup. Try different exposure times: e.g. 10, 25, 50, 75 seconds, and look (with a microscope) at the resulting wafers to determine optimal exposure time. Underexposure will leave photoresist within the features. Overexposure will cause dilation of features or removal of features entirely.
- 18** Post bake exposed wafer on hot plate to 115°C for 90 seconds.
- This step provides thermal energy to continue photochemical reactions initiated by UV light.
- 19** Place wafer in SPR developer solution on rotator for 40s.
- Using a consistent time is important so the wafer is not over- or under-developed. The photoresist that was exposed through the mask will visibly dissolve.
- 20** Wash wafer in water for 40 seconds on rotator, two times
- Wash the wafer in both dishes of water. Water washes away the developer solution and solubilized photoresist.
- 21** Dry wafer with nitrogen or air.
- 22** Inspect wafer under microscope to confirm generation of desired features.

Replica molding of PDMS stamps

- 23** Place wafer in 150 mm Petri dish
- 24** Pour PDMS on wafer and cure for at least 4 hours at 65°C.
- 25** Cut out PDMS stamps.
- Use a utility knife to carefully cut a rectangle in the PDMS that is over the glass wafer but outside of the micropattern. After cutting a few times around the

rectangle, gently peel the PDMS rectangle away from the wafer. At this point the PDMS rectangle will have large unpatterned edges around the micropattern. The edges should be trimmed away and the micropatterned area can be cut into one or multiple PDMS stamps. Ideally, the PDMS stamp(s) should be cut so channels that will be formed between the PDMS stamp features and PIPAAm are open, which enables better contact between the PDMS stamp features and substrate. Notch the back (non-feature face) of the PDMS stamp to mark its orientation.

SUPPORT PROTOCOL 2: Preparation of PIPAAm-coated coverslips

This Support Protocol provides instructions for fabricating PIPAAm-coated glass coverslips as target substrates for μ CP of ECM proteins. PIPAAm is a thermoresponsive polymer that is hydrophilic below its lower critical solution temperature (LCST) at around 35°C and hydrophobic above 35°C. Therefore, ECM protein patterns μ CP on PIPAAm can be released in a process termed SIA by immersing the substrate in warm water at 40°C and allowing the water to cool to room temperature, which dissolves the PIPAAm layer (Feinberg and Parker, 2010). This property is used in Basic Protocol 2 to transfer the ECM patterns from PIPAAm to the target topographic surfaces (Sun et al., 2015).

The thickness of the PIPAAm layer needed depends on the depth and geometry of the topographic substrate that will be patterned. Our group has previously used a 10 wt% solution of PIPAAm in butanol to perform PoT on features of microridges and trenches with a trench depth-to-width aspect ratio of less than 0.5 (Sun et al., 2015). To perform PoT with topographic micro-trench features with depth-to-width aspect ratio of greater than 0.5, we used a 40 wt% solution of PIPAAm in butanol (Sun et al., 2015). For 10 wt% and 40 wt% PIPAAm solutions, different volumes and spin-coating speeds should be used (Table 1), which result in PIPAAm coatings with different thicknesses.

Materials

- 25 mm circular number 1 coverslips
- Coverslip rack
- Ethanol (200 proof)
- Sonicator (Branson 3510)
- Oven (65°C)
- Spin-coater (Specialty coating systems G3P-8)
- 10 wt% or 40 wt% PIPAAm solution (see recipe)
- Micropipettes and pipette tips
- Positive displacement pipette (optional)
- Kimwipes
- 150 mm diameter Petri dish

For cell culture studies, UV light in biosafety cabinet

Prepare coverslips for coating

- 1 Place coverslips in coverslip rack.
- 2 Immerse rack of coverslips in ethanol.
- 3 Sonicate coverslips in ethanol for 60 minutes.
- 4 Dry coverslips by leaving rack in an open biosafety cabinet with the blower on for 30 minutes.
- 5 Store cleaned coverslips on the rack in a container to protect from dust. Coverslips can be stored for weeks. If they look dirty they can be re-sonicated.

Apply PIPAAm coating

- 6 Place coverslip on spin-coater chuck.
- 7 Apply 200 μ l 10 wt% PIPAAm, or 250 μ l 40 wt% PIPAAm to the coverslip.
Since 40 wt% PIPAAm is viscous, it may be easier to use a positive displacement pipette. Always cap the tube of PIPAAm solution between pipetting steps, because butanol will evaporate and the concentration will change.
- 8 Spin-coat at 2000 RPM for 60 sec for 40 wt% PIPAAm, or, spin-coat at 6000 RPM for 60 seconds for 10 wt% PIPAAm.
Spin-coating removes excess solution and leaves a thin, uniform layer. When spin-coating 10 wt% PIPAAm, the butanol will evaporate almost entirely during the spin-coating process.
- 9 Place PIPAAm coated coverslips in a Petri dish.
For 40 wt% PIPAAm coverslips, place them in the dish on top of a Kimwipe, to prevent the PIPAAm from sticking them to the polystyrene and leave the dish covered on the bench overnight to allow the butanol to evaporate. 10 wt% PIPAAm coverslips can be used 10 minutes after coating.

Sterilize coverslips (optional)

- 10 For cell culture studies, sterilize spin-coated PIPAAm coverslips before patterning. Place coverslips in Petri dish and treat with UV light in biosafety cabinet for 45 minutes.

SUPPORT PROTOCOL 3: Labeling of ECM proteins (LAM, FN, COL IV) with SE-conjugated dyes

This Support Protocol provides instruction to fluorescently label ECM proteins by conjugating fluorophores to lysine residues. SE-conjugated fluorophores are mixed with

buffered ECM proteins and react with amines on lysine residues, and then the mixture is dialyzed to purify the fluorophore-conjugated protein.

Materials

SE buffer (see recipe)

Fluorophore-conjugated SE stock (see recipe)

Frozen ECM protein aliquot(s) (FN, COL IV, or LAM, see recipes)

Slide-a-lyzer dialysis kit (dialysis cassettes, needles, syringes) (Thermo Fisher PI66332)

Microcentrifuge tubes

Sterile 1X phosphate-buffered saline (PBS)

Sterile distilled water

1. Thaw frozen ECM aliquot(s).
2. Add 400 μ l stock ECM (1 mg/ml), 200 μ l SE buffer, and 20 μ l protein tag stock to a microcentrifuge tube. Incubate at room temperature for 1 hour.
Because ECM proteins are mechanosensitive, very gently invert and revert the tube one time to mix the solution. Avoid mechanical agitation.
3. Fill a 200 ml beaker with SE buffer.
4. Hydrate a dialysis cassette in the SE buffer beaker for 5 minutes.
5. Assemble the needle and syringe (from slide-a-lyzer dialysis kit).
6. Pull about 1 ml of air into the syringe, then draw the labeled protein mixture up from the microcentrifuge tube.
7. Insert the syringe needle into the dialysis cassette port.
Do not insert the needle too deep or it may puncture the membrane. Make note of which syringe port is used. The cassettes are designed so ports can only be used once. Using a port multiple times may cause sample leakage.
8. Invert the syringe and cassette, and push the air out of the syringe and into the cassette.
9. Revert the syringe and cassette, and push protein solution into the cassette.
10. Draw air out of the cassette until the protein solution spreads across the cassette.
Adding air, then protein, then removing air facilitates spreading the protein solution in the cassette to increase the surface area for dialysis.
11. Replace the cassette in the beaker of SE buffer. Protect from light and incubate at 4°C for 2 hours.

