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Protocol for Micropatterning of Cell Monolayers into Islands of Desired Size and Shape V.2

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We use this protocol and it's working

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Disclaimer

The user of this protocol should review it and establish proper procedures for safely completing the protocol. This includes use of personal protective equipment, use of fume hoods or biosafety cabinets, and reviewing safety data sheets for proper procedures for handling materials. Although this protocol describes some steps related to safety, the steps described in this protocol are not intended to describe in detail all procedures for safe operation.

Abstract

The following protocol is used to confine cell monolayers to user-defined geometries on hydrogels. The protocol uses methods of soft lithography with polydimethylsiloxane (PDMS). The successful protocol creates islands of cells on compliant substrates with user defined size and shape. These cell islands can be used to study a variety of questions in cell mechanics, mechanobiology, and collective cell migration using experimental methods such as optical microscopy, digital image correlation, particle image velocimetry, cell tracking, cell segmentation, traction force microscopy, and monolayer stress microscopy.

Materials

Materials and Equipment for Section I

- Vector graphics editing software, such as Adobe Illustrator or Inkscape. (The instructions below show sample images from Illustrator, but other software will work as well.)
- High resolution printer with at least 600 DPI resolution. (Many print shops have printers with sufficient resolution.)

Materials for Section II

- Silicon Wafers (test grade, polished on one side, 50.8 mm diameter)
- SU-8 3035 Photoresist (Kayaku Advanced Materials NC2021868)
- SU-8 Developer (Kayaku Advanced Materials NC99011158)
- Silane (TRIDECAFLUORO-1,1,2,2-TETRAHYDROOCTYL) SILANE (SIT8173)
- Plastic petri dish
- Aluminum foil
- Glass petri dish
- Isopropyl alcohol
- Soft bristled toothbrush (look for ultra-fine bristles)
- Mold release (Mann Ease Release 200, optional)

Equipment for Section II

- OmniCure S1500 UV Curing System and light guide
- UV Radiometer/Photometer (320-400 nm spectral range)
- UV blocking safety glasses
- Photomask
- Hot plate
- Razor blade
- Glass sheet (4 × 4 in or larger will work)
- Shaker plate
- Vacuum chamber
- Fume hood

Materials for Section IIIa

- Patterned Silicon Wafer
- Polydimethylsiloxane (PDMS) (Sylgard 184)
- Weighing boats

Equipment for Section IIIa

- Spin Coater
- 60°C Oven or hotplate

Materials for Section IIIb

- Biopsy punch

- Hollow punch (such as for punching holes in soft materials, ~16 mm diameter, optional)
- Razor blade
- Polydimethylsiloxane (PDMS) (Sylgard 184)
- Weighing boat
- Aluminum foil

Equipment for Section IIIb

- 60°C oven or hotplate

Materials for Section IV

- PDMS Masks
- Microscope slides
- Polyacrylamide (PA) gels (or substrate to be patterned)
- 0.1 M HEPES buffer
- Sulfo-SANPAH (ProteoChem c1111-100), diluted to a concentration of 50 mg/mL in 0.1 M HEPES
- Collagen I (Corning 354249 High Concentration Rat Tail), diluted to a concentration of 0.1 mg/mL in sterile ultrapure water
- Aluminum foil

Equipment for Section IV

- Plasma treater
- 365 nm UV lamp
- Biosafety cabinet
- Cell culture microscope

Materials for Section V

- Collagen treated polyacrylamide gels
- Cell culture medium
- Cells
- Bovine Serum Albumin (BSA), suspend at 3% w/v concentration in serum-free cell culture medium
- 1x Phosphate buffered saline (PBS)

Equipment for Section V

- Biosafety cabinet
- Cell culture incubator



Safety warnings

- ❗ The user of this protocol should review it and establish proper procedures for safely completing the protocol. This includes use of personal protective equipment, use of fume hoods or biosafety cabinets, and reviewing safety data sheets for proper procedures for handling materials. Although this protocol describes some steps related to safety, the steps described in this protocol are not intended to describe in detail all procedures for safe operation.

Summary

- 1 The following protocol is used to confine cell monolayers to user-defined geometries on hydrogels. First, a photomask with the desired micropattern is designed and printed, as detailed in Section I of this protocol. Next, SU-8 negative photoresist is applied, cured, and developed on a silicon wafer (Section II). Polydimethylsiloxane (PDMS) is then spin-coated onto the wafer and allowed to cure in a 60°C oven overnight (Section IIIa). Cured PDMS is then peeled off the silicon wafer and cut into square masks to be used for collagen treating (Section IV). Collagen treated hydrogels are then seeded with cells (Section V).

Example results can be seen in the accompanying manuscript.

1.1

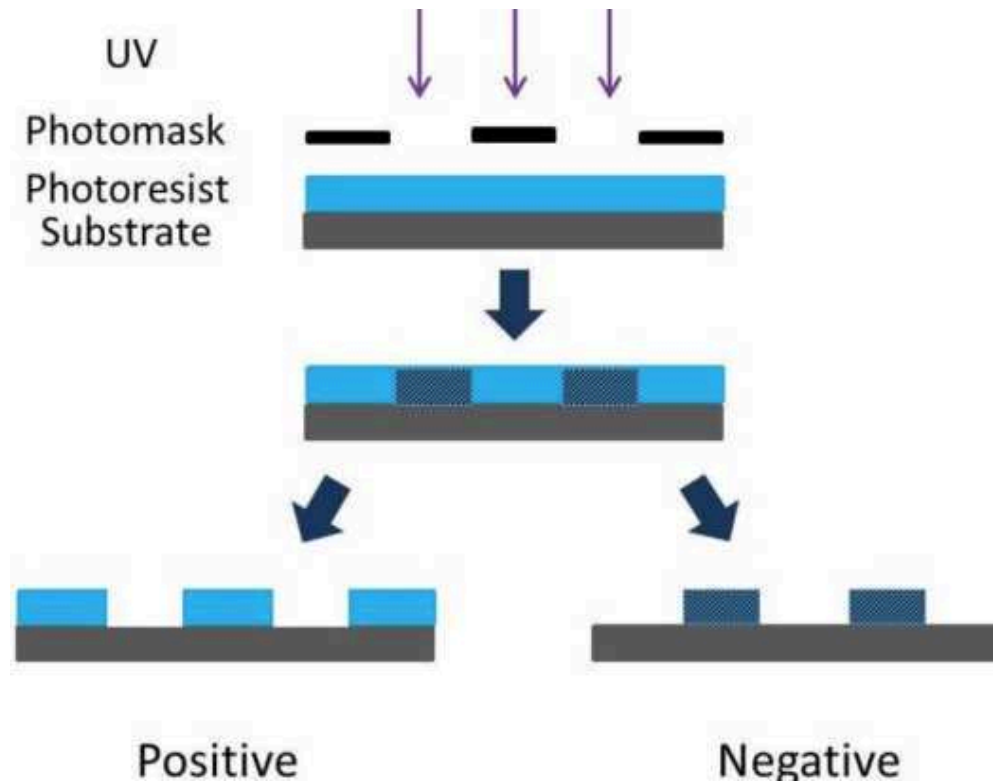


Figure 1. Illustration of SU-8 negative photoresist. Regions of the SU-8 not covered by the photomask cure/harden when exposed to UV light, forming surface features on a silicon wafer substrate. This patterned silicon wafer then acts as an inverse mold for PDMS, creating holes in the PDMS sheet.

- 2 **Note for Micropatterning of Large Features (≥ 1 mm)**
For micropatterning large circles (≥ 1 mm), a simpler procedure can be used by punching holes with a biopsy punch. For this case, see Section IIIb, which replaces Sections I-IIIa.
- 3 **Note about Safety**

The user of this protocol should review it and establish proper procedures for safely completing the protocol. This includes use of personal protective equipment, use of fume hoods or biosafety cabinets, and reviewing safety data sheets for proper procedures for handling materials. Although this protocol describes some steps related to safety, the steps described in this protocol are not intended to describe in detail all procedures for safe operation.

Section I: Design of the Photomask

- 4 Note: Instructions written in Section I describe the procedure using Adobe Illustrator. Other software can be used as well, though the exact details will be different.
- 5 Create a new document in Illustrator. Choose CMYK color mode (File > Document Color Mode) and select units of mm (File > Document Setup).
- 6 Use the ellipse tool to draw a circle with the radius of the silicon wafer chip to be used (we typically use a 50.8 mm diameter wafer). Select the feature and set both stroke and fill to black (Properties > Appearance)

6.1

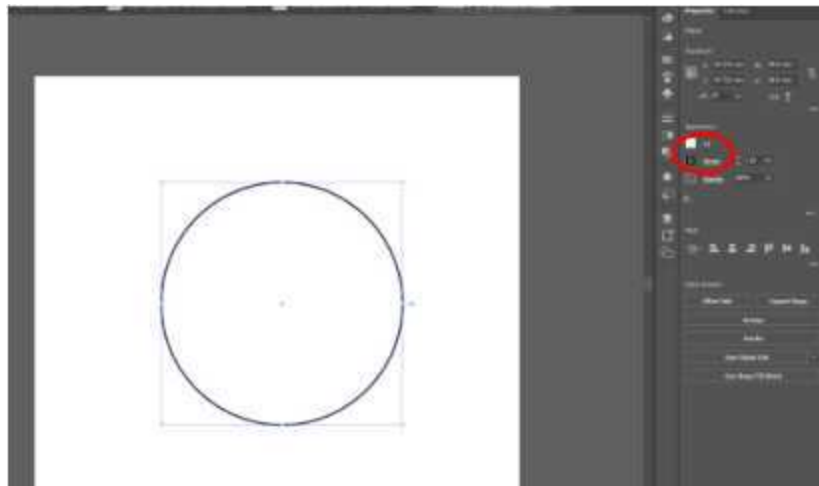


Figure 2. Circular base feature with a diameter of 50.8 mm. Fill and stroke settings located in the appearance section of feature properties is shown by the red circle.

- 7 Use additional drawing tools to generate the surface feature to be patterned. Select the feature and set the stroke to 0 pt. Set both stroke and fill to white (white features will cure during fabrication).

7.1

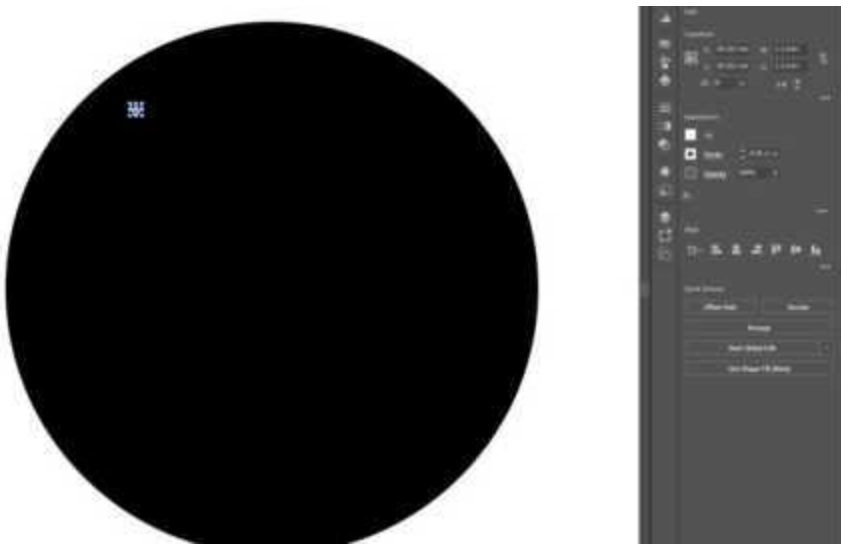


Figure 3. Initial white W feature drawn on the black base feature.

