HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2017 March 01.

Published in final edited form as:

Methods Mol Biol. 2017 ; 1493: 321–343. doi:10.1007/978-1-4939-6448-2_24.

Photolithography-Based Substrate Microfabrication for Patterning Semaphorin 3A to Study Neuronal Development

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Summary

Protein micropatterning techniques, including microfluidic devices and protein micro-contact printing, enable the generation of highly controllable substrates for spatial manipulation of intracellular and extracellular signaling determinants to examine the development of cultured dissociated neurons *in vitro*. In particular, culture substrates coated with proteins of interest in defined stripes, including cell adhesion molecules and secreted proteins, have been successfully used to study neuronal polarization, a process in which the neuron establishes axon and dendrite identities, a critical architecture for the input/output functions of the neuron. We have recently used this methodology to pattern the extracellular protein Semaphorin 3A (Sema3A), a secreted factor known to control neuronal development in the mammalian embryonic cortex. We showed that stripe patterned Sema3A regulates axon and dendrite formation during the early phase of neuronal polarization in cultured rat hippocampal neurons. Here, we describe microfabrication and substrate stripe micropatterning of Sema3A. We note that same methodologies can be applied to pattern other extracellular proteins that regulate neuronal development in the embryonic brain, as Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), and Netrin-1. We describe modifications of these methodologies for stripe micropatterning of membrane-permeable analogs of the second messengers cyclic AMP (cAMP) and cyclic GMP (cGMP), intracellular regulators of neuronal polarization that might act downstream of Sema3A.

Keywords

Nanofabrication; protein stripe micropatterning; microfluidics; micro contact printing; Semaphorin 3A; neuronal development; axon and dendrite polarization; poly(dimethylsiloxane) (PDMS)

1. Introduction

In mammalian embryonic development, neuronal cells polarize to create the distinct cellular compartments of axon and dendrites that inherently differ in form and function (1–3). The

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initial establishment of axon and dendrite polarity may depend on intrinsic determinants within the neuron and extrinsic factors from its environment (1–3). In the developing mammalian embryonic cortex, Semaphorin 3A (Sema3A), a secreted protein of the class III semaphorin superfamily, is expressed in a descending gradient across the cortical layers, with the highest expression at the pial surface (4–6). The Sema3A gradient was shown to function as a chemoattractant for orienting apical dendrites of cortical pyramidal neurons toward the pial surface and to have an opposite effect on axon development (5). Furthermore, Sema3A was demonstrated to guide radial migration of these neurons (6). Recently we have shown that in cultured rat hippocampal neurons, localized Sema3A differentially regulates axon and dendrite initiation during the early phase of neuronal polarization (7). The highly localized exposure to Sema3A was achieved by plating dissociated hippocampal neurons on substrates patterned with stripes of Sema3A.

Photolithography-based microfabrication techniques are widely used to generate highly controllable substrates that are patterned with proteins of interest for broad applications in cell and developmental biology (8–12). Substrate stripe patterning has been used to study and manipulate early events in neuronal development, e.g. axon and dendrite polarization and directed axon outgrowth (axon pathfinding) (13, 14). These studies have used stripe patterning of cell adhesion proteins (15), secreted extracellular proteins that regulate neuronal development in the developing embryonic brain (7, 13, 14), as well as membrane permeable analogs of intracellular signaling determinants (13, 16).

Here, we describe the microfabrication and stripe micropatterning of a glass substrate with Semaphorin3A (Sema3A). We describe two methods for the stripe patterning of Sema3A, using microfluidics (17, 18) and micro-contact printing (9, 19, 20), both of which utilize replicas of an elastomeric polymer poly(dimethylsiloxane) (PDMS), patterned with 50 μm wide stripes spaced 50 μm apart, with the depth of 3–5 μm. The design and dimensions of the micropatterns are important and should be tailored for the specific experimental needs. We found the 50 μm wide stripes with the 50 μm spacing to be optimal for the examination and manipulation of early stages of cultured rodent hippocampal neuronal development (7, 13, 16). Furthermore, the depth of the microchannels would determine the amount as well as the uniformity in thickness of the protein layer that is deposited on the glass coverslip by microfluidics, and therefore needs to be determined experimentally. We found depths of 3–5 μm to be optimal to study neuronal development. This micropatterned PDMS replica can be used to flow Sema3A patterning solution by microfludics through the microchannels, while reversibly sealing the PDMS on poly-L-lysine (PLL) coated glass coverslip. Alternately, the ridges in the micropattern can be used for Sema3A micro-contact printing on the glass coverslip. Under both patterning methods, the protein is adsorbed to the glass most likely by protein surface forces including van der Waals, hydrophobic and electrostatic interactions, with van der Waals most likely being dominant (21). To visualize the stripes by fluorescence, fluorescently-conjugated BSA is added to the stripe patterning solution, unless a fluorescently-conjugated version of the protein of interest is available. The availability of fluorescently-conjugated BSA with the entire spectrum of emission wavelength, from the UV to the far infrared, allows flexibility and compatibility with specific needs of immunohistochemistry. We further describe modifications of these methodologies for stripe micropatterning of membrane-permeable analogs of the second messengers cyclic AMP

(cAMP) and cyclic GMP (cGMP), intracellular regulators of neuronