Video Article

Expanding Nanopatterned Substrates Using Stitch Technique for Nanotopographical Modulation of Cell Behavior

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URL: <http://www.jove.com/video/54840>

DOI: [doi:10.3791/54840](https://doi.org/10.3791/54840)

Keywords: Bioengineering, Issue 118, Nanopattern, stitch technique, electron beam lithography, soft lithography, nanoimprinting lithography, cell modulation, polydimethylsiloxane, polystyrene

Date Published: 12/8/2016

Citation: Wang, K., Leong, K.W., Yang, Y. Expanding Nanopatterned Substrates Using Stitch Technique for Nanotopographical Modulation of Cell Behavior. *J. Vis. Exp.* (118), e54840, doi:10.3791/54840 (2016).

Abstract

Substrate nanotopography has been shown to be a potent modulator of cell phenotype and function. To dissect nanotopography modulation of cell behavior, a large area of nanopatterned substrate is desirable so that enough cells can be cultured on the nanotopography for subsequent biochemical and molecular biology analyses. However, current nanofabrication techniques have limitations to generate highly defined nanopatterns over a large area. Herein, we present a method to expand nanopatterned substrates from a small, highly defined nanopattern to a large area using stitch technique. The method combines multiple techniques, involving soft lithography to replicate poly(dimethylsiloxane) (PDMS) molds from a well-defined mold, stitch technique to assemble multiple PDMS molds to a single large mold, and nanoimprinting to generate a master mold on polystyrene (PS) substrates. With the PS master mold, we produce PDMS working substrates and demonstrate nanotopographical modulation of cell spreading. This method provides a simple, affordable yet versatile avenue to generate well-defined nanopatterns over large areas, and is potentially extended to create micro-/nanoscale devices with hybrid components.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54840/>

Introduction

A number of recent findings reveal that substrate nanotopography has pronounced influence on cell behavior, from cell adhesion, spreading and migration, to proliferation and differentiation¹⁻⁶. For instance, a smaller cell size and lower proliferation rate have been observed in cells cultured on deep nanogratings, even leading to apoptosis although the cell alignment, elongation and migration were enhanced, compared with the flat controls^{2,7-10}. Moreover, nanotopography has been shown to facilitate the differentiation of stem cells into certain lineages such as neuron^{2,11,12}, muscle¹³, and bone^{3,4}. In addition, because of increasing concerns on the toxicity of engineered nanomaterials^{14,15}, there is a need to incorporate nanotopography into physiologically relevant *in vitro* models for accurate risk assessment of nanomaterials. To fulfill the biochemical and molecular biology analyses, enough cells are needed to be grown on a large area of nanopatterned substrate. However, conventional nanofabrication techniques have limitations to generate highly defined nanopatterns over a large area.

Self-assembly including colloid lithography¹⁶ and polymer demixing¹⁷ can readily generate large-area nanostructures at low costs. Because self-assembly relies on interactions between the assembling elements such as colloidal particles and macromolecules, and possible interactions between these elements and substrate, it cannot be a stand-alone method of producing nanostructures with precise spatial positioning and arbitrary shapes¹⁸. The accompanied high density of defects is also a drawback. Precise spatial control of nanopatterns can be achieved by employing templated self-assembly, which uses top-down lithographic approaches to provide the topographical and/or chemical template to guide the bottom-up assembly of the assembling elements¹⁹⁻²¹. Alternative nanofabrication techniques such as step-and-flash lithography²² and a roll-to-roll nanoimprinting lithography²³ have been developed but have limited use because of their sophisticated process or the requirement of specialized equipment. Nevertheless, a template or a master mold with defined nanoscale patterns is needed for templated or alternative nanofabrication techniques.

Such templates and master molds are conventionally generated by using focused electron, ion, or photon beam lithography. For instance, electron beam lithography (EBL)²⁴ and focused ion beam lithography²⁵ can generate defined patterns with a sub-5 nm resolution. Two-photon lithography has demonstrated a feature size as small as 30 nm²⁶. Although the focused beam lithography techniques enable generation of well-defined nanoscale structures, the capital investment and the time-consuming, costly process restrict their widespread use in academic research²⁷. Therefore, it is highly desirable to develop enabling yet affordable techniques to produce a large area of nanopatterned surfaces with high fidelity.