- 8 Use the patterning tool (or copy and paste) to generate the white surface pattern. Ensure there is 1 mm between each feature. Note:
- It appears easiest to place the first feature at a desirable (x, y) location (Properties > Transform) and then paste each additional feature at a 1mm offset. Then copy and paste groupings of the feature to generate a large pattern.
 - Be sure to leave feature-free room of at least a few mm around the edge to ensure easy removal of the PDMS mask.

8.1

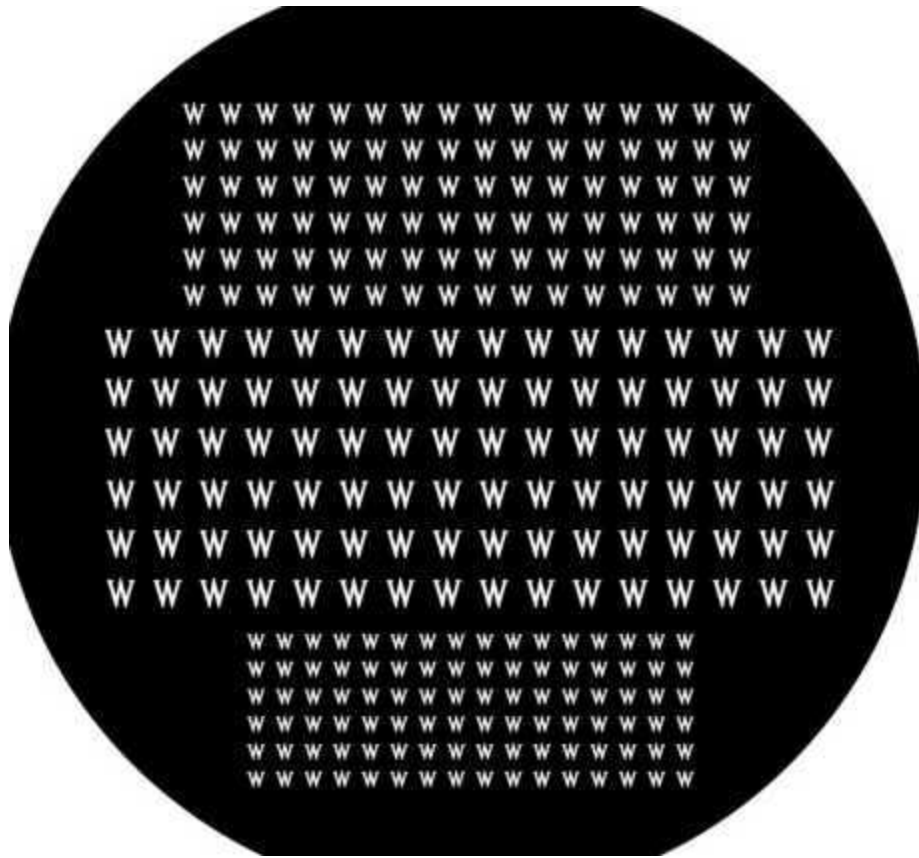


Figure 4. Patterned white W feature of various sizes. White within the photomask will develop into extruded features while black will dissolve during silicon chip fabrication

- 9 Print the photomask on a transparent sheet. Note:
- Since a high-resolution printer will be needed (at least 600 dots per inch), masks can be printed at a print shop.
 - If higher resolution features are needed, a photomask can be ordered from a company that offers high resolution printing. The smaller the features, the higher the printing resolution will need to be.

Section II: Fabrication of Patterned Silicon Wafer

- 10 First, we will apply SU-8 to a silicon wafer, which is described in the next few steps. Begin by heating the hot plate to 65 °C.
- 11 Place the silicon wafer on a scale and add 2 mg of SU-8 3035 to the center of the wafer for every μm of feature height desired. Note:
- It is best to keep features between 300 and 400 μm tall. There are different formulations of SU-8 that can achieve features of different heights, but this protocol has been optimized to use SU-8 3035, with a target height between 300 and 400 μm tall.

- Different wafer sizes can be used, but wafer size is limited by the size of the spin coater chuck available.

- 12 Place the wafer directly on the hot plate and wait several minutes until the SU-8 becomes less viscous. Use a razor blade to spread the SU-8 such that it covers most of the wafer. Leave ~5 mm of room around the edge so that the SU-8 does not run off the chip. Ensure that the SU-8 is spread evenly with no holes or mounds.



Figure 5. Image of SU-8 after spreading it on a silicon wafer using a razor blade.

- 13 Cover the wafer with an opaque lid and bake the SU-8 at 95°C for 80 minutes.
- Note: A petri dish lid wrapped in aluminum foil works well to cover the wafer as it bakes. Place the lid directly over the wafer on the hotplate.
- 14 Let the wafer come completely back to room temperature before removing it from the hot plate. This wafer can be left overnight, but make sure it is left covered on a flat surface.
- Note:
- Bake times will need to be adjusted for SU-8 thicker than 400 μm .
 - It is important for the wafer to return to room temperature slowly, as rapid cooling can introduce thermal stress between the SU-8 and the wafer resulting in cracking, peeling, and delamination.
- 15 The next few steps will cure the SU-8 and develop it. Begin by preheating the OmniCure for at least 20 minutes before use.

- 16 Place the wafer on a flat, mobile surface (use a small glass sheet for this). Place the photomask on top of the wafer and tape it down to the surface. Make sure the photomask is aligned with the SU-8 on the wafer ink side down.
- Note: If features are very small, or the SU-8 is thin, an additional glass sheet can be placed on top of the photomask to prevent unwanted redirection of light during the exposure process.