12. Fill another beaker with sterile 1X PBS, and transfer the dialysis cassette. Protect from light and incubate at 4°C for 2 hours.
13. Fill a beaker with sterile distilled water, and transfer the dialysis cassette. Protect from light and incubate at 4°C overnight.
14. With a needle and syringe, using a different syringe port, add air to the cassette.
15. Without removing the needle from the cassette, draw up the labeled protein, then remove the needle.
16. Calculate the concentration of fluorescently labeled protein based on the final volume and initial amount of protein. Aliquot the labeled protein and store at -20°C.

BASIC PROTOCOL 2: Modified SIA technique for PoT of ECM proteins

PoT involves transfer of 2D ~5 nanometer thick patterns of ECM proteins onto topographically complex substrates, which enables independent control of physical and chemical surface properties. For PoT, a flat sacrificial PIPAAm layer patterned with ECM proteins (Fig. 3A, E, I), generated in Basic Protocol 1, is placed against a topographically complex substrate (Fig. 1F–G). In Basic Protocol 2, through a modified SIA process (Feinberg and Parker, 2010), PIPAAm swelling (Fig. 1H–I) followed by PIPAAm dissolution transfers the 2D pattern onto the contours of the substrate topography (Fig. 1J), where the protein layer adheres through hydrophobic interactions (Sun et al., 2015). The swelling and dissolution of PIPAAm to transfer protein patterns to topographic substrates (Sun et al., 2015) is a modification of SIA of ECM protein nanofibers from PIPAAm substrates (Feinberg and Parker, 2010). PIPAAm is a thermoresponsive polymer that is hydrophilic below its LCST around 32–35°C and relatively hydrophobic above its LCST. The LCST varies with molecular weight and polydispersity, which can vary batch-to-batch depending on supplier. Above the LCST, intramolecular hydrogen bonding of PIPAAm leads to shrinkage and insolubility in water, whereas below the LCST, intermolecular hydrogen bonding of PIPAAm with water results in PIPAAm's swelling and solubility in water (Lin et al., 1999). This property of PIPAAm has been exploited for a range of tissue engineering applications including formation of cell sheets (Takahashi et al., 2013), *in vitro* engineering of ECM protein nanofibers (Feinberg and Parker, 2010), and protein transfer in PoT (Sun et al., 2015), as described here.

PoT can be performed on many microtopographies, including ridge-and-trench topographies (Figure 3 B–C, F–G, J–K) and heterogeneous and isotropic topographies, such as paper and sandpaper (Fig. 3 D,H, L) (Sun et al., 2015). These results suggest PoT may be applicable to patterning nanotopographies also (Sun et al., 2015). Further, results with PoT printing of bovine serum albumin (Fig. 3M) and immunoglobulin G (Fig. 3N) show that PoT can be used to pattern a range of proteins beyond ECM proteins.

Since cells will eventually be seeded onto PoT printed substrates (Fig. 3O), the drying of substrates, rinsing and aspiration steps should be performed in a biosafety cabinet. Dishes should be covered when they are removed from the cabinet to maintain sterility.

Both for sterility purposes and to protect the microscale substrate features, tweezers should be used to handle substrates, by contacting the substrate sides. It is important to avoid applying pressure to or scratching the substrate features.

Materials

Topographic PDMS substrates (see Support Protocol 4 or 6)
Ethanol (200 proof)
Distilled water
Sonicator (Branson 3510)
Nitrogen or compressed air
Tweezers
PIPAAm-coated coverslips patterned with ECM protein (from Basic Protocol 1)
35 mm diameter Petri dishes
37°C oven
Kimwipes
12 well plate
65°C oven
Vacuum grease (optional)

Clean and prepare topographic PDMS substrates for PoT

- 1 Sonicate topographic PDMS substrates (see Support Protocol 4 or 6) in 50% ethanol in distilled water for 30 minutes.

Fill a beaker with enough 50% ethanol to cover the substrates and transfer substrates to the beaker. Sonicate for 30 minutes. Substrates should be dried within ten minutes after sonication is complete. Otherwise, they will take up ethanol and swell.
- 2 Dry substrates in nitrogen or compressed air stream.

Substrates must be completely dry so ethanol does not dissolve the PIPAAm or affect the protein pattern.

Swelling and dissolution of PIPAAm to transfer pattern to topography

- 3 Place each PIPAAm coverslip patterned with ECM protein (from Basic Protocol 1) in a 35 mm Petri dish.

- 4 Bring the topographic PDMS substrate, feature-side down, into contact with patterned PIPAAm coverslip, with correct orientation if necessary.
- 5 Press down with tweezers around the edges and in the center of the substrate.
- 6 Gently add 40° C distilled water into the Petri dishes around the substrates. The final water level should be about halfway up the substrate height. Substrates that float or come unstuck at this point should be discarded.
- 7 Incubate the substrates/patterns in a 37°C oven for 3 hours.
This step allows for the water to infiltrate in and around the PIPAAm layer.
- 8 Remove substrates/patterns from incubator, leave at room temperature overnight.
This step cools the PIPAAm, causing it to swell and dissolve, resulting in protein transfer to the topographic substrate.
- 9 Check water infiltration.
Look at substrates through the bottom of the dishes. If there is a bubble in the center of the substrates, the water has not completely infiltrated the substrates and dissolved the PIPAAm, so leave the substrates overnight again to allow for full infiltration.

Rinsing steps to remove any residual PIPAAm

- 10 Carefully peel substrates off of coverslips.
- 11 Dry the sides and back of the substrates.
Carefully use a Kimwipe to wick moisture away. Press the back of the substrate into the Kimwipe to dry it. The back of the substrate needs to be dry so it can adhere to the well plate substrate during rinsing steps. If desired, small amounts of vacuum grease could be added to the corners of the back of the substrate to promote adherence.
- 12 Transfer the substrates to a 12 well plate so they are feature side up.
Using tweezers, press the corners of each substrate to adhere the PDMS to the 12 well plate. This prevents substrates from floating when water is added.
- 13 Add sterile distilled water to each well to cover the substrates.
- 14 Cover the dish and place on a rotator for ten minutes.
- 15 Aspirate the water from each well and add fresh water, cover dish and place on rotator for 10 more minutes.
- 16 Repeat previous step.
- 17 Place in 65°C oven for 12 minutes. If there is any PIPAAm still left, it will be insoluble from the temperature increase and appear white. If there is residual PIPAAm, perform more rinsing and rotating steps, then warm up the dish to check for PIPAAm again.

- 18 At this point, PoT is complete and any excess PIPAAm is removed. Imaging or cell seeding can be performed on the PoT-printed substrate.

SUPPORT PROTOCOL 4: Generation of PDMS substrates with 10–100 μ m deep topographic features for use as substrates in PoT

This Support Protocol describes photolithography techniques using SU-8 photoresist to generate wafers for making PDMS substrates with 10–100 μ m deep topographic features as substrates for PoT of ECM proteins. The silicon wafers generated with this Support Protocol can be used to make PDMS substrates multiple times, though they may periodically require re-silanization to prevent PDMS from adhering strongly to the wafer surface. Recommended parameters for coating wafers with different photoresist thicknesses vary depending on the specific type of SU-8 photoresist. For example, an SU-8-2015 photoresist layer can vary from 13 to 38 μ m thick by spin-coating from 4000 to 1000 RPM (Microchem). When layer thickness is increased, the soft-bake time, exposure energy, post-bake time, and development time increase as well (Microchem). For specific details, readers are referred to Microchem's SU-8 datasheets (Microchem; Microchem). Note that this protocol should be performed in a cleanroom to minimize defects and that process parameters may vary depending on the equipment used.

Materials

4" silicon wafers
SU-8 2015 or SU-8-2050 photoresist (Microchem)
Photomask (Photosciences Inc., acquire during step 2)
UV lamp
Spin-coater
SU-8 developer (Microchem)
Isopropanol
Nitrogen or compressed air
Vacuum desiccator
Trichloro(1H,1H,2H,2H-perfluorooctyl)silane
Sylgard PDMS 184 (see recipe)

Mask design and acquisition

- 1 Design photomask features with Computer Aided Design (CAD) software.
SU-8 is a negative photoresist, meaning UV light will pass through transparent mask regions and render the photoresist insoluble, which will dictate resulting feature geometry.
- 2 Use CAD file to make/order photomask.

Prepare workspace

- 3 Warm hotplates to 150°C and 95°C. If necessary, warm a hotplate to 65°C as well (for post-baking of SU-8-2050 coated wafers).

Photolithography steps

- 4 Leave 4" silicon wafer on the 150°C hotplate for 15 minutes.
This step dehydrates the wafer.
- 5 Place the wafer on the spin-coater chuck.
- 6 Pour SU-8 photoresist onto the wafer. Use SU-8-2015 or SU-8 2050 depending on desired feature height.

Pour in concentric circles beginning at the center of the wafer. Keep the bottle close to the wafer during pouring to minimize bubble formation. Cover about 2/3 of the wafer with SU-8.
- 7 Spin the wafer at recommended speed to generate desired SU-8 thickness. For example, to generate a ~13 µm thickness layer of SU-8 2015, spin at 500 rpm for 10 seconds, then at 4000 rpm for 30 seconds (Microchem).
- 8 Soft bake the wafer according to the parameters for desired thickness. For example, 2-3 minutes at 95°C for 13 µm thickness.

This step evaporates solvent.
- 9 Expose wafer to UV light through photomask with appropriate exposure energy. For example, 140mJ/cm² for 13 µm thickness.

SU-8 is a negative photoresist, so the exposed regions will remain. The exposure step produces acids, which drive crosslinking of the photoresist during the post-baking step (Microchem).
- 10 Post bake wafer, for example, 3–4 minutes at 95°C for 13 µm thickness.
- 11 Place wafer in SU-8 developer for recommended time (example 2–3 minutes for 13 µm thickness).
- 12 Rinse with isopropanol. If a white film forms during rinsing, the wafer needs to be developed longer and should be developed for 30 more seconds, then rinsed with isopropanol again.
- 13 Dry wafer with nitrogen or air.
- 14 Silanize wafer under vacuum with Trichloro(1H,1H,2H,2H-perfluorooctyl)silane overnight to decrease PDMS adhesion.

Replica molding of PDMS stamps

- 15 Place wafer in a 150 mm Petri dish.
- 16 Pour PDMS on the wafer.
- 17 Degas the PDMS in the vacuum desiccator for 10 minutes.

- 18 Cure PDMS in 65°C oven for at least four hours
- 19 Cut the PDMS around the outside of the silicon wafer. Remove the wafer and adhered PDMS from the dish. Cut and peel the PDMS that infiltrated underneath the wafer, and then peel the PDMS from the top of the wafer. Similarly to cutting out PDMS stamps in Support Protocol 1, cut the patterned area into individual PDMS substrate(s). Cut the substrates so that the features will form channels where water will be able to infiltrate during PoT. Notch the substrates to mark their orientation.

SUPPORT PROTOCOL 5: Generation of PDMS topographic substrates from ROUGH Materials

This protocol describes the production of topographic PDMS substrates without using photolithography techniques. PDMS substrates with varying microtopographies can be prepared by replica molding against different materials. Previously, our group has generated PDMS substrate microtopographies of varying roughness for PoT from A4 paper, and 150- and 220-grit sandpaper (Sun et al., 2015).

Materials

Paper or sandpaper

Sylgard PDMS 184 (see recipe)

100 mm diameter Petri dish

Utility knife

Razor blade

Scissors

65°C oven

1. Using scissors or utility knife, cut out a piece of paper or sandpaper that will fit in a Petri dish.
2. Place the paper or sandpaper in the bottom of the Petri dish.
3. Pour PDMS onto the paper.
4. Cure at 65°C for at least four hours.
5. Cut the PDMS and peel it off of the paper, then cut into individual substrate(s). If necessary, notch the back of the substrates to remember their orientation. The PDMS that was against the paper will have the negative topography of the paper surface.

SUPPORT PROTOCOL 6: Cell seeding on PoT-printed substrates

This protocol describes steps for culturing cells on PoT-printed substrates.

Materials

PoT-printed substrates

1% Pluronics (see recipe)

1X PBS

Cell suspension in media

Centrifuge

Hemocytometer

Incubator for cell culture

1. Move Petri dish containing PoT-printed substrates from the end of Basic Protocol 2 to the biosafety cabinet. To ensure sterility, leave in biosafety cabinet with UV light on for 15 minutes.
2. Aspirate water and add 1% Pluronics solution to cover PoT-printed substrates. Incubate with Pluronics for 15 minutes.

Pluronics prevents nonspecific adsorption of molecules to the PDMS in the areas not patterned with ECM proteins.
3. Aspirate Pluronics and rinse PoT-printed substrates three times with sterile 1X PBS.
4. Count cells with hemocytometer and determine cell suspension concentration. Dilute to the seeding concentration. 20,000 cells/cm² can be used for sparse cell seeding, 200,000 cells/cm² can be used for dense cell seeding.

If researchers are interested in a specific interaction with the patterned ECM proteins, they may wish to use serum-free media or media which is depleted of components like FN or vitronectin which may confound certain experimental results.
5. Seed cells in the wells/on the PoT-printed substrates.
6. Incubate and allow cells to attach and spread. The duration of this step is dependent on the goal of the experiment.

REAGENTS AND SOLUTIONS

Use sterile distilled water in all recipes and protocol steps.

40 wt% PIPAAm in butanol

- 20 ml 1-butanol
- 8 g PIPAAm (Polysciences 21458-10)
- Add 10 ml butanol to a 50 ml conical tube
- Add PIPAAm to the conical tube

- Add the other 10 ml butanol to the conical
- Vortex vigorously and leave overnight
- Store up to 3 months at room temperature

10 wt% PIPAAm in butanol

- 10 ml 1-butanol
- 1 gram PIPAAm (Polysciences 21458-10)
- Add 5 ml butanol to 15 ml conical tube
- Add PIPAAm to conical tube
- Add the other 5 ml butanol to the conical
- Vortex vigorously and leave overnight
- Store up to 3 months at room temperature

Frozen FN aliquots (1mg/ml)

- 5 mg lyophilized fibronectin, Corning 356009
- 5 ml sterile distilled water.
- Add water to the container of FN powder with a p1000 micropipette by repeatedly ejecting water to form a drop on the pipette tip and wicking the drop onto the inside top of the FN container.
- Do not swirl or agitate the FN solution, this can cause the FN to polymerize. Leave the FN solution at room temperature for 30–60 minutes.
- With a micropipette, dispense FN solution into 25 200 µl aliquots and freeze at –20°C. Smaller aliquots (e.g. 50 µl) can be prepared based on personal preference.
- Store for up to 3 months

Frozen COL IV aliquots (1mg/ml)

- 5 mg collagen IV [insert supplier]
- 5 ml water
- Add water to the lyophilized COL IV
- Incubate at room temperature for 30 minutes
- Dispense COL IV solution into aliquots and freeze at –20°C
- Store for up to 3 months

Frozen LAM aliquots (1mg/ml)

- 1 mg laminin (Thermo Fisher 23017015) comes as 1 mg/ml liquid solution

- Aliquot and freeze at -20°C
- Store for up to 3 months

Guanidine hydrochloride (8M)

- 7.642 grams guanidine hydrochloride
- Add water to a final volume of 10 ml
- Store at room temperature for up to 6 months

Fluorophore-conjugated SE stock

- SE label (e.g. Alexa 555 succinimidyl ester (Thermo Fisher A20009), 1mg
- 100 μl DMSO
- Add 100 μl DMSO to the vial of SE label powder.
- Make 10 10 μl aliquots and store protected from light at -80°C for up to 6 months

SE Buffer

- 4.1 gram sodium bicarbonate
- 500 ml distilled water
- Add bicarbonate to water on stir plate, mix for 5 minutes or until dissolved
- Sterile filter
- Store at 4°C for up to 6 months

1% Pluronics solution

- 5 gram Pluronics F-127 (P2443, Sigma)
- 500 ml distilled water
- Mix on stir plate overnight
- Sterile filter
- Store at 4°C for up to 6 months

Sylgard PDMS 184

- Dow Corning 4019862 PDMS 184 kit
- 20 grams PDMS 184 base resin
- 2 grams PDMS 184 curing agent
- Centrifugal mixer (Thinky ARE 250)
- Vacuum desiccator

- Mix in centrifugal mixer (2000 RPM for 2 minutes) and degas (2000 RPM for 2 minutes). Alternately, mix by hand for 10 minutes by stirring with a serological pipette, and degas in vacuum desiccator
- Once the PDMS base resin is mixed with the curing agent, it will begin to cure. PDMS 184 will cure within four hours at 65°C. Prepared PDMS mixtures should be kept at room temperature until used. They should be used (i.e. poured onto wafers) within three hours of mixing and protected from dust.

COMMENTARY

Background Information

Precise control of ECM adhesive area or topography is valuable in many biological applications. Soft lithography techniques to generate elastomeric stamps and structures initially developed by Whitesides and coworkers (Kumar and Whitesides, 1993) have been used for many applications, including μ CP of materials, as topographically micropatterned substrates for cell culture, and for microfluidics applications (Ruiz and Chen, 2007). Here, we present PoT, a protocol for μ CP of ECM proteins on a sacrificial PIPAAm substrate followed by transfer of these protein patterns from the PIPAAm onto topographically micropatterned PDMS substrates.

In μ CP, a stamp (conceptually similar to an ink stamp) is used to generate 2D patterns of molecules, with nanoscale thickness and nano- to millimeter-scale lateral dimensions, on smooth and flat substrates. Initially μ CP was used to stamp alkanethiols onto gold layers, which were then etched to generate patterns of gold (Kumar and Whitesides, 1993) for microelectronics and for microcrystal formation (Kumar et al., 1994). Across several fields, μ CP was extended to many materials, including other metals, solvents, salts, polymers, and biomolecules (Ruiz and Chen, 2007). Several important studies optimized μ CP techniques for protein molecules (Bernard et al., 1998; James et al., 1998; Tan et al., 2004). Feinberg and coworkers used a SIA approach involving μ CP of ECM proteins on PIPAAm to generate ECM protein nanofibers (Feinberg and Parker, 2010). This approach was modified to perform PoT of complex topographies (Sun et al., 2015) as discussed in this unit.

The influence of topography on cellular behavior has been studied for decades. For example, Weiss described alignment of cells cultured on anisotropic collagen fibrils from fish scales (Weiss, 1959) and Brunette described geometry-dependent alignment of fibroblasts cultured on micromachined silicon wafers (Brunette, 1986). These micro- and nanoscale topographies are important for basic science and tissue engineering studies because they mimic physical cues in the microenvironment, such as ECM fibril topography. With the development of precise photolithography and soft lithography techniques, many studies have aimed to explore how micro- and nanotopographies influence cellular behavior (Bettinger et al., 2009). For example, microtopographies have provided novel insights on how cells behave in 3D microenvironments, such as confined spaces (Stroka et al., 2014) and migration tracts (Kraning-Rush et al., 2013).