We have reported a simple, cost-effective stitch technique for generating a large area of nanopatterned surface from a small well-defined mold²⁸. This protocol provides step-by-step procedure from replication of poly(dimethylsiloxane) (PDMS) molds using an EBL-written pattern, to

assembly of multiple PDMS molds into a single large mold, to generation of a master mold on polymeric such as polystyrene (PS) substrates, to production of working substrates. With the expanded nanopatterned substrates, we demonstrated nanotopographical modulation of cell spreading.

Protocol

1. Replication of PDMS Molds from an EBL Mold

1. Fabricate silicon mold²⁹
 1. Spin coat 200 μ l polymethyl methacrylate (PMMA) solution on a 1 $\times$ 1 cm silicon (Si) substrate at 2,500 rpm for 1 min to form a thin film.
 2. Bake the PMMA film on the Si substrate at 180 °C for 2 min.
 3. Write the designed nanopattern on the PMMA film by using a focused electron beam at an area dose of 300 μ C/cm².
 4. Develop the PMMA nanopattern in developer for 80 sec.
 5. Deposit the PMMA nanopattern with a nickel layer of 50 nm in thickness using an E-beam evaporator at an output voltage of 10 kV, emission current of 0.5 mA and a deposition rate of 0.5 Å/sec.
 6. Lift off the PMMA part in 20 ml remover at 80 °C for 20 min.
 7. Reactive ion etch (RIE) the nanopattern into the Si substrate to get a Si mold of the desired depth.
NOTE: Gas mixture of tetrafluoromethane (CF₄)/oxygen (O₂) (90%/10%) at an inductively coupled plasma (ICP) power of 400 W and RIE power of 150 W is used to etch Si substrate to a depth of 560 nm.
2. Silanize Si mold
 1. Put a glass coverslip and the Si mold in a 100 mm PS Petri dish and transfer them in a glass desiccator located in a fume hood.
 2. Drop 10 μ l 1H,1H,2H,2H-Perfluorooctyltrichlorosilane on the coverslip.
Caution: 1H,1H,2H,2H-Perfluorooctyltrichlorosilane may cause skin corrosion and serious eye damage. Wear appropriate personal protective equipment (PPE).
 3. Cover the Petri dish partially.
 4. Keep the desiccator under vacuum for 5 hr in a fume hood to complete the silanization of the Si mold.
3. Prepare PDMS prepolymer
 1. Weigh 10 g PDMS resin and 1.05 g curing agent in a disposable weighing boat.
 2. Mix the PDMS prepolymer thoroughly by using a plastic spoon.
 3. Degas the PDMS prepolymer in a plastic desiccator under vacuum for about 20 min till a clear mixture is observed.
4. Replicate PDMS molds
 1. Put the silanized Si mold in a 60 mm Petri dish.
 2. Pour the PDMS prepolymer on the Si mold in the Petri dish.
 3. Place the Petri dish in a plastic desiccator and degas for about 10 min till all bubbles disappear.
 4. Transfer the Petri dish to a hotplate and cure the PDMS prepolymer at 70 °C for 4 hr.
 5. Peel off the PDMS mold from the Si mold carefully by using tweezers.
NOTE: PDMS molds can be stored at ambient conditions for up to one week. After curing, there are some uncrosslinked PDMS resin molecules and residual curing agent in the PDMS molds³⁰. The low molecular weight molecules will gradually diffuse out and accumulate at the surface over time. This affects the topographical and mechanical properties of the PDMS surface³¹. The diffusion is not significant within one week.

2. Stitching of PDMS Molds into a Large, Single Mold

1. Prepare multiple PDMS molds by repeating Step 1.4.
NOTE: Weigh same amount of PDMS mixture each time to obtain PDMS molds of same thickness.
2. Determine the orientation of anisotropic PDMS nanopatterns such as nanogratings under an optical microscope and mark it on the backside of the PDMS molds with a marker pen.
NOTE: It is not necessary to mark the orientation of isotropic nanotopography such as nanopillars.
3. Clean a Si substrate with ethanol in a fume hood and dry it with compressed air.
4. Trim off the unpatterned areas of each PDMS mold with a blade.
NOTE: For the PDMS molds that will be placed at the periphery of the stitched mold, only the unpatterned areas in contact with others should be trimmed off.
5. Place the trimmed PDMS mold with the nanopattern face-down on the mirror side of the Si substrate and then align other molds close to but not touching the surrounding mold(s).
6. Prepare a PDMS adhesive layer
 1. Cast 1 g degassed PDMS prepolymer (PDMS resin and curing agent ratio: 10:1.05) on a clean glass slide (7.5 cm $\times$ 2.5 cm) to form a 0.5 mm thick layer.
 2. Bake the PDMS layer at 100 °C on a hotplate for 3-5 min. Use a needle to touch the layer and ensure that the layer is partially but not completely cured.
NOTE: Partially cured PDMS cannot flow like uncured PDMS prepolymer, but it is sticky compared with cured PDMS.
7. Place the PDMS layer on the backside of aligned PDMS molds and quickly invert this assembly and transfer it to the hotplate.