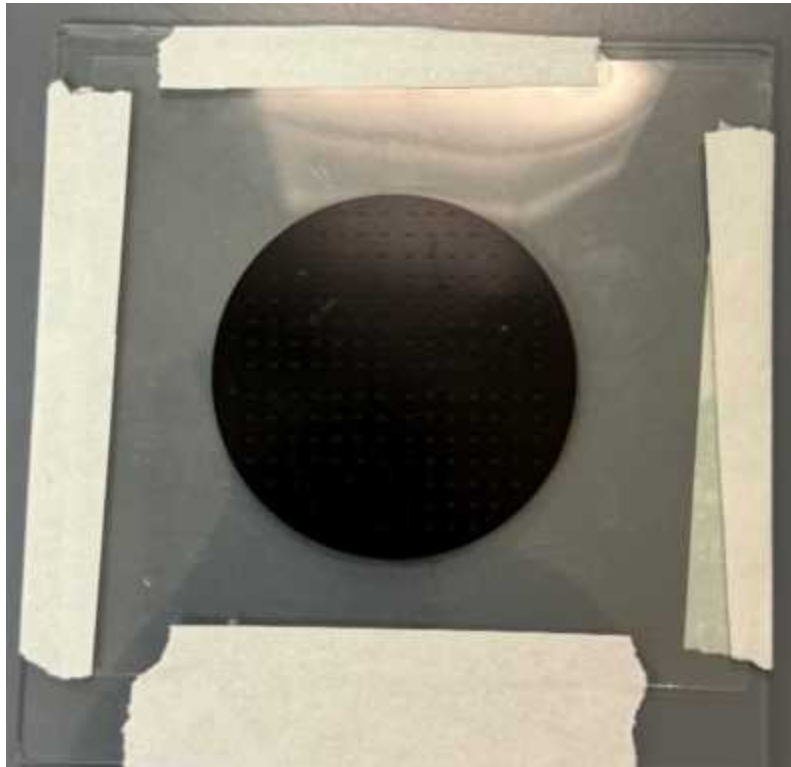


Figure 6. Photomask covering the SU-8-coated silicon wafer, held in place by tape on a glass sheet.

- 17 Place the OmniCure light guide ~10 inches above the wafer. Use the radiometer to measure the exposure energy, and calculate the needed exposure time using the equation, $t = E/I$, where t is the exposure time, E is the target exposure energy (from manufacturer) in mJ/cm^2 , and I is the UV irradiance (intensity, measured with radiometer) in $\mu\text{W}/\text{cm}^2$. Note:
- Target exposure energy will change based on SU-8 thickness. For a $400\ \mu\text{m}$ thick SU-8 layer, a UV irradiance of $8600\ \mu\text{W}/\text{cm}^2$, an exposure time of 125 s works well.
 - Too long of an exposure time will result in features larger than intended, and too short will result in no features developing.
 - Wear UV-blocking safety glasses while exposing.

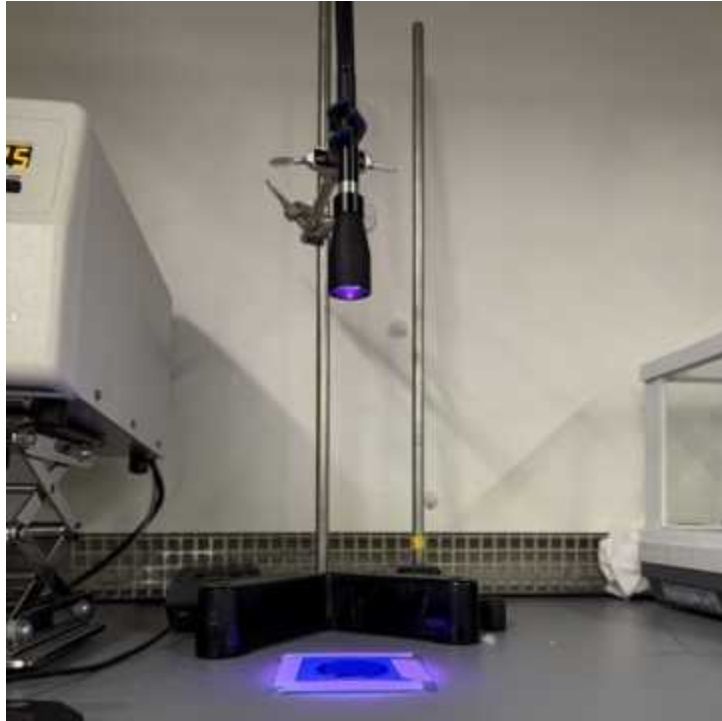


Figure 7. OmniCure S1500 UV system curing the features defined by the photomask onto the SU-8 coated chip.

- 18 Remove the photomask from the wafer. Place the wafer on the hot plate and cover it with a lid. Bake the wafer at 65°C for 5 minutes and then 95°C for 40 minutes (for a 400 μm thick SU-8 layer). Allow the wafer to return to room temperature before proceeding to next step. Note:
 - Bake time will need to be altered based on SU-8 thickness.
 - If there are ripples that form on the surface of the chip during the post-exposure bake, then the chip was heated too quickly during the post-exposure bake. Manually increase the temperature until the desired temperature is achieved (a ramp rate of 10°C every 10 minutes works well). There exist hot plates that can be programmed with temperature ramping, but they're expensive and not commonly available in biology labs, so manual ramping is sufficient.
- 19 **In fume hood (for all next steps):** Place the wafer into a glass petri dish. Pour SU-8 developer into the dish until it covers the wafer.
- 20 Place the dish onto a shaker plate. Set the oscillation speed such that a small wave circulates around the dish, washing over the SU-8-covered wafer.



Figure 8. Silicon wafer developing in SU-8 developer solution on shaker plate.

- 21 Develop on the shaker plate until there is no more uncured SU-8 visible (only features are left). This will take ~1-2 hrs for a 400 μm tall SU-8 layer. Note:
- To test whether the SU-8 has fully developed, remove the wafer from the developer and spray it with isopropyl alcohol (IPA). If any white clouding forms on the wafer, it is not completely developed and needs more time.
 - A soft bristled toothbrush can be used to help brush away uncured SU-8 during the development process.