Because patterning of adhesive cues and patterning of topographic cues both drive cellular behaviors, many approaches seek to present both chemical and topographical ECM cues to cells, both for studying the combined influences of multiple ECM cues and for tissue engineering applications. For example, different approaches have combined topographical and chemical ECM cues to direct behavior of neurons (Greene et al., 2011), endothelial cells (Feinberg et al., 2008), and pre-osteoblasts (Charest et al., 2006). However, it is difficult to achieve independent control over microtopography and patterned chemical cues on the same substrate. The approaches developed to date have had a number of limitations. If the depth-to-width aspect ratio of the features is sufficiently low, then the elastomeric PDMS is soft enough to conform to the topography (Bietsch and Michel, 2000). For μ CP onto deeper topographic features, an option is to increase the pressure applied during stamping or decrease the stamp elastic modulus. However, this can result in deformation of the features in the PDMS stamp used for μ CP and/or deformation of the feature in the substrate, which results in loss of pattern fidelity. To address this, researchers have used multilayer stamps having rigid topographic features coupled to a soft layer so the stamp can conform better to substrate topographies (Odom et al., 2002; Schmid and Michel, 2000). The μ CP of molecules onto sacrificial substrates, followed by substrate dissolution, has also been used to pattern some topographic substrates (Fernandez et al., 2011; Yu et al., 2012). However, these approaches are limited to patterning substrate topographies with low aspect ratio features. The PoT approach presented here is unique in that it transfers an ECM protein pattern to a topographically patterned substrate with high fidelity and independent control over the orientation of the ECM patterning relative to the topography. Further, PoT is able to conform to topographic features with aspect ratios of greater than 2.5, far exceeding what is possible with other approaches.

Critical Parameters and troubleshooting

Basic Protocol 1—Ideally, μ CP will reliably reproduce the pattern of the PDMS stamp features onto the substrate. Unexpected results in μ CP can occur because of improper technique or poorly made PDMS stamps. Before use, PDMS stamps and wafers should be inspected with microscopy to ensure that the mask pattern was properly replicated to the wafer, and from the wafer to the PDMS stamp.

Micropatterns that are distorted relative to stamp feature patterns can result from improper technique or stamp design. Stamp collapse or sagging occurs when recessed areas of the PDMS stamp also contact the PIPAAm substrate during μ CP. This can result from improper PDMS stamp design, specifically if PDMS stamp features are too far apart then the recessed areas between them can sag and touch the PIPAAm. This typically results from applying too much pressure to the PDMS stamp, which can be fixed by applying less pressure. Another problem is that features with aspect ratios that are high (>2) can collapse, causing feature distortion. This can be fixed by making stamps with lower aspect ratio features.

Alternatively, sometimes the pattern does not fully transfer to the PIPAAm substrate, resulting in patterned features that appear thinner, heterogeneous, and/or grainy. Not all of the pattern will be perfect every time, but there should be good agreement between PDMS stamp features and pattern geometry. One reason for incomplete transfer is poor contact

between the PDMS stamps and PIPAAm. A possible reason for this is not applying enough pressure to the PDMS stamp. When the PDMS stamp is initially applied to PIPAAm, it will be either in or out of contact with the PIPAAm. This could mean more pressure should be applied to induce contact between the PDMS stamp and the PIPAAm. If only some of the regions of the PDMS stamp are in contact with PIPAAm, there will be subtle optical differences in their appearance. A check is to gently tap the corner of the PDMS stamp with the point of a tweezer. If the PDMS stamp corner looks different after tapping, it was not in contact, but has come into contact with the PIPAAm after tapping. Another good check is to gently bump the PDMS stamp laterally with the tweezers. If the contact is good, the coverslip and the PDMS stamp will move laterally together. Additionally, PDMS stamps should be cut so channels formed by the PDMS stamp features are open, which prevents an air bubble from forming and thus enables better contact between the PDMS stamp features and PIPAAm. For this reason, PDMS stamps that can trap air in pockets, such as crisscross mesh patterns, are more difficult to use successfully in μ CP on PIPAAm. Further, instead of simply dropping the PDMS stamp on the PIPAAm, the PDMS stamp should be applied as described in Basic Protocol 1 Step 13, by contacting the PDMS stamp edge to the coverslip and then gently tilting the PDMS stamp into contact. Also, a biosafety hood should be used to minimize dust and particles. Dust on the substrate or PDMS stamp will interfere with μ CP by forming a physical barrier to contact and pattern transfer.

Prior to performing Basic Protocol 2 to transfer an ECM micropattern onto topography, the pattern should be checked for fidelity with microscopy. Only good-quality micropatterns should be used for Basic Protocol 2.

Note that the concentration of ECM protein solution applied to the stamp may be varied in μ CP. This will also affect the concentration of ECM protein transferred to PIPAAm. Because there are nonlinear relationships between solution concentration and adsorption, it is important to quantify the final densities of ECM protein after μ CP and PoT. Typically, we use 50 μ g/ml for FN, 100 μ g/ml for LAM, and 100 μ g/ml for COL IV. FN adsorption to PDMS surfaces is known to increase nonlinearly with both time and concentration (Toworfe et al., 2004). We previously found that there was a nonlinear increase in the thickness of the fibronectin layer transferred onto PIPAAm with increasing concentration of fibronectin in solution incubated on the PDMS stamps (Feinberg and Parker, 2010). Note that after protein incubation, not all protein will transfer from the PDMS stamp to the PIPAAm, so the relationship between solution concentration and PIPAAm surface coverage is complex. Additionally, protein transfer can be inconsistent when a PDMS stamp is used for μ CP for the very first time, but subsequent μ CP should produce good results.

Basic Protocol 2—Several parameters contribute to the success of protein pattern transfer from PIPAAm to the topographically patterned substrate during PoT, including (i) limited protein fiber extensibility, (ii) the ability of water to infiltrate the topography and cause PIPAAm swelling and dissolution, and (iii) the PIPAAm coating thickness.

Limited protein fiber extensibility: The patterned protein layer generated in Basic Protocol 1 by μ CP onto PIPAAm cannot transfer with full fidelity to all possible microtopographies. Our data suggests that for PoT of FN patterns onto microscale ridges and trenches, a trench

depth-to-width aspect ratio of 2.4 is the limit where the pattern can be transferred to the topography as a continuous structure (Sun et al., 2015), likely because of failure strain of FN fibrils (Klotzsch et al., 2009). Different proteins may have different failure strains. Therefore, if PoT does not generate expected results, particularly if there appear to be “broken” patches of protein in high aspect-ratio regions, consider the impact of the protein extensibility and the topographic feature aspect ratio. We have performed PoT using ECM pattern lines ranging from 100 nm to 1 cm wide and microridges separated by trenches ranging from 2 μ m to 200 μ m deep and up to 200 μ m wide, while keeping within the depth-to-width limitations previously noted (Sun et al., 2015).

