

Video Article

Creating Two-Dimensional Patterned Substrates for Protein and Cell Confinement

Dawn M. Johnson¹, Natalie A. LaFranzo¹, Joshua A. Maurer¹

¹Department of Chemistry, Washington University in St. Louis

Correspondence to: Joshua A. Maurer at maurer@wustl.edu

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Abstract

Microcontact printing provides a rapid, highly reproducible method for the creation of well-defined patterned substrates.¹ While microcontact printing can be employed to directly print a large number of molecules, including proteins,² DNA,³ and silanes,⁴ the formation of self-assembled monolayers (SAMs) from long chain alkane thiols on gold provides a simple way to confine proteins and cells to specific patterns containing adhesive and resistant regions. This confinement can be used to control cell morphology and is useful for examining a variety of questions in protein and cell biology. Here, we describe a general method for the creation of well-defined protein patterns for cellular studies.⁵ This process involves three steps: the production of a patterned master using photolithography, the creation of a PDMS stamp, and microcontact printing of a gold-coated substrate. Once patterned, these cell culture substrates are capable of confining proteins and/or cells (primary cells or cell lines) to the pattern.

The use of self-assembled monolayer chemistry allows for precise control over the patterned protein/cell adhesive regions and non-adhesive regions; this cannot be achieved using direct protein stamping. Hexadecanethiol, the long chain alkane thiol used in the microcontact printing step, produces a hydrophobic surface that readily adsorbs protein from solution. The glycol-terminated thiol, used for backfilling the non-printed regions of the substrate, creates a monolayer that is resistant to protein adsorption and therefore cell growth.⁶ These thiol monomers produce highly structured monolayers that precisely define regions of the substrate that can support protein adsorption and cell growth. As a result, these substrates are useful for a wide variety of applications from the study of intercellular behavior⁷ to the creation of microelectronics.⁸

While other types of monolayer chemistry have been used for cell culture studies, including work from our group using trichlorosilanes to create patterns directly on glass substrates,⁹ patterned monolayers formed from alkane thiols on gold are straight-forward to prepare. Moreover, the monomers used for monolayer preparation are commercially available, stable, and do not require storage or handling under inert atmosphere. Patterned substrates prepared from alkane thiols can also be recycled and reused several times, maintaining cell confinement.¹⁰

Video Link

The video component of this article can be found at <https://www.jove.com/video/3164/>

Protocol

1. Preparation of the Patterned Master (Figure 1)

1. Center the silicon wafer on the spin-coater and rinse the wafer with acetone during the initial step of the two-cycle spin program in Table 1. The acetone will evaporate during the second step of the spin program leaving a clean, dry wafer.
2. Apply approximately 1 mL AZ9245 photoresist/in (in diameter) to the wafer and spin-coat using the conditions described in Table 1.
3. Soft-bake the photoresist-coated wafer at 110°C for 2 m using a high-uniformity hotplate.
4. Photopattern the substrate using either a direct-write photolithography system or a mask aligner system and appropriate mask. Masks can be purchased from a number of commercial sources. Additionally, transparencies printed with UV absorbent ink, taped to an optical flat, may be used to produce patterns with large features.
5. Develop the patterned wafer in 1:2 400K developer:semi-conductor grade deionized water for 1 m 45 s with gentle agitation. Rinse thoroughly with semi-conductor grade deionized water and dry with a stream of nitrogen (N₂) gas. Pattern development can be checked using a microscope with a UV filter. If the pattern is not fully developed, the wafer can be returned to the developing solution for additional time.

Note: For best results, photopatterning should be carried out in a cleanroom environment.

2. Preparation of PDMS Stamp (Figure 2)

1. Prepare a 10:1 by weight resin:hardener mixture of Sylgard 182 (PDMS) and completely cover the master (photopatterned wafer) with the mixture in a disposable petri dish.
2. De-gas the PDMS-covered master in a vacuum dessicator until no bubbles are visible and allow the stamp to cure in an oven at 65°C for 1.5 h. Prior to curing, it is important make sure the master is at the bottom of the dish as it may rise to the surface during the de-gassing step.
3. Cut the PDMS stamp out of the master and trim to the appropriate size. Store the stamp in a covered container (feature side up) to protect it from dust and debris.

