

MICROPATTERNING CELL-SUBSTRATE ADHESION ON LINEAR POLYACRYLAMIDE-GRAFTED GLASS

Materials

1. Acetic acid (glacial)
2. Acrylamide solution (40% w/v, Bio-Rad, Hercules, CA) stored at 4°C.
3. Ammonium peroxydisulfate (APS; Bio-Rad) stored desiccated at room temperature. Prepare 10% APS solution freshly.
4. Bind-silane from either Amersham Biosciences (Silane A174) or Sigma (M6514).
5. Bind-silane Working Solution,

Bind-silane	3 ul
95% ethanol	950 ul
Acetic acid	50 ul
Total Volume	1 ml
6. ECM protein of interest, typically fibronectin, collagen, or laminin. Fluorescently labeled ECM solution.
7. Ethanol (95%; 190 Proof, ACS/USP grade)
8. FluoSpheres carboxylate-modified microspheres (0.1 μm , Molecular Probes, Eugene, OR)
9. 1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 8.5, filtered and stored at 4°C.
10. Microposit Developer MF-319 (MicroChem, Newton, MA).
11. Microposit Remover 1165 from MicroChem (Newton, MA).
12. *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED; Bio-Rad) stored at 4°C.
13. PBS, pH 7.4, filtered and stored at room temperature.
14. SPR-220.3 positive photoresist (MicroChem, Newton, MA).
15. Tetramethylammonium hydroxide solution (TMAH) in water (25% w/v) (*optional, for replacing Microposit Developer*).

16. Triton X-100 (*optional, for replacing Microposit Developer*)
17. Diamond-tip pen.
18. Coverslips for micropatterning and a smaller one for covering the patterned area.
19. UV light source for photoresist.
20. Photomask with the desired pattern. If the experiment demands a resolution of the pattern better than 10 μm , it is necessary to custom order the photomask, e.g. from Advance Reproductions Corp (North Andover, MA). Otherwise, the mask may be ordered as an inexpensive transparency film from a number of companies (e.g. CAD/Art Services, Bandon, OR).
21. Razor blade or a pair of fine forceps.
22. Spin processor (Laurell; North Wales, PA); alternatively, an inexpensive low-speed tabletop centrifuge may be modified to hold coverslips and placed in a fume hood for spin coating.

Procedure (unless specified, all the steps are to be performed at room temperature)

1. Mark one side of 45 x 50 mm No.1 coverslips with a diamond tip pen.
2. Pass coverslips over the inner flame of a Bunsen burner with the marked side facing down to increase its hydrophilicity.
3. Working in a fume hood, apply ~30 μl of Bind-silane working solution onto the flamed side of coverslips with a plastic pipet tip and smear it evenly on the surface with the same pipet tip. Excess may be removed with Kimwipes[®]. Allow Bind-silane to react for 3 min.
4. Rinse coverslips with ethanol and wipe with Kimwipes[®] to remove any residual reagent. Allowed to air dry. *Activated coverslips are stable at room temperature for at least three months.*
5. Bake activated coverslip on a heat block at 115°C for 2 min. Allow the coverslips to cool to the room temperature.
6. Working in a fume hood, spread positive photoresist SPR-220, 180 μl for a 45 x 50 mm coverslip, uniformly across the activated side of the coverslip with a spin processor at 5000 rpm for 30 second. *An old bench-top centrifuge may modified to generate similar results.*