Ability of water to infiltrate topography: The PoT technique depends on the PIPAAm swelling and dissolving for pattern transfer to occur. Our group has performed PoT onto microridges generated by replica molding of PDMS, rough substrates generated from paper and sandpaper masters, and onto flat PDMS substrates as a control (Sun et al., 2015). Note that when substrates with microridges are used, they must be prepared so that water can infiltrate into the channels (trenches) between the microridges that are formed between the substrate and the PIPAAm. For situations where these types of channels do not exist, it is necessary to wait for the PIPAAm itself to hydrate by absorbing water, which can take multiple days in some cases.

PIPAAm coating thickness: The thickness of PIPAAm needed for PoT increases with the aspect ratio and/or depth of the topographic features. Our group has previously used 10 wt% PIPAAm (molecular weight=40 kDa) solution in butanol to perform PoT with topographic ridge features with a depth to width aspect ratio of less than 0.5 (Sun et al., 2015). To perform PoT with topographic ridge features with depth: width aspect ratio of greater than 0.5, we used 40 wt% PIPAAm solution in butanol (Sun et al., 2015). PIPAAm layer thickness increased with the PIPAAm solution concentration that was used (Sun et al., 2015). PIPAAm concentration and spin-coating speed can be varied to deposit more or less PIPAAm to perform PoT on deeper features. The concentration of PIPAAm needed will also change if a different molecular weight PIPAAm is used (unpublished observations).

Anticipated results

Basic Protocol 1—With fluorescent microscopy, researchers should be able to observe patterned protein layer on the PIPAAm substrate, which matches the features of the PDMS stamp that was used to generate the protein pattern.

Support Protocol 1 and 4—Micropatterns on the wafer should be visible to the eye. For example, a 1 cm square area patterned with 20 μ m wide lines that are 20 μ m apart will appear different than an unpatterned area of the wafer. Under a stereomicroscope, the patterns on the wafer should be visible. The features of the PDMS stamps made from the wafers should also be visible with a stereomicroscope. When the PDMS stamp is cut and placed on its edge, the cross section of the features will be visible under phase contrast microscopy.

Support Protocol 2—40 wt% PIPAAm coated coverslips will appear different to the eye than uncoated glass coverslips. When warm water is added to both 40 wt% and 10 wt% coated PIPAAm coverslips, the PIPAAm is water insoluble and will appear whitish when illuminated, such as by a phase microscope lamp. As the water cools the PIPAAm will dissolve and will become clear.

Support Protocol 3—Protein removed from the dialysis cassette in the final steps of labeling will have taken on the color of the label. For example, proteins tagged with Alexa Fluor 555 conjugates will appear as a pink solution to the eye. A good check is to perform μ CP of the labeled protein and observe the fluorescence. This can even be done with flat, featureless PDMS blocks used as stamps, which will generate a fluorescent rectangle when stamped. Another check is spectrophotometry.

Basic Protocol 2—With fluorescent microscopy, researchers should be able to observe the patterned layer from Basic Protocol 1, which has been transferred to the topographic PDMS substrate and follows the substrate's topographic contours. Ideally confocal imaging can be used so that the focal planes of the contours can be imaged and reconstructed.

Support Protocol 5—The expected result is PDMS substrates that have the negative geometry of the master used to make them.

Support Protocol 6—The expected result is that cells will adhere to the ECM proteins on the substrate. This may allow for observation of cellular behaviors influenced by topographic and chemical ECM cues.

Time considerations

Basic Protocol 1—Since sonication takes 30 minutes, and protein adsorption and transfer take one hour each, 3 hours is a good estimate for total time for μ CP on PIPAAm.

Support Protocol 1—Time to acquire the photomask after designing it will vary based on the supplier. Making glass wafers will take 1–2 hours depending on how many are made. Curing PDMS will take a minimum of 4 hours at 65°C.

Support Protocol 2—It takes about 90 minutes to clean and dry coverslips, which can be stored afterwards. It takes 2 minutes to spin-coat each coverslip with PIPAAm. 10 wt% PIPAAm coverslips can be used 10 minutes after coating, but 40 wt% PIPAAm coverslips must dry overnight before use. PIPAAm coverslips should be treated with UV light for 45 minutes to sterilize them before patterning protein for eventual use with cells.

Support Protocol 3—Because of the 1 hour incubation followed by multiple two hour dialysis steps and one overnight dialysis step, protein labeling and dialysis takes around 24 hours.

Basic Protocol 2—The first part takes 30 minutes to sonicate substrates, followed by 3 hours of incubation. Then, it will take 1–2 days for water to fully infiltrate and dissolve PIPAAm, and another hour for washing steps.

Support Protocol 4—Time to acquire the photomask after designing it will vary based on the supplier. Making wafers will take 1–2 hours depending on how many are made, followed by silanization overnight. Curing PDMS will take 4 hours at 65°C.

Support Protocol 5—It takes 4 hours to cure PDMS at 65°C.

Support Protocol 6—45 minutes to sterilize the substrates. It will take about 30 minutes to seed cells. The culture time is dependent on the experimental goals.