3. Preparation of the Patterned Gold Substrate (Figure 3)

1. Prepare 25 mm no. 1 round glass coverslips for metal deposition by treating them with oxygen plasma for 10 m. Rinse twice with 18.2 MΩ deionized water followed by twice with ethanol, drying with a stream of N₂ gas between each step.
2. Using a multi-pocket electron-beam deposition system, deposit 50 Å titanium followed by 150 Å gold. Do not vent the evaporator between deposition of the titanium layer and gold layer. Alternatively, gold-coated coverslips may be purchased from a variety of suppliers, however, it is important that the metal layers are prepared by electron-beam evaporation and not thermal evaporation. If purchased from an outside source, gold substrates may be cleaned by plasma oxidation or "piranha" (7:3 Conc. H₂SO₄:30% H₂O₂)¹¹ prior to use.

Note: "Piranha" solution is explosive in the presence of organic compounds.

3. Prepare the stamping solution, 10 mM hexadecanethiol in absolute ethanol, and the backfilling solution, 1 mM glycol-terminated thiol in absolute ethanol.
4. Rinse the stamp with ethanol and dry thoroughly with N₂ gas. Apply stamping solution to the PDMS stamp dropwise until fully coated. Dry the stamp thoroughly with N₂ gas. Proceed to 5a or 5b as appropriate based on the features of the PDMS stamp.
5.
 - a. Gently press the stamp onto the gold substrate and allow the monolayer to form for 15 s.
 - b. Place the gold substrate into a petri dish containing 18.2 MΩ deionized water, ensuring the substrate is submerged. Gently press the stamp onto the gold substrate and allow the monolayer to form for 15 s.
6. Rinse the stamped substrate twice with ethanol, drying with N₂ gas after each rinse and place the substrate in a petri dish.
7. Cover the substrate with backfilling solution and seal the dish with parafilm to prevent evaporation.
8. Allow the background monolayer to form in the dark for 12-14 h.
9. Remove the patterned coverslip from the backfilling solution and rinse twice with ethanol, drying with N₂ gas after each rinse.

4. Applying Protein and Cells to the Patterned Substrate

1. Place the patterned coverslip in a small petri dish or cell culture chamber and cover with 500 µL to 1 mL of Dulbecco's Phosphate Buffered Saline (DPBS). The DPBS must completely cover the substrate during protein incubation to ensure even protein coverage.
2. Add a concentrated solution of protein to the DPBS and mix the solution by pipeting several times. Incubate the protein mixture with the substrate at 37°C for 1 h. Final concentrations for laminin and fibronectin are typically 12 µg/mL and 20 µg/mL, respectively. Protein can be labeled with an amine reactive fluorescent dye to allow for easy pattern visualization. However, labeled protein should be mixed 1:1 with unlabeled protein as protein labeling can interfere with biological activity.
3. After incubation, rinse the substrate thoroughly with DPBS (4-5x) to remove unbound protein, taking care not to dry the substrate or bring it through the air-water interface. After the first three rinses, add approximately 500 µL of complete cell growth media to maintain a wet substrate.
4. Replace the growth media used to rinse the substrate with fresh media.
5. Dissociate and count cells for plating onto the substrate. Either dissociated primary cells, such as hippocampal neurons, or immortalized cell lines, such as CHO-K1 cells, can be utilized.
6. Plate dissociated cells onto the substrate. Typically 30 to 200 cells/mm² are used.