7. Baking at 115°C for 90 sec. *After baking, photoresist coated coverslips can be stored at room temperature in the dark for up to 3 months.*
8. Place the photomask over the coverslip and sandwich them between two pieces of glass plates with paper clamps to ensure tight contact between the mask and the glass surface. Expose the assembly to 365nm UV light. *The contact is particularly important if the light source is not well collimated. In addition, it is critical to make sure that the patterned side on the photomask is facing the coverslip. For illumination with a less than uniform light source, the assembly of photomask and coverslip may be placed on an Orbit Shaker. Rotation at about 70 rpm during exposure creates a uniform average illumination across the surface. The exact exposure time depends on the optical condition and must be calibrated for each setup.*
9. Remove coverslip from the assembly and bake at 115°C for another 90 sec. Allow the coverslip to cool to the room temperature.
10. Immerse the coverslip in Microposit Developer MF-319 in a glass Petri dish in a chemical fume hood, and shake for 45 sec. Optimal timing and mixing conditions are affected by the exposure condition and should be controlled carefully. *Developing in TMAH working solution (2.45% TMAH with 0.1% Triton X-100) generates results comparable to those using Microposit Developer MF-319.*
11. Rinse the coverslip extensively in deionized water. Allow to air dry. The pattern of photoresist should be visible under a microscope.
12. Prepare a mixture of 200 µl of 40% acrylamide, 10 µl of 1 M HEMPES (pH 8.3), and 752.5 µl of deionized water to obtain a final concentration 8% acrylamide. Degas for 20 min. *Start to prepare acrylamide solution and degas when processing **photolithography** so that degassing can be accomplished after finishing step 11.*
13. After degassing, add 15 µl TEMED and 22.5 µl 10% APS, mix thoroughly, and pipet immediately onto the glass surface patterned with SPR-220. Cover with a 25 x 25 mm coverslip, or a suitable size matching the desired pattern and allow 20 min for acrylamide to polymerize,
14. Remove the top coverslip carefully with a razor blade or a pair of fine forceps. Rinse the surface thoroughly with deionized water to remove any unreacted acrylamide. *Linear PAA coated coverslip may be stored in water at least overnight.*
15. Remove SPR-220, patterned by UV exposure and development, by immersing in deionized water for 1 min with agitation then in Microposit Remover 1165 at room temperature for 5 min or until the coverslip became clear.
16. Wash the surface with deionized water thoroughly to remove Microposit Remover and allow the surface to air dry. Surface patterning may be observed as preferential wetting of the area covered with PAA when rinsing gently with water. *At this stage,*

micropatterned coverslip is very stable and may be stored at room temperature for at least 3 months.

17. Mount patterned coverslip onto whatever observation chamber to be used for cell culture.
18. (optional) Add ECM coating solution, with a fluorescent dye if necessary (such as Alexa Fluor carboxylic acid, succinimidyl ester SE, 568nm or 488nm; prepared in DMSO at 10 mg/ml and stored at -20°C, avoid exposure to light), and incubate for at least 30 min with gently shaking at room temperature. Adding fluorescent dye will allow the pattern to be visible under fluorescence optics. Otherwise the adhesive pattern may be detectable only through cell behavior.
19. Rinse the substrate with PBS three times.
20. Sterilize the surface under UV for 15 min in a biosafety cabinet before plating cells.

Troubleshooting

Step 11. Poor patterning

Make sure that the side coated with SPR-220 is facing the patterned side of photomask during exposure. Make sure that the contact between photomask and coverslip in step 8 is tight. After photolithography, examine substrate under a microscope and adjust exposure and development timing accordingly. Over- or under-development causes the photoresist-covered region to be larger or smaller than the pattern on the photomask, respectively, while poor contact between the photomask and the coverslip causes fuzzy edges. Adjust the time of development by trial and error. If the developing time is much shorter than 30 seconds or longer than 60 seconds, then the exposure time should be decreased or increased, respectively.

Step 15. PAA bound SPR-220 comes off as a sheet instead of gradually dissolving in Microposit Remove 1165 solution.

This problem is almost exclusively due to underdevelopment of photoresist. After exposure and development, examine the pattern under a microscope to make sure that the exposed region is totally clear. Residual SPR-220 makes the coverslip appear cloudy, and causes linear PAA to coat onto a thin film of SPR-220 instead of activated cover glass surface. Extend developing time and/or exposure time accordingly. Check expiration date of Microposit Developer MF-319, order new lot if necessary. TMAH working solution may gradually become less effective after one month. Prepare fresh TMAH working solution if necessary.

Step 20. No clear sign of cells following the pattern

This problem is almost always associated with step 15, where linear PAA coats residual SPR-220 rather than glass surface and comes off as a sheet. After photolithography, it is important to examine carefully the coverslip under a microscope to make sure that the region free of SPR-220 is indeed clear. Residual SPR-220 on the coverslip appears cloudy. The problem may be resolved by extending developing time or exposure time accordingly.

Step 20. Poor cell attachment

This is most likely due to an inappropriate amount of TEMED and APS, which is different from that used typically in preparing PAA gels. Insufficient TEMED and APS generate long linear polymers of PAA that might extend onto adhesive areas.

Reference

Guo, W.-H. and Wang, Y.-L. (2009) Micropatterning cell–substrate adhesions using linear polyacrylamide as the blocking agent. In *Live Cell Imaging* (R.D. Goldman, J.R. Swedlow, D.L. Spector, eds), pp. 43-52. Cold Spring Harbor Lab Press, Cold Spring Harbor, NY.