21.1

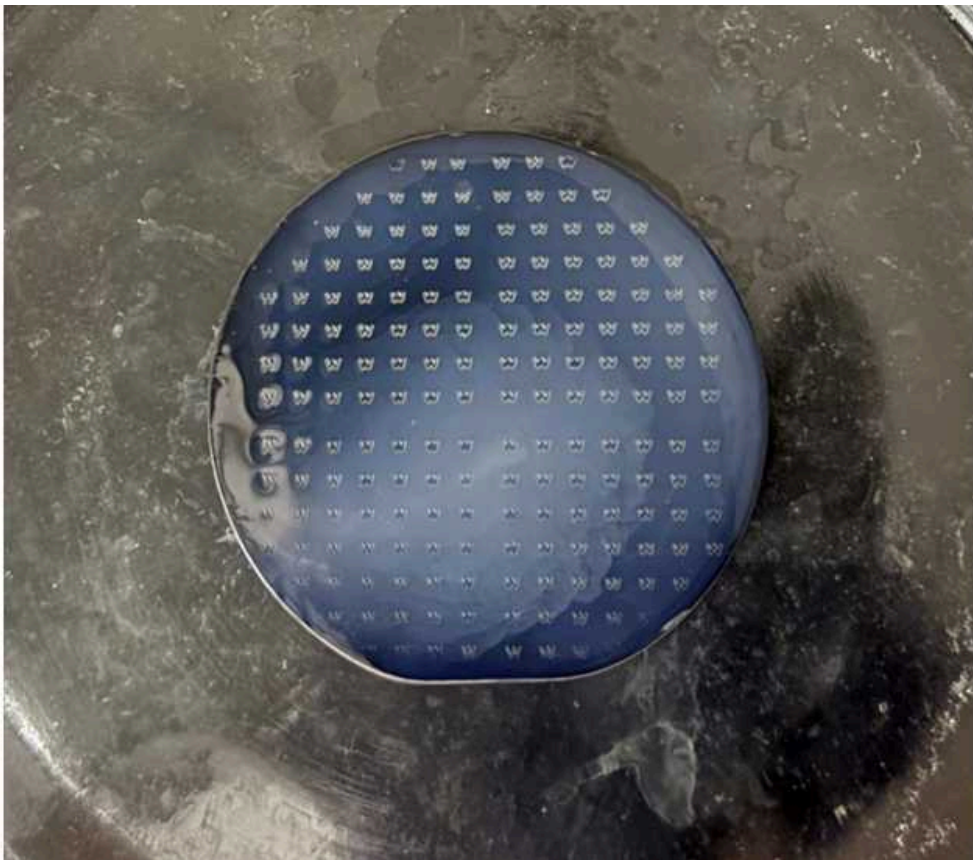


Figure 9. White clouding formed on silicon wafer after spraying with IPA during development, indicating more time in the SU-8 developer is required.

- 22 Once the SU-8 is completely developed, submerge in IPA in a petri dish for 5 - 10 minutes and blow dry with air.
- 23 Add 100 μ L (TRIDECAFLUORO-1,1,2,2-TETRAHYDROOCTYL) SILANE to a petri dish and place it in a vacuum chamber.
- 24 Place the wafer in the chamber next to the petri dish filled with (TRIDECAFLUORO-1,1,2,2-TETRAHYDROOCTYL) SILANE and pull vacuum for 10 minutes, then turn off vacuum let sit for 10 minutes. The silane will evaporate in the chamber and be deposited on the surface of the wafer. Silane should now be visually deposited onto the surface of the wafer and the wafer is ready to be used for micropatterning. This will look like cloudiness on the surface of the chip.
- 24.1 Mold release (Mann Ease Release 200) is an alternative option if silane and/or vacuum is unavailable. Spray the patterned surface of the silicon wafer with the release and spread it with a soft brush.

24.2



Figure 10. Wafer and (TRIDECAFLUORO-1,1,2,2-TETRAHYDROOCTYL) SILANE filled petri dish in desiccator chamber.

25 Tips and Common Mistakes

- When spreading the SU-8, be sure to leave room around the edge (a few mm will be enough) to ensure the SU-8 does not run off. This will cause uneven features across the chip. This will also make the PDMS mask easier to remove.
- The pattern should begin to be visible at 65°C during the post-exposure bake. If the pattern is not visible, then it means that the SU-8 was not exposed to UV long enough and the exposure time needs to be increased. Alternatively, if the features are larger than the design, then the SU-8 was over exposed.
- A sign of good crosslinking within the SU-8 is a slight amber hue in the hardened SU8 features.

Section IIIa: Fabrication of PDMS Masks

- 26 Mix ~1.5 grams of PDMS in a 10:2 ratio of base to curing agent in a weigh boat. Be sure to mix the PDMS extremely well. Place mixture in vacuum chamber and desiccate for ~10 minutes to remove bubbles.

- 27 Place the silicon wafer on the spin coater and pour 3/4 of the PDMS mixture onto the center of the wafer.
- 28 Spin coat at 400 rpm for 15-20 seconds. Check to see that the PDMS covers the entire wafer but is not taller than the surface features. The goal is to create a through-hole membrane, therefore the height of the PDMS layer must not exceed the height of the SU-8 posts. If the PDMS layer is higher than the SU-8 features, the holes will not form making the patterning no longer feasible.
- If PDMS is spread too thin, add more. If PDMS is too thick, spin coat for more time.
 - Features should be visible above the features on the chip.
 - The spin speed will need to be adjusted based on feature height.

28.1

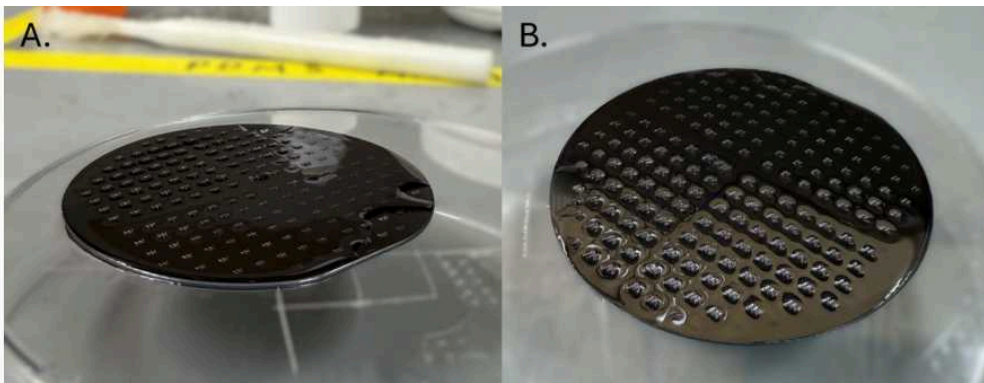


Figure 11. Silicon chip with cured and developed SU-8 features acting as an inverse mold for PDMS. (A) PDMS layer exceeding the height of the SU-8 posts, indicating the chip should spin for a longer time. (B) SU-8 posts protruding through the PDMS layer as desired.