5. Representative Results:

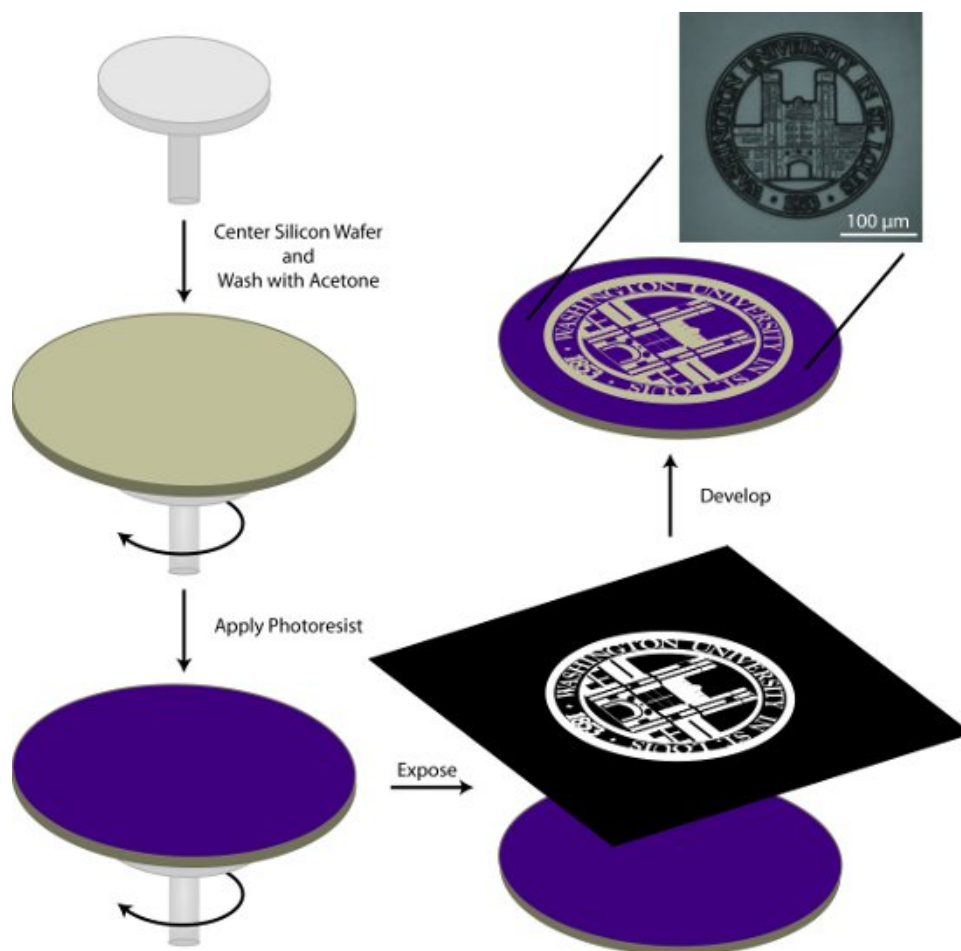


Figure 1. General schematic for the photolithographic preparation of a patterned master. In this process, a silicon wafer is cleaned with acetone, coated with photoresist, exposed to the pattern of interest, and the pattern is developed.

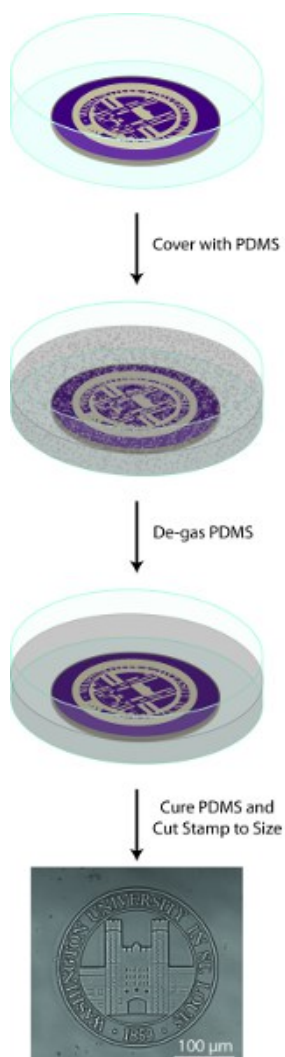


Figure 2. General schematic for PDMS stamp preparation. In this process, the patterned master is covered with Sylgard (10:1 resin:hardener), de-gassed in a vacuum desiccator, cured in an oven at 60°C, and cut to size.