8. Apply a compressive force (5 kPa) using a metal block on the top of assembly to ensure a good contact between the PDMS adhesive layer and the backside of PDMS molds, and cure the PDMS adhesive layer at 100 °C for 1 hr.
NOTE: Carefully adjust the position of the metal block to avoid the tilt of the assembly.
9. Remove the metal block and peel off the single, stitched PDMS mold from the Si substrate.

3. Generation of a Master Mold on PS Substrates

Note: The stitched PDMS mold immobilized on a glass slide can be used to generate a master mold on a PS plate or a PS thin film, from which working nanopatterned substrates can be produced.

1. Generate a master mold on a PS plate
 1. Prepare a PS plate
 1. Dry the PS pellets in a vacuum oven at 80 °C for two days.
 2. Preheat a press machine at 230 °C.
 3. Assemble an aluminum plate, a polytetrafluoroethylene (PTFE) sheet and the aluminum spacer in an order from bottom to top.
 4. Load 3.5 g PS pellets in the aluminum spacer with a square opening of 3 cm (L) × 3 cm (W) × 0.3 cm (H).
NOTE: The spacer is approximately 0.1 cm thicker than the PDMS molds, and thus the final nanopatterned PS substrate is about 0.1 cm thick.
 5. Place another PTFE sheet and then another aluminum plate on the aluminum spacer.
 6. Place the assembly in the press machine.
 7. Preheat the PS pellets at 230 °C for 30 min.
 8. Apply a compressive pressure (0.1 MPa) on the assembly for 5 min.
 9. Release the pressure and then reapply a compressive pressure of 0.5 MPa on the assembly.
 10. Repeat Step 3.1.1.9 with a pressure increase of 0.5 MPa till the desirable pressure of 1.5 MPa is reached.
 11. Turn off the heater of the press machine and cool it down below 70 °C at a constant pressure of 1.5 MPa.
 12. Take the assembly out and store the PS plate in a vacuum oven at 80 °C to prevent moisture from re-entering the PS plate.
 2. Nanoimprint the stitched PDMS mold into PS plate
 1. Place the PS plate in an aluminum spacer set on a 3-inch Si wafer.
NOTE: The inner dimensions of the spacer are the same as those of the PS plate so the PS plate fits right in the spacer.
 2. Heat the PS plate on a hotplate at 250 °C for 30 min.
 3. Put the stitched PDMS mold with nanopatterns face-down on the molten PS plate.
NOTE: One side of the PDMS mold is put in touch with the surface of the PS plate first and another side is lowered gradually in contact with the PS surface to avoid the formation of air bubbles at the interface.
Caution: The surface of the hotplate is hot. Wear thermogloves during nanoimprinting process.
 4. Place an aluminum plate on the glass slide of the stitched PDMS mold.
 5. Apply a compressive pressure (12.5 kPa) by using metal blocks on the aluminum plate and wait for 3 min.
NOTE: Make sure that the aluminum plate is not tilted.
 6. Lift and replace the metal block from the aluminum plate and increase the compressive pressure to 25 kPa.
 7. Repeat Step 3.1.2.6 with the pressure increased to 50 kPa.
NOTE: This step is to remove the air trapped between the PDMS mold and PS plate.
 8. Maintain the temperature of the hotplate between 240 and 250 °C under the consistent pressure of 50 kPa for 15 min.
 9. Turn off the hotplate and cool down the whole setup.
NOTE: A fan can be used to expedite the cooling process.
 10. Remove the metal blocks after the temperature is below 50 °C, and carefully peel off the stitched PDMS mold from the PS plate.
NOTE: The PS substrate has the reverse nanopatterns and can be used as a master mold to produce working PDMS substrates.
2. Generate a master mold on a PS thin film
 1. Prepare a PS thin film
 1. Dissolve 1 g PS in 10 ml toluene in a fume hood.
Caution: Toluene can cause skin irritation and serious eye damage, and may cause damage to organs through prolonged or repeated exposure. Wear appropriate PPE.
 2. Spin-coat 1 ml PS solution on a 2-in wafer at 2,500 rpm for 1 min to form ~1 μm thick PS thin film.
 3. Evaporate toluene from the film by setting the PS film on Si wafer in a fume hood for 3 days.
 4. Anneal the PS thin film in a vacuum oven at 80 °C overnight.
 2. Nanoimprint the PDMS mold on a PS thin film
 1. Put the stitched PDMS mold with nanotopography face-down on the PS thin film, which is set on a hotplate.
 2. Apply a compressive pressure of 12 kPa on the PDMS mold by using metal blocks on the glass side of the PDMS mold.
 3. Increase the temperature of the hotplate to 180 °C and maintain it for 15 min.
Caution: The molten PS film can function as a lubricant. Pay attention to prevent the metal blocks from sliding off.
 4. Turn off the hotplate and cool down the whole setup.
NOTE: A fan can be used to expedite the cooling process.
 5. Remove the metal blocks after the temperature drops below 50 °C, and carefully peel off the stitched PDMS mold from the PS film.
NOTE: The nanopatterned PS film will serve as a master mold to produce working PDMS substrates.