- 29 Place the wafer into a 60°C oven or on a hotplate heated to 60°C and let the PDMS cure overnight.
- 30 **Next day:** Use tweezers to peel the cured PDMS off the wafer and place it flat on a dish, ensuring the side that was in contact with the wafer remains face-down on the dish (smooth side down). The result will be a PDMS sheet will have clean holes in the membrane.
- If PDMS does not come off clean it (1) may be because the PDMS is too thin, (2) the wafer may not have been properly treated with silane or (3) the PDMS was not mixed well.
 - Due to the height of the SU-8 features, the PDMS sheet should be thick enough to be handled easily and not fold over on itself. If folding occurs, it can be helpful to place the PDMS in a shallow container of ethanol and use tweezers to unfold the sheet or increase the amount of PDMS spun onto the chip.



- 31 Cut the PDMS sheet into square masks that fit on the surface of the polyacrylamide (PA) gel used for collagen treatment.
- 32 Remove excess/uncured PDMS from the wafer by spraying IPA and gently scrubbing with a soft brush.
- 33 **Tips and Notes**
 - If the PDMS covers the top of the features, then there is no way for the ECM to reach the surface of the gel. This can be prevented by proper spin coating.
 - A good quality mask will peel off the chip cleanly.

Section IIIb: Alternative protocol for large (≥ 1 mm) circular islands

- 34 If 1 mm or larger circular islands are desired, then biopsy punches can be used to make the masks. No silicon wafers or SU-8 are required, meaning all steps above (i.e., all of Sections I-IIIa) can be replaced by the brief procedure below. These masks can then be used in the same manner for the remainder of the protocol.
 - 34.1 Prepare PDMS as described above and spread the PDMS in a thin layer on a petri dish.
 - 34.2 Place the dish into a 60°C oven and let the PDMS cure overnight.
 - 34.3 Cut the PDMS into squares, then using a biopsy punch, punch holes into the PDMS sheet.
 - If circular masks are desired, a hollow punch can be used to punch large circles in the PDMS sheet.
 - It helps to do this on a piece of aluminum foil, so that you can visually see if the punch went all the way through.

34.4

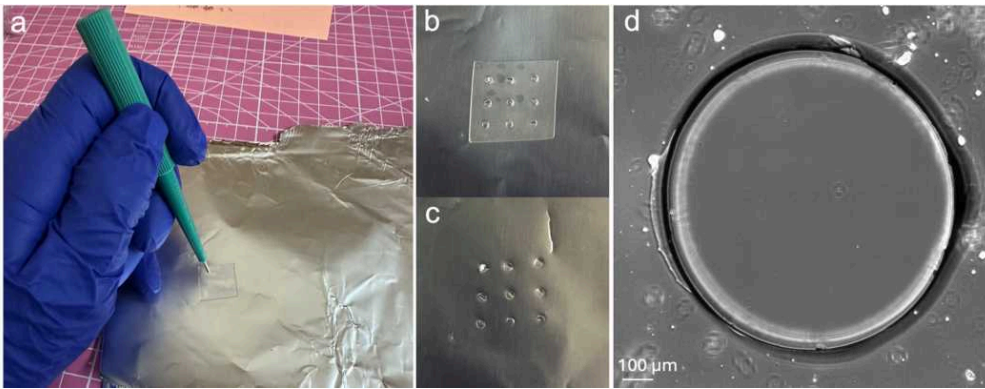


Figure 12. Biosy punch method for creating 1 mm circular islands. **(a)** Biopsy punch being used to create a square-shaped PDMS mask. **(b)** PDMS mask with biopsy punched holes. **(c)** Foil after mask has been removed, showing biopsy punch made it completely through the PDMS. **(d)** Image of the hole created in the PDMS by the biopsy punch.

Section IV: Collagen Treating

- 35 Make polyacrylamide gels, or desired substrate to be patterned. A procedure for polyacrylamide gel fabrication can be found in Saraswathibhatla et al., Sci. Data, 2020, 7, 197 (<https://doi.org/10.6084/m9.figshare.12378218>).
- 36 Place the PDMS masks on the edge of a microscope slide such that both its top and bottom surface are exposed (make sure the smooth side is facing down).
- 37 Place the slide on a petri dish to ensure both sides of the masks remain exposed. Masks tend to droop, so a petri dish works well to elevate the masks.

37.1



Figure 13. PDMS masks placed on the edge of a glass microscope slide elevated by a petri dish for O₂ plasma treatment.

- 38 Plasma treatment of PDMS masks with O₂ on high power for 5 minutes.
- 39 **In biosafety cabinet:** For each 250 mm² of gel area, make 1.5 mL of diluted sulfo-SANPAH (dilute to 1 mg/mL in 0.1 M HEPES). Keep the BSC lamp off as much as possible. SANPAH is sensitive to light.
- Note: The amount of diluted sulfo-SANPAH prepared will need to be increased or decreased in proportion to the surface area of the gel.
- 40 When polymerizing polyacrylamide gels, we place a coverslip on top to ensure uniform thickness. Remove the coverslips from polyacrylamide gels and then aspirate water.
- Note : More information on the preparation of the polyacrylamide gels can be found in the accompanying manuscript
- 41 Allow gels to dry for a few minutes, until you can clearly see the boundary of the gel. Inspect the gels, make sure they did not tear/rip when removing the coverslip.

41.1

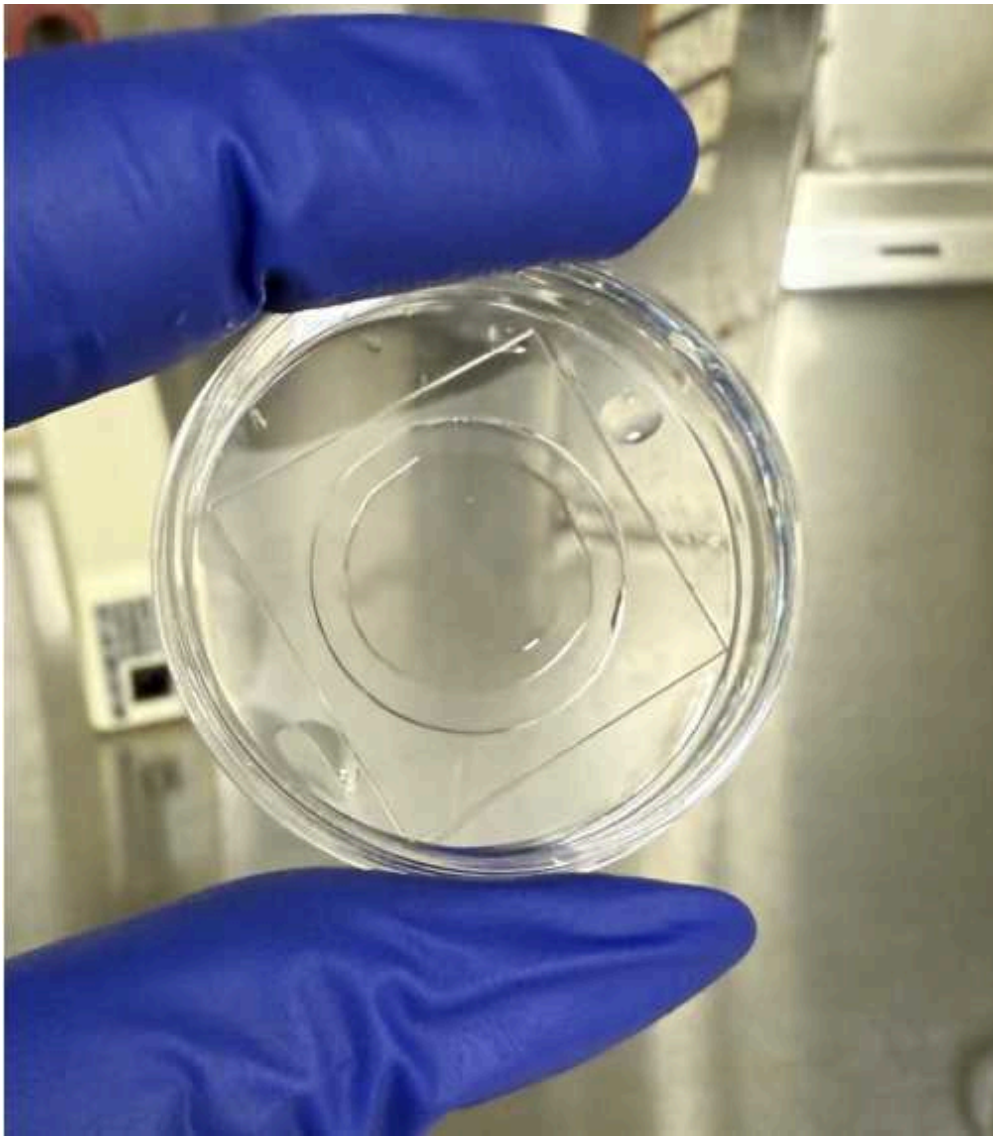


Figure 14. Clear polyacrylamide hydrogel ($E = 6$ kPa) clearly defined on a glass bottom petri dish with no visible tears.

- 42 Once the top surface of a gel is dry, place the smooth side of the PDMS mask on the gel and gently tap it down with tweezers. If the mask is not adhering, the gels need more time to dry.
- Water in the interior of the gel should not dry. When the interior of the gel begins to dry, the gel becomes more opaque.

42.1

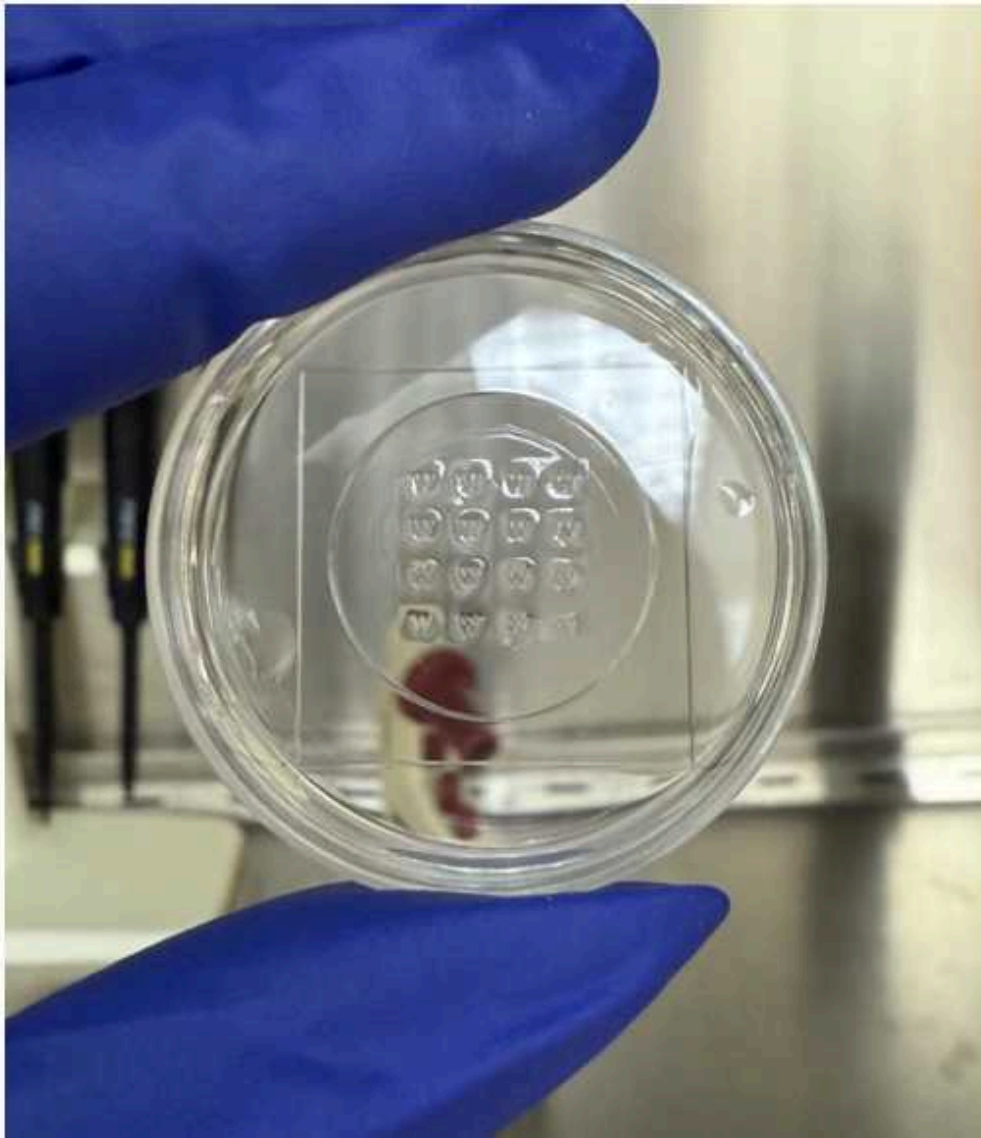


Figure 15. PDMS mask adhered to the surface of a clear polyacrylamide hydrogel.

- 43 Add 1 mL of the diluted (50 mg/mL) sulfo-SANPAH to each gel in a large droplet. Try to keep all of the liquid on top of the mask, but it will still work if the droplet runs out of the mask.
- 44 Using 200 μ L of the diluted sulfo-SANPAH, pipette the air bubbles out of the holes of the mask by pipetting up and down. Inspect your mask in a cell culture microscope. Air bubbles appear as bright spots when looking through a microscope.
 - Ensure that the pipette tip does not touch the mask when pipetting, or the mask may fall off the gel.

44.1

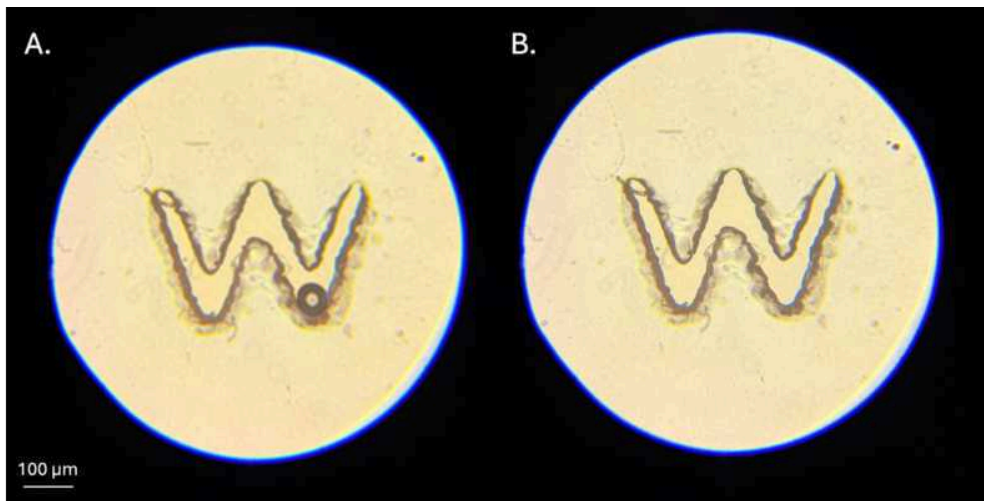


Figure 16. (A) Air bubble in a W-shaped feature of a PDMS mask during SANPAH treatment. (B) Bubble removed by pipetting sulfo-SANPAH up and down.

- 45 Once all bubbles are removed from the gels, place them under a UV lamp for 12 minutes.
- Note: The UV lamp in a BSC can be used as well; just make sure the gels are close to the UV bulb (within a few inches) for adequate exposure.

45.1



Figure 17. Gels exposed to 365 nm UV light with diluted sulfo-SANPAH solution, binding SANPAH to the gel surface and enabling subsequent collagen cross-linking.

- 46 Aspirate off the diluted sulfo-SANPAH, then add 500 mL of fresh diluted SANPAH solution to each gel. Remove any bubbles that may have formed. Expose to UV for 6 minutes.

- 47 Aspirate the diluted sulfo-SANPAH and add 1 mL of 0.1 mg/mL collagen to each gel. Remove air bubbles by pipetting up and down.
- 48 Place the gels in a dish and wrap it with aluminum foil. Store overnight at 4°C.
 - Do not allow collagen to sit on gels for more than 1 day. If gels are needed later, replace collagen solution with 1x phosphate buffered saline.

Section V: Seeding Cells

- 49 Aspirate collagen solution from polyacrylamide gels and add 1 mL of 1x PBS to each gel.
- 50 Remove the mask from each gel with tweezers and discard them. Aspirate the PBS from the gels.
- 51 Add 1 mL of BSA solution to each gel and place the gels in the cell culture incubator for 1 hour to warm to 37°C.
- 52 Aspirate off BSA solution and add 400 µL of suspended cells to each gel. The cell seeding density will need to be altered for each pattern and cell type. It is recommended to start with a concentration of 2.5×10^6 cells/mL, which, for gel area 250 mm², is equal to 10,000 cells per mm².
- 53 Allow cells to adhere for 40 – 60 minutes in the incubator, then aspirate solution from gel and add 2 mL of fresh cell culture medium.
 - Note: Adhesion time may need to be altered for cell type.
- 54 Place gels in the incubator overnight to come to confluence.

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