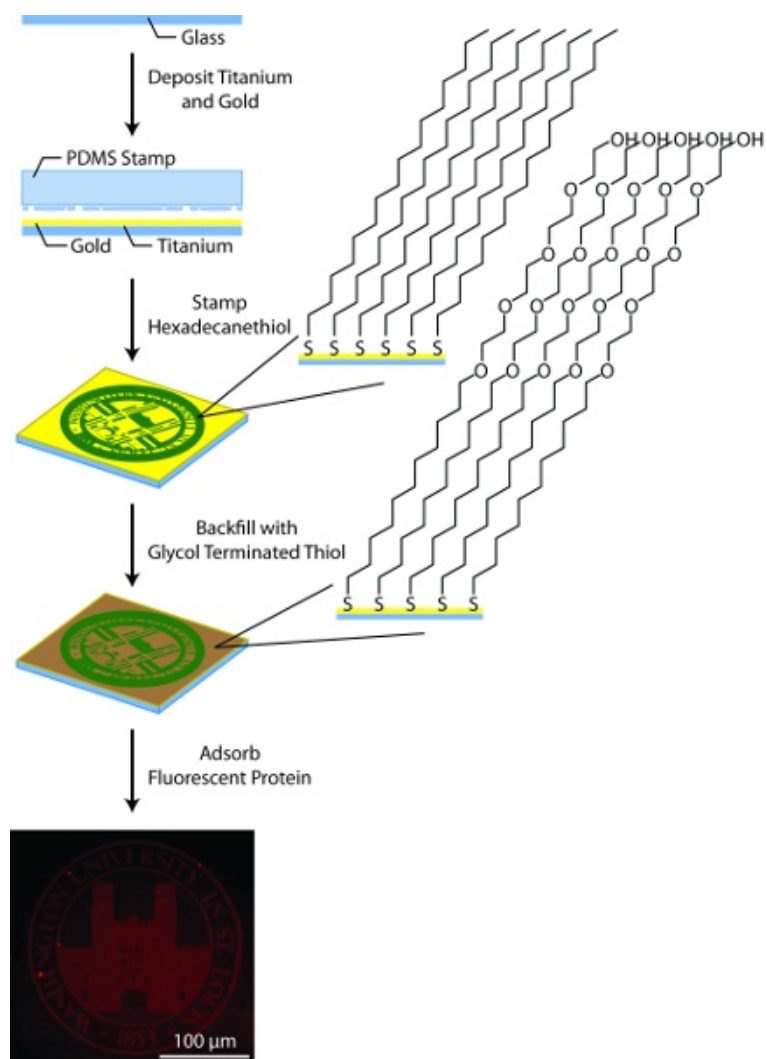


Figure 3. General schematic for substrate patterning. In this process, glass substrates are coated with titanium (50Å) and gold (150Å) using an electron beam evaporator, patterned by microcontact printing hexadecanethiol using a PDMS stamp, backfilled with glycol terminated alkane thiols, and coated with fluorescently-labeled protein.

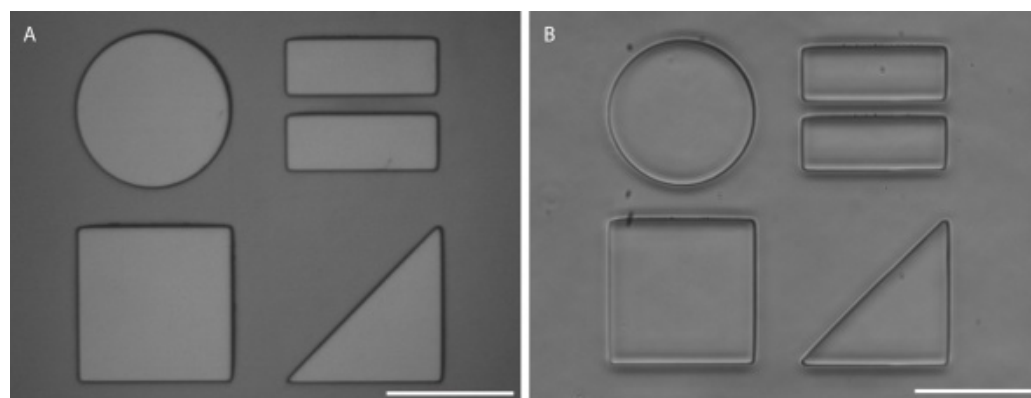


Figure 4. Patterned master (A) and PDMS stamp (B) prepared using the described methods. Scale bars are 100μm.