4. Nanotopographical Modulation of Cell Behavior

Note: Human epithelial cells are cultured on the representative nanotopographies to demonstrate nanotopographical modulation of cell spreading.

1. Cast PDMS working substrates from the master mold generated from either Step 3.1 or 3.2 depending on the application.
 2. Using a hollow steel arch punch, cut the nanopatterned PDMS substrates into discs to fit the configuration of a specific multi-well plate (e.g., 24-well plate).
 3. Use tweezers to place the PDMS discs into the wells of a multi-well plate.
 4. Sterilize the PDMS substrates by using 70% ethanol and then UV exposure, each for 30 min.
 5. Wash the PDMS substrates with 1x sterile phosphate buffered saline (PBS) three times.
 6. Coat the PDMS substrates with extracellular matrix protein (*i.e.*, 20 $\mu\text{g}/\text{ml}$ fibronectin) for 30 min at room temperature.
 7. Rinse the PDMS substrates three times with sterile PBS, each for 5 min.
 8. Suspend human lung cancer cell A549 in Dulbecco's modified eagle medium with 10% fetal bovine serum and count the cells using a hemocytometer.
 9. Plate the cells at a seeding density of 2,000 cells/ cm^2 on the PDMS substrates and culture them at 37 °C in a humidified atmosphere containing 5% CO_2 for one day.
 10. Wash the cells with PBS three times.
 11. Fix the cells in a mixture of 4% paraformaldehyde and 2% glutaraldehyde in PBS for 4 hr and dehydrate cells using a CO_2 critical point dryer for scanning electronic microscopic observation²⁹.
- Caution: Paraformaldehyde and glutaraldehyde may cause severe skin burns and eye damage. Operate in a chemical hood and wear appropriate PPE.

Representative Results

The stitch technique can generate a large area of nanopatterned substrates with high fidelity. **Figure 1a** and **1b** display the large area of nanopatterns transferred from the stitched PDMS mold to PS plate and PS thin film on a Si substrate, respectively. The comparison between the original EBL-written mold (**Figure 1c**) and the final PDMS working substrate (**Figure 1d**) confirms that the EBL-written nanopatterns can be faithfully transferred to the working substrate. Nanotopography of various geometry and dimensions can be used to modulate cell behavior. As demonstrated in **Figure 2** with A549, an adenocarcinomic basal epithelial cell line as model cells, the anisotropic nanogratings can elongate the cells along the nanograting direction compared with the multipolar morphology that A549 cells display on the isotropic nanopillars.