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LITERATURE CITED

- Alford PW, Nesmith AP, Seywerd JN, Grosberg A, Parker KK. Vascular smooth muscle contractility depends on cell shape. *Integrative biology : quantitative biosciences from nano to macro*. 2011; 3:1063–1070. [PubMed: 21993765]
- Bernard A, Delamarche E, Schmid H, Michel B, Bosshard HR, Biebuyck H. Printing patterns of proteins. *Langmuir*. 1998; 14:2225–2229.
- Bettinger CJ, Langer R, Borenstein JT. Engineering substrate topography at the micro- and nanoscale to control cell function. *Angewandte Chemie*. 2009; 48:5406–5415. [PubMed: 19492373]
- Bietsch A, Michel B. Conformal contact and pattern stability of stamps used for soft lithography. *Journal of Applied Physics*. 2000; 88:4310–4318.
- Broaders KE, Cerchiari AE, Gartner ZJ. Coupling between apical tension and basal adhesion allow epithelia to collectively sense and respond to substrate topography over long distances. *Integrative biology : quantitative biosciences from nano to macro*. 2015; 7:1611–1621. [PubMed: 26507156]
- Brunette D. Fibroblasts on micromachined substrata orient hierarchically to grooves of different dimensions. *Experimental cell research*. 1986; 164:11–26. [PubMed: 3956588]
- Charest JL, Eliason MT, Garcia AJ, King WP. Combined microscale mechanical topography and chemical patterns on polymer cell culture substrates. *Biomaterials*. 2006; 27:2487–2494. [PubMed: 16325902]
- Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. *Science*. 1997; 276:1425–1428. [PubMed: 9162012]
- Chou L, Firth JD, Uitto VJ, Brunette DM. Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. *J Cell Sci*. 1995; 108(Pt 4):1563–1573. [PubMed: 7615675]
- DiMilla PA, Stone JA, Quinn JA, Albelda SM, Lauffenburger DA. Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *The Journal of cell biology*. 1993; 122:729–737. [PubMed: 8335696]
- Doyle AD. Generation of 3D Collagen Gels with Controlled Diverse Architectures. *Current protocols in cell biology/editorial board, Juan S Bonifacino ... [et al]*. 2016; 72:10 20 11–10 20 16.
- Driscoll MK, Sun X, Guven C, Fourkas JT, Losert W. Cellular contact guidance through dynamic sensing of nanotopography. *ACS nano*. 2014; 8:3546–3555. [PubMed: 24649900]
- Feinberg AW, Alford PW, Jin H, Ripplinger CM, Werdich AA, Sheehy SP, Grosberg A, Parker KK. Controlling the contractile strength of engineered cardiac muscle by hierarchal tissue architecture. *Biomaterials*. 2012; 33:5732–5741. [PubMed: 22594976]
- Feinberg AW, Parker KK. Surface-initiated assembly of protein nanofabrics. *Nano letters*. 2010; 10:2184–2191. [PubMed: 20486679]
- Feinberg AW, Wilkerson WR, Seegert CA, Gibson AL, Hoipkemeier-Wilson L, Brennan AB. Systematic variation of microtopography, surface chemistry and elastic modulus and the state

dependent effect on endothelial cell alignment. *Journal of biomedical materials research Part A*. 2008; 86:522–534. [PubMed: 17994556]

Fernandez JG, Samitier J, Mills CA. Simultaneous biochemical and topographical patterning on curved surfaces using biocompatible sacrificial molds. *Journal of biomedical materials research Part A*. 2011; 98:229–234. [PubMed: 21548017]

Greene AC, Washburn CM, Bachand GD, James CD. Combined chemical and topographical guidance cues for directing cytoarchitectural polarization in primary neurons. *Biomaterials*. 2011; 32:8860–8869. [PubMed: 21885117]

Guido S, Tranquillo RT. A methodology for the systematic and quantitative study of cell contact guidance in oriented collagen gels. Correlation of fibroblast orientation and gel birefringence. *J Cell Sci*. 1993; 105(Pt 2):317–331. [PubMed: 8408268]

James C, Davis R, Kam L, Craighead H, Isaacson M, Turner J, Shain W. Patterned protein layers on solid substrates by thin stamp microcontact printing. *Langmuir*. 1998; 14:741–744.

Kim J, Staunton JR, Tanner K. Independent Control of Topography for 3D Patterning of the ECM Microenvironment. *Advanced materials*. 2016; 28:132–137. [PubMed: 26551393]

Klotzsch E, Smith ML, Kubow KE, Muntwyler S, Little WC, Beyeler F, Gourdon D, Nelson BJ, Vogel V. Fibronectin forms the most extensible biological fibers displaying switchable force-exposed cryptic binding sites. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:18267–18272. [PubMed: 19826086]

Kranning-Rush CM, Carey SP, Lampi MC, Reinhart-King CA. Microfabricated collagen tracks facilitate single cell metastatic invasion in 3D. *Integrative biology : quantitative biosciences from nano to macro*. 2013; 5:606–616. [PubMed: 23388698]

Kumar A, Biebuyck HA, Whitesides GM. Patterning self-assembled monolayers: applications in materials science. *Langmuir*. 1994; 10:1498–1511.

Kumar A, Whitesides GM. Features of gold having micrometer to centimeter dimensions can be formed through a combination of stamping with an elastomeric stamp and an alkanethiol "ink" followed by chemical etching. *Applied Physics Letters*. 1993; 63:2002–2004.

Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*. 2009; 139:891–906. [PubMed: 19931152]

Lin SY, Chen KS, Liang RC. Thermal micro ATR/FT-IR spectroscopic system for quantitative study of the molecular structure of poly (N-isopropylacrylamide) in water. *Polymer*. 1999; 40:2619–2624.

Lu P, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harbor perspectives in biology*. 2011; 3

Microchem. SU-8 2000 Permanent Epoxy Negative Photoresist Processing Guidelines for SU-8 2000.5, SU-8 2002, SU-8 2005, SU-8 2007, SU-8 2010 and SU-8 2015

Microchem. SU-8 2000 Permanent Epoxy Negative Photoresist Processing Guidelines for SU-8 2025, SU-8 2035, SU-8 2050 and SU-8 2075. [*This reference does not have a pre-defined format and therefore uses the "Generic" format]

Odom TW, Love JC, Wolfe DB, Paul KE, Whitesides GM. Improved pattern transfer in soft lithography using composite stamps. *Langmuir*. 2002; 18:5314–5320.

Palchesko RN, Szymanski JM, Sahu A, Feinberg AW. Shrink Wrapping Cells in a Defined Extracellular Matrix to Modulate the Chemo-Mechanical Microenvironment. *Cellular and molecular bioengineering*. 2014; 7:355–368. [PubMed: 25530816]

Parker KK, Brock AL, Brangwynne C, Mannix RJ, Wang N, Ostuni E, Geisse NA, Adams JC, Whitesides GM, Ingber DE. Directional control of lamellipodia extension by constraining cell shape and orienting cell tractional forces. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2002; 16:1195–1204. [PubMed: 12153987]

Pelham RJ Jr, Wang Y. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:13661–13665. [PubMed: 9391082]

Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC medicine*. 2006; 4:38. [PubMed: 17190588]