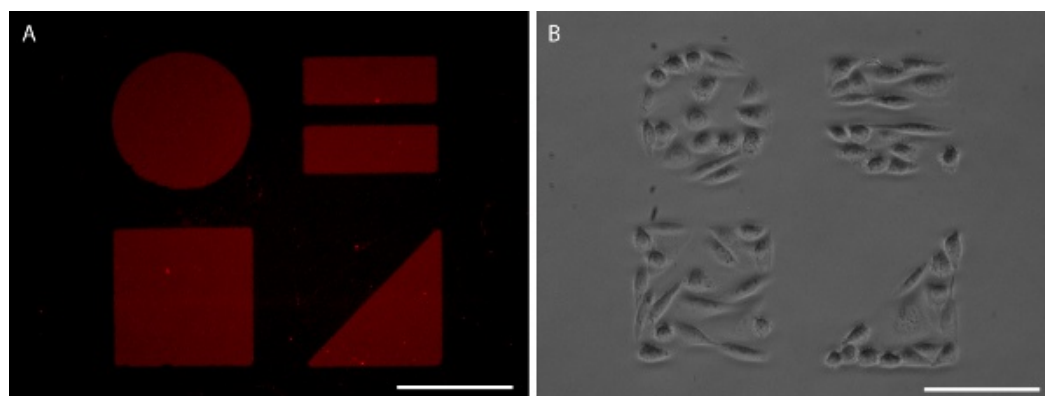


Figure 5. Patterned SAMs visualized with AlexaFluor 647-labeled fibronectin (A) and CHO-K1 cell confinement (B). Scale bars are 100 μ m.

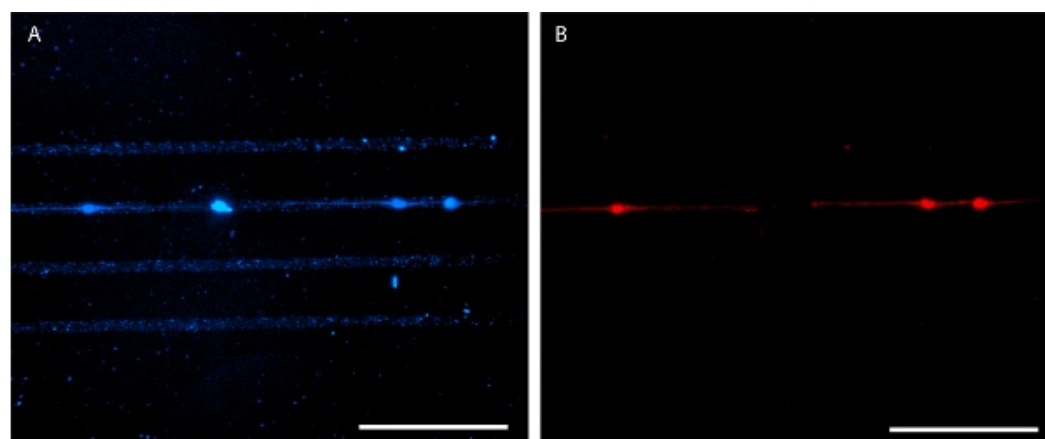


Figure 6. Patterned laminin seeded with E18 mouse hippocampal neurons at 4 days *in vitro*. AlexaFluor 350-conjugated anti-laminin antibody is used for pattern visualization (A) and E18 mouse hippocampal neurons are stained with MitoTracker Red 580 (B). Scale bars are 100 μ m.