Figure 1. Generation of a large area of nanopatterned substrates using stitch technique. (a,b) Optical images of the nanopatterns transferred to PS plate and PS thin film, respectively. The arrows indicate the polymer raise in the interstices of the stitched PDMS molds. (c,d) SEM images of the nanopatterns on EBL mold and final PDMS working substrate, respectively. The scale bars are 1 μm . [Please click here to view a larger version of this figure.](#)

Figure 2. Nanotopographical modulated cell spreading of A549 cells. A549 cells grown on (a) nanogratings of 500 nm in line width, 500 nm in spacing and 560 nm in height and (b) nanopillars of 500 nm in diameter, 450 nm in edge-to-edge spacing and 560 nm in height, respectively. The scale bars are 10 µm. [Please click here to view a larger version of this figure.](#)

Discussion

We present a simple, affordable, yet versatile method to generate a large area of nanopatterned substrate. To faithfully expand highly defined nanopatterns, great attention should be paid to several critical steps. The first one is to trim the multiple PDMS molds. Unpatterned areas of the PDMS molds need to be removed. Additionally, the sidewalls of the molds should be cut vertically as perfect as possible to minimize the gaps between the molds. Collectively, the portion of unpatterned areas in the final stitch mold can be reduced. Secondly, the nanopatterned surface of these PDMS molds needs to be aligned without any distortion on the silicon substrate. Because the PDMS nanostructures are prone to deform, it is critical to place the nanopatterned surfaces against a mirror side of the silicon substrate gently and evenly (avoid trapping air between the PDMS mold and silicon surface). The PDMS molds will be aligned as close as possible to, but not touching the neighboring molds to further minimize the unpatterned portion of the final stitch mold. Otherwise, the touched nanostructures will deform during nanoimprinting. Thirdly, the thickness of the PDMS molds may vary from batch to batch, and thus it is critical to make the thickness uniform in addition to making the thickness of each mold uniform by leveling the small Si mold perfectly before PDMS casting. Although the variation in thickness across the PDMS molds can be compensated by adjusting the thickness of the PDMS prepolymer (adhesive) layer cast on a glass slide, a thick prepolymer layer could be problematic. The prepolymer may be pulled through the interstices between the PDMS molds to the patterned surface by the capillary force, and consequently damage the nanopatterns. The thickness variation can be minimized by preparing the same amount of PDMS mixture when casting from the EBL mold. As a result, a thin PDMS prepolymer layer can be used. Alternatively, partially curing the prepolymer layer will increase its viscosity, and thus reduce its raise and eventually eliminate the possible damage of nanopatterned surfaces.

The stitch technique is limited by elastomeric nature of PDMS. Although soft lithography has been applied to replicate feature sizes as small as 2 nm³² and, in principle, can reach a resolution of less than 0.5 nm¹⁸, the nanoscale PDMS features cannot be replicated flawlessly when the aspect ratio of height to width is too high (> 2) or too low (< 0.2). The nanofeatures may collapse when the aspect ratio is too high, or result in insufficient relief when the PDMS stamp of a < 0.2 ratio is used³³. Moreover, multiple PDMS molds cannot be stitched seamlessly because of the interstices and incomplete trimming of the PDMS molds, and thus there are unpatterned and misaligned areas (in particular for continuous nanopatterns such as nanogratings). Given the small percentage of the defected area over the total surface area, the stitch technique still provides a simple and affordable way to produce a large area of nanopatterned substrates. In addition, when the stitched mold is nanoimprinted into the polymer substrate, molten polymer might flow into the interstice, resulting in an uneven surface (**Figure 1a**). The uneven surface makes it challenging to collect samples for cellular or molecular biological analyses. In microfluidic applications, the raise also causes an incomplete sealing when microchannels are sealed against the patterned substrate. The uneven surface problem can readily be solved by applying polymer thin film technique to minimize the raise through tuning the film thickness (**Figure 1b**).

Although the stitch technique needs a defined master mold for the expansion, it is simple and affordable compared with other techniques such as step-and-flash lithography and a roll-to-roll nanoimprinting lithography. The stitch technique requires only hot plates and a way of exerting compressive forces during stitching and nanoimprinting processes, but not expensive equipment. Moreover, the stitch process can be conducted in a clean environment, but not necessarily in a cleanroom.

The stitch technique is also versatile. In addition to expanding an identical nanopattern to a large area, the stitch technique can be applied for the molds consisting of micro- and/or nanoscale features of various shapes, dimensions and arrangements. To this regard, a combinatory library of micro-/nanotopographies can be built to provide high-throughput platform for investigate cell-topography interactions. This simple, affordable and versatile stitch technique can potentially be extended to create micro-/nanoscale devices with hybrid components.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was partly supported by NSF CBET 1227766, NSF CBET 1511759, and Byars-Tarnay Endowment. We gratefully acknowledge use of the West Virginia University Shared Research Facilities which are supported, in part, by NSF EPS-1003907.

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