- Rehfeldt F, Engler AJ, Eckhardt A, Ahmed F, Discher DE. Cell responses to the mechanochemical microenvironment—implications for regenerative medicine and drug delivery. *Advanced drug delivery reviews*. 2007; 59:1329–1339. [PubMed: 17900747]
- Renault J, Bernard A, Bietsch A, Michel B, Bosshard H, Delamarche E, Kreiter M, Hecht B, Wild U. Fabricating arrays of single protein molecules on glass using microcontact printing. *The Journal of Physical Chemistry B*. 2003; 107:703–711.
- Ruiz SA, Chen CS. Microcontact printing: a tool to pattern. *Soft Matter*. 2007; 3:168–177.
- Schmid H, Michel B. Siloxane polymers for high-resolution, high-accuracy soft lithography. *Macromolecules*. 2000; 33:3042–3049.
- Stroka KM, Jiang H, Chen SH, Tong Z, Wirtz D, Sun SX, Konstantopoulos K. Water permeation drives tumor cell migration in confined microenvironments. *Cell*. 2014; 157:611–623. [PubMed: 24726433]
- Sun Y, Duffy R, Lee A, Feinberg AW. Optimizing the structure and contractility of engineered skeletal muscle thin films. *Acta biomaterialia*. 2013; 9:7885–7894. [PubMed: 23632372]
- Sun Y, Jallerat Q, Szymanski JM, Feinberg AW. Conformal nanopatterning of extracellular matrix proteins onto topographically complex surfaces. *Nature methods*. 2015; 12:134–136. [PubMed: 25506720]
- Szymanski JM, Feinberg AW. Fabrication of freestanding alginate microfibers and microstructures for tissue engineering applications. *Biofabrication*. 2014; 6:024104. [PubMed: 24695323]
- Takahashi H, Shimizu T, Nakayama M, Yamato M, Okano T. The use of anisotropic cell sheets to control orientation during the self-organization of 3D muscle tissue. *Biomaterials*. 2013; 34:7372–7380. [PubMed: 23849343]
- Tan JL, Liu W, Nelson CM, Raghavan S, Chen CS. Simple approach to micropattern cells on common culture substrates by tuning substrate wettability. *Tissue engineering*. 2004; 10:865–872. [PubMed: 15265304]
- Thery M. Micropatterning as a tool to decipher cell morphogenesis and functions. *J Cell Sci*. 2010; 123:4201–4213. [PubMed: 21123618]
- Toworfe GK, Composto RJ, Adams CS, Shapiro IM, Ducheyne P. Fibronectin adsorption on surface-activated poly(dimethylsiloxane) and its effect on cellular function. *Journal of biomedical materials research Part A*. 2004; 71:449–461. [PubMed: 15481053]
- Weiss P. Cellular Dynamics. *Reviews of Modern Physics*. 1959; 31:11–20.
- Win Z, Vrla GD, Steucke KE, Sevcik EN, Hald ES, Alford PW. Smooth muscle architecture within cell-dense vascular tissues influences functional contractility. *Integrative biology : quantitative biosciences from nano to macro*. 2014; 6:1201–1210. [PubMed: 25363686]
- Yu H, Xiong S, Tay CY, Leong WS, Tan LP. A novel and simple microcontact printing technique for tacky, soft substrates and/or complex surfaces in soft tissue engineering. *Acta biomaterialia*. 2012; 8:1267–1272. [PubMed: 21945825]
- Zhang Y, Lo CW, Taylor JA, Yang S. Replica molding of high-aspect-ratio polymeric nanopillar arrays with high fidelity. *Langmuir*. 2006; 22:8595–8601. [PubMed: 16981781]
- Zhao Y, Zeng H, Nam J, Agarwal S. Fabrication of skeletal muscle constructs by topographic activation of cell alignment. *Biotechnology and bioengineering*. 2009; 102:624–631. [PubMed: 18958861]

Significance Statement

Cells are integrated into tissues by the extracellular matrix (ECM), which consists of fibrillar and sheet-like biopolymer networks that support cell adhesion, direct cell migration, regulate signaling molecules, and contribute to tissue mechanics. The topographical and biochemical structure of the ECM are important regulators of cell behavior during both homeostasis and disease pathogenesis. Engineering these ECM cues at the nano- and microscale has thus become an important *in vitro* tool for understanding cell-ECM interactions. Patterning on Topography is a technique for engineering cell culture substrates with independent nanoscale control of chemical and topographical ECM cues. This provides an approach for understanding how cell responses are regulated by chemical and physical cues for basic science, tissue engineering and medical device applications.

Basic Protocol 1: Microcontact printing of ECM protein patterns on PIPAAm

Basic Protocol 2: Modified surface-initiated assembly approach to conformally transfer ECM protein pattern to topographic surface

Figure 1.

Schematic showing PoT process. (A) Incubation of ECM solution causes ECM protein to adsorb to the PDMS stamp surface. (B) Rinsing the stamp removes excess protein. (C) Contacting the stamp to a glass coverslip with a spin-coated PIPAAm layer transfers adsorbed protein to PIPAAm, which generates an ECM pattern on PIPAAm (D and E) matching the stamp features. (F and G) To transfer the protein pattern on PIPAAm to a topographically complex surface, contact the surface to the ECM pattern. (H and I) PIPAAm swells to “push” and transfer the ECM pattern to the topographic surface, which results in (J) a PoT-printed surface.

Figure 2.

(A) Setup for SPR photolithography consisting of UV lamp, lab jack with platform, and rotator. (B) Large rectangular piece of glass with soft material around its edges (C) Glass piece with soft material, mask, and stage secured together with large binder clips.

Figure 3.

Results of PoT with 20 μm wide and 20 μm spaced (20 $\times$ 20) lines patterns (A) FN lines patterned on PIPAAm with μCP . (B) and (C) PoT printing of FN onto variable aspect ratio microridges using 20 $\times$ 20 topographic ridges of (B) 15 μm depth and (C) 24 μm depth. (D) PoT onto 220-grit sandpaper. (E–H) Maximum intensity projections of (E) μCP FN on PIPAAm, (F) PoT-printed FN on 15 μm deep ridges, (G) PoT-printed FN on 24 μm deep ridges, and (H) PoT-printed FN on 220-grit sandpaper. (I–L) Orthogonal views of (I) μCP FN on PIPAAm, (J) PoT-printed FN on 15 μm deep ridges, (K) PoT-printed FN on 24 μm deep ridges, and (L) PoT-printed FN on 220-grit sandpaper. (M) PoT printing of bovine serum albumin onto 32 μm deep ridges. (N) PoT printing of immunoglobulin G onto 32 μm deep ridges. (O) Cardiomyocytes cultured on PoT-printed FN. Blue: nuclei (DAPI), green: FN, red: α -actinin. Scale bars are 20 μm . Distances shown in projection views are given in μm . Dashed red lines in (E–H) indicate the locations of cross-sections shown in (I–L).

Table 1
Parameters for the spin-coating and use of 10 wt% and 40 wt% PIPAAm solution (Sun et al., 2015).

Concentration	Topography	PIPAAm layer thickness (dry)	Volume	Spin Speed	Time until use
40 wt%	Aspect ratio >0.5	~5.5 μm	250 μl	2000 RPM	Dry overnight
10 wt%	Aspect ratio <0.5	~1 μm	200 μl	6000 RPM	Dry for 10 minutes