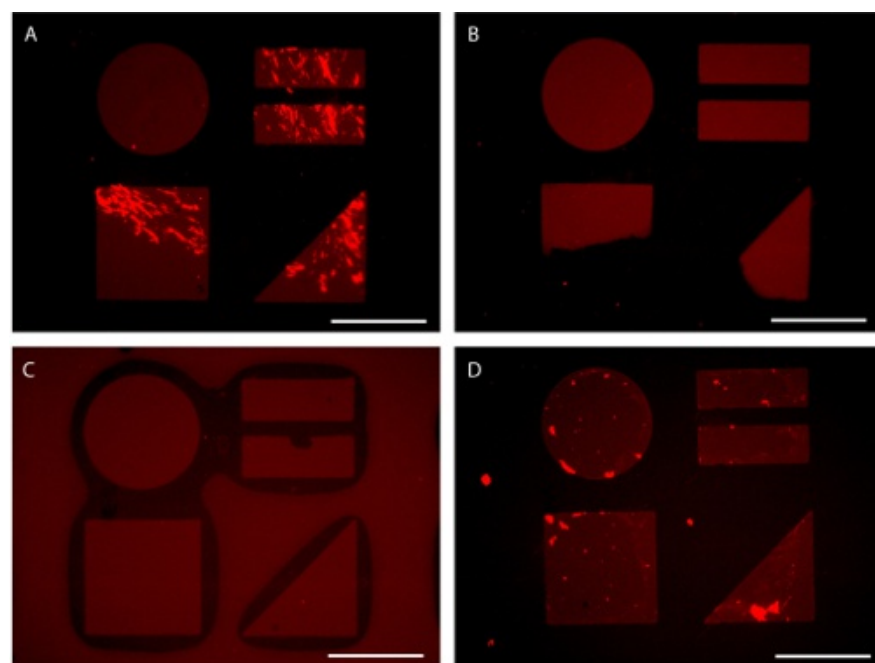


Figure 7. Potential pitfalls in patterned substrate preparation visualized by AlexaFluor 647-conjugated fibronectin adsorption. (A) Insufficient mixing leads to uneven protein adsorption. (B) Uneven application of pressure during stamping leads to partial pattern transfer. (C) Excessive pressure during stamping can lead to stamp collapse. (D) Exposure of patterned surface to the air water interface during rinsing can result in background protein adsorption. Scale bars are 100 μ m.

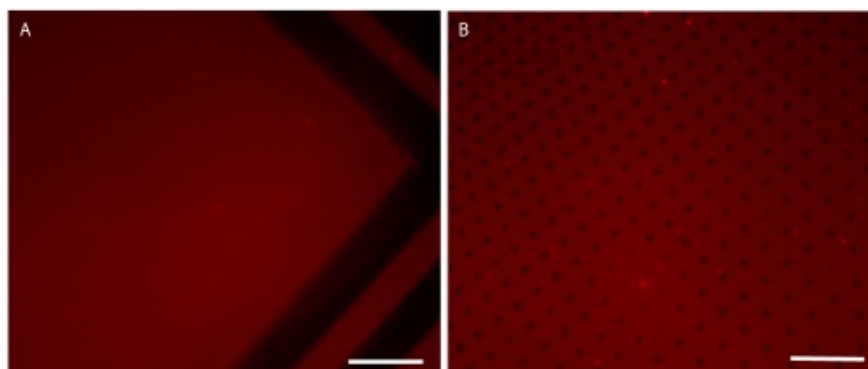


Figure 8. Submerged patterning can produce patterns with small features that are difficult to print by conventional microcontact printing in air. Images (A) and (B) show different regions of the same pattern, printed with the same PDMS stamp in air (A) or deionized water (B). 10 μ m-wide support lines which surround the pattern (added to help prevent stamp collapse) are seen in (A), however, the smaller dot features as shown in (B) are not seen. This demonstrates that printing in air works well for larger features, but printing in water may be necessary for patterns with smaller features. Scale bars are 20 μ m.

Cycle	Acceleration Rate (rpm/s)	Final Speed (rpm)	Time (s)
1	500	1000	5
2	3800	3800	30

Table 1. Two-cycle spin program used to create a 4.5 μ m thick coating of AZ9245 on a silicon wafer.

To prepare PDMS stamps for the formation of patterned substrates, a master in photoresist is first fabricated (Figures 1 and 4A). The master is the inverse of the stamp and is created using either a direct-write lithography system or a mask aligner. When a positive photoresist, such as AZ9245, is used for master production, the resist-coated wafer is exposed to light with the same pattern that will appear on the final substrate. While it is not always possible, it has been reported that the ideal aspect ratio (feature size to resist thickness) for PDMS stamp masters is 1:2.¹³ We have found that aspect ratios of 1:40 are possible, depending on the nature of the pattern. AZ9245 coated silicon wafers under the conditions described here give photoresist with a nominal thickness of 4.5 μ m. We have found that this thickness of AZ9245 can be used to produce PDMS masters with features ranging from >100 μ m to 2 μ m.

PDMS stamps are cast from Sylgard 182 (or Sylgard 184) using the master fabricated from photoresist (Figure 2). Photoresist masters can be used multiple times to create many copies of the same stamp. After hardening the PDMS, stamps are removed from the master using a razor blade and the resulting stamp can be visualized under a microscope by placing stamp feature side down on a glass coverslip (Figure 4B).

Proper stamping results in a sharp, clear protein pattern that can be visualized by application of fluorescently labeled protein (Figures 3 and 5). Alternatively, immunohistochemistry may be used to visualize the protein pattern after cell fixation (Figure 6). Cell growth is well confined to the protein pattern for both immortalized cell lines and primary cells (Figures 5 and 6).

