PREPARATION OF A MICROPATTERNED RIGID-SOFT COMPOSITE SUBSTRATE FOR PROBING CELLULAR RIGIDITY SENSING

Materials

1. Coverslip (45x50mm #1; Fisher Scientific, Pittsburgh, PA)
2. Diamond-tip pen
3. Bunsen burner
4. Bind silane working solution: Mix 950μL 95% ethanol and 50μL of 95% glacial acetic acid and add 3μL of Bind-Silane (γ-methacryloxypropyltrimethoxysilane; GE Healthcare, Uppsala, Sweden) to form the working solution
5. Ethanol (C_2H_5OH) 95%
6. Acrylamide solution (40% w/v; BioRad, Hercules, CA)
7. Bis-acrylamide solution (2% w/v; BioRad)
8. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; BioRad)
9. Ammonium persulfate aqueous solution (APS, 10% w/v; BioRad) prepared fresh
10. 10x and 1x phosphate buffered saline (PBS, pH 7.4)
11. Aqueous sucrose solution (50% w/v)
12. Rain-X
13. Coverslip (#2, 25mm circular or square; Fisher Scientific)
14. Razor blade
15. Tweezers
16. Heating block set to 95˚C
17. SU-8 2002 (MicroChem Corp., Newton, MA)
18. SU-8 developer (MicroChem Corp.)
19. Spin coater

20. UV light source for crosslinking SU-8

21. Orbital shaker

22. Photomasks, on inexpensive transparency films for patterns larger than 10 μm or chrome plated lime glass for smaller features

23. Glass Petri dishes

Procedure

Making the polyacrylamide gel base

1. Mark the edge of one side of a 45 x 50 mm No. 1 coverslip with a diamond tip pen and pass the marked side through the plasma of a Bunsen burner flame to render the surface hydrophilic.

2. In a fume hood, apply ~30 μl Bind-silane solution onto the flamed side of the coverslip and spread solution evenly across the surface.

3. After ~15 minutes at room temperature when the surface has completely dried, rinse the treated side with 70% ethanol to remove residual reagent and allow to air dry. *Activated coverslips are stable at room temperature for at least 3 months.*

4. Determine the appropriate concentration of acryalmide and bis-acrylamide necessary for the desired gel stiffness. Prepare solution by mixing calculated volumes of acrylamide and bis-acrylamide, 10x PBS and distilled water to a total volume of 1 mL. Degas solution for 30 minutes.

5. While degassing the solution, prepare the top coverslip by coating the surface with either Rain-X (to increase surface hydrophobicity) or 50% sucrose solution (to serve as a separation layer) to facilitate later removal from the gel surface. Ensure complete coverage of the coverslip surface with either treatment. *Sucrose coating is recommended for very soft gels to prevent introduction of gel strain during coverslip removal.*
    - To coat with Rain-X: Spread Rain-X solution across the coverslip surface and wipe away with a Kimwipe. Rinse with distilled water and wipe until clear.
    - To coat with sucrose solution: mark one side of the coverslip with a magic marker to facilitate identification. Pass the unmarked side through the plasma of a Bunsen burner. Pipette ~100μL 50% w/v sucrose solution on the flamed side of the coverslip. Place the coverslip in a spin coated and spin the sample at 5000 rpm for 15 seconds.

6. After degassing polyacrylamide solution, add 6 μL freshly prepared 10% APS and 4 μL TEMED. Mix thoroughly and pipette a 20 μL drop onto Bind-silane activated coverslip.

7. Cover with prepared top coverslip with coated side facing the acrylamide solution and ensure the solution spreads uniformly beneath the top coverslip. Allow solution to polymerize 30

minutes at room temperature. *When using sucrose coated top coverslip, the coverslip assembly should be turned upside down during polymerization to avoid higher density sucrose solution settling into the polyacrylamide.*

8. Remove the top coverslip; the method depends on which treatment was used in step 5. *Slow careful removal of the top coverslip is essential to preventing gel cracks which can compromise micropatterning.*
    - For Rain-X coated coverslips: flood the coverslip surface with distilled water and wait at least 15 minutes for the water to seep into the gel. Use a razor blade to slowly lift the top coverslip off the acrylamide gel.
    - For sucrose coated coverslips: immerse the coverslip-gel sandwich into hot distilled water in a Petri dish to remove the sucrose solution. *The coverslip should release in 20-30 minutes. Be sure to keep water hot.*

9. Equilibrate gel in distilled water for 30 minutes on a shaker, to remove any dissolved substances and help prevent crystal formation during subsequent drying.

10. Remove ~1 mm along the very edge of the gel using a razor blade. Rinse the gel with water to remove any bits of polyacrylamide gel. This removes any lips that are often present along the edge, which can interfere with proper micropattering.

11. Allow the gel to air dry for at least a few hours or overnight. *Complete drying of the gel is essential for micropatterning.*

Micropatterning the Polyacrylamide Gel

12. Bake coverslips on a temperature regulated heating plate for 1 min at 95°C to ensure that the polyacrylamide hydrogel is completely dry. Allow coverslip to cool to room temperature.

13. In a fume hood, spread ~300 μL of SU-8 2002, a negative photoresist, across the surface of the dehydrated polyacrylamide gel. Spin the coverslip at 5000rmp for 20 seconds in a spin coater.

14. Bake the coverslip on a heating plate at 95°C for 2-3 minutes then allow the coverslip to cool to room temperature. *Using SU-8 2002, this procedure should create a uniform layer ~2 μm in thickness.*

15. Place desired photomask over the coverslip and expose assembly to 365 nm UV light. The exposure time depends on the intensity of the light. For a light power of 100 nJ cm^{-2} at the sample, the optimal exposure time should be around 60-90 seconds. *Make sure that the photomask is in tight contact with the photoresist, which may be achieved by clamping the two together using binder clips.*

16. Bake the coverslip on a heating plate at 95°C for 2-3 minutes then allow the coverslip to cool to room temperature.

17. Immerse the coverslip in SU-8 developer in a glass Petri dish placed on an orbital shaker inside a chemical fume hood for approximately 60-90 seconds.

18. Remove the coverslip from developer and rinse with briefly with 95% ethanol from a squirt bottle before immersing the coverslip in a separate Petri dish of ethanol for ~30 seconds. *After air drying the micropattern should be visible and there should be no speckles or films between the intended pattern areas, which would indicate residual SU-8 from incomplete development. Remove any remaining SU-8 by immersing in developer for approximately 10 seconds followed by ethanol rinsing as described above.*

19. For immediate use, wash the coverslip in PBS for an hour on a shaker to rehydrate the gel and remove residual developer. For later use, bake the coverslip for 4 hours at 95°C to evaporate residual developer. *It is important to ensure complete removal of any residual developer. Coverslips can be stored in a desiccator for up to 3 weeks.*

Coating the Surface with ECM Proteins and Cell Seeding

20. If the substrate is stored in dehydrated form, rehydrate the gel in PBS for 1 hour at room temperature. Sterilize under the UV of a biosafety cabinet for 15-20 minutes.

21. Incubation of the coverslip with an extracellular matrix protein (e.g. 10 μg/mL fibronectin in PBS) for approximately 20 minutes at room temperature. This allows adsorption of the protein to promote cell adhesion. *SU-8 is known to have a biofouling surface, which will passively adsorb proteins from serum containing media to allow cell adhesion. Coating with extracellular matrix proteins further enhances cell adhesion.*

22. Equilibrate substrate with culture media for at least 30 minutes in a CO_2 incubator.

23. Plate cells at the desired density or at a high density then replace media after cells have adhered to a significant number of patterned areas. Cells should attach to SU-8 patterned areas within 15 minutes, although a longer period may be required if the adhesive areas are small.

Note: While the SU-8 pattern and adhered cells are easily visible at a low magnification with phase contrast or bright field optics, the use of high magnification, oil immersion lens may be limited by the combined thickness of the hydrogel and photoresist. A lens of long working distance is essential, and better resolution may be achieved with a water immersion lens to avoid spherical aberration. It is also noteworthy that SU-8 emits autofluorescence when excited at 488 nm, therefore fluorophores with a long wavelength are preferred over probes such as GFP.

Troubleshooting

Although the present method may seem straightforward, the following problems may arise from improper execution of a few crucial steps.

(1) *Poor micropatterning*: After development, the micropattern may not look as expected. Poor contact between the photomask and SU-8 creates ill-defined borders of the micropattern. Debris on a dirty photomask, or beading of the edge of polyacrylamide or SU-8 (from poor spin coating) can create space between the photomask and SU-8. In addition, over-exposure can cause SU-8 areas to appear larger than expected with poorly defined edges, while over developing would cause SU-8 areas to appear smaller with rounded corners.

(2) *Poor association of SU-8 with the polyacrylamide surface*: During development, the micropattern may become detached from the polyacrylamide surface. SU-8 is most likely underexposed or insufficiently heated after exposure. Underexposure prevents proper formation of crosslinks within SU-8 all the way down to the polyacrylamide surface for anchoring the photoresist to the gel surface and preventing detachment during development. Insufficient baking after UV exposure also prevents exposed regions of SU-8 from curing properly. In addition, too thick a layer of polyacrylamide may slow down heat conduction and require a longer period of baking.

(3) *Adhesion of cells to supposedly non-adhesive surfaces of polyacrylamide*: After plating, cells may attach to the polyacrylamide surface between SU-8 covered regions. Most likely SU-8 is under-developed, which causes a thin film of unexposed SU-8 to remain on the surface of polyacrylamide. This residual film then adsorbs proteins and mediates cell adhesion. The problem may be rectified by additional treatment with SU-8 developer as noted in Step 10.

(4) *Cracking of the gel beneath SU-8*: Cracks appear on the hydrogel surface after rehydration. The most likely cause is adhesion of the top coverslip to the gel during removal, possibly due to incomplete coverage of the surface of top coverslip with Rain-X or sucrose solution. In addition to ensuring complete coverage, increasing the concentration of bis-acrylamide relative to acrylamide (while maintaining the same elastic modulus) may alleviate this problem.

(5) *Poor cell adhesion*: During plating, cells may require a prolonged period of time to attach to the SU-8 surface. Treatment of SU-8 with an extracellular matrix protein (Step 21) or increasing the time of incubation should help.

Reference

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Wong, S., Guo, W.-H., and Wang, Y.-L. (2014) Fibroblasts probe substrate rigidity with filopodia extensions before occupying an area. *Proc. Natl. Acad. Sci. USA* 111:17176-17181.