While this technique is easily mastered, several common problems may arise. The application of protein without sufficient mixing of the concentrated protein solution in the DPBS can lead to uneven protein patterns (Figure 7A). Improper stamping can lead to partial pattern transfer or stamp collapse (Figure 7B-C). In addition, exposing the patterned substrate containing adsorbed protein to air can disrupt the monolayer causing decreased resistance in the background (Figure 7D). Patterns comprised of very small features (< 5 μ m) and high aspect ratios often require the use of submerged microcontact printing. In this procedure (3.5b) water is used as a barrier to prevent hexadecanethiol from depositing onto the substrate outside of the pattern (Figure 8).¹⁴

Discussion

A number of issues can arise in the lithographic production of the master used for PDMS stamp creation. Underexposure of the resist-coated wafer results in hazy and indistinct patterns and overexposure of the resist-coated wafer results in enlarged or missing features. In general, masters with large feature sizes (>10 μ m) are relatively easy to pattern and develop, while masters with smaller features can require extensive optimization of photopatterning and development parameters (beyond the parameters recommended by the photoresist manufacturer). The most difficult masters to produce combine both large and small features.

Fabrication of a PDMS stamp from a master is straightforward using the procedure described in section 2. However, care must be taken to completely mix the two components of Sylgard 182. Failure to achieve complete mixing of the components will result in an inability to harden the stamp and likely destruction of the master. Additionally, the master may shatter when separating it from the PDMS stamp if excess force is used or twisting occurs during separation. If the master does break in the process of removing the stamp, it may still be possible to use the stamp after cleaning with ethanol. However, this stamp may produce patterns with defect sites.

While microcontact printing of SAMs using a PDMS stamp is versatile and may be useful in many applications, a number of crucial steps must be followed to achieve successful protein and cell confinement. To obtain good protein and cell confinement, gold substrates must be prepared by electron-beam deposition, since thermal evaporation leads to rough gold surfaces that do not support proper monolayer formation. Moreover, we have found that how the glass substrate is prepared for titanium and gold deposition also affects the quality of the gold substrate and monolayer stability. While glass substrates can be cleaned for deposition using a wide variety of techniques, including boiling methanol, "piranha",¹¹ and

"buzzard" solution,¹² in our hands coverslips prepared by plasma oxidation are far superior to coverslips cleaned using other methods. We have observed that solvent, acid, and base-based cleaning methods give monolayers with decreased temporal stability and an increased number of defect sites.

The exact stamping technique required to produce optimal protein and cellular patterns varies based on the size and shape of the features to be stamped and aspect ratio of the stamp. Typically, the exact stamping technique for each new stamp must be optimized and stamping technique improves after several trials. Applying too much pressure during stamping will lead to stamp collapse, which is prone to happen with stamps containing larger aspect ratios (Figure 7C). Alternatively, applying too little pressure results in partial pattern transfer (Figure 7B). It is also possible to take advantage of stamping technique to slightly alter the features of the pattern. For example, we have found that features can be slightly increased in size by stamping for longer periods of time and others have reported that the dimensions of certain features can be reduced using submerged microcontact printing techniques.¹⁵ Often air bubbles become trapped beneath the stamp when performing submerged stamping; however, this technique may give the best results when small features in conjunction with large aspect ratios are desired.

There are many advantages to using microcontact printing in conjunction with self-assembled monolayer chemistry. When directly printing proteins onto substrates, conformational changes have been shown to occur¹⁶ attributable to the step where proteins are dried during the stamping process. This drying step is thought to lead to protein aggregation and denaturation, which in turn affects protein function. In the SAM system, proteins adsorb to the substrate from a buffered aqueous solution, identical to how substrates are typically prepared for cell culture. In addition, microcontact printing of proteins does not achieve cell confinement for extended periods of time, due to the lack of protein resistant background molecules.¹⁷ In contrast, the use of a glycol background monolayer provides for excellent confinement.⁵ In cases where the use of a gold substrate is either not possible or undesirable, trichlorosilane monomers can be utilized instead to allow for protein and cell confinement.⁹ While there are methods for the confinement of proteins and cells that do not rely on microcontact printing, these methods tend to be more labor intensive and are not amenable to producing the large numbers of identical substrates typically required for biological studies.^{18,19}

Disclosures

No conflicts of interest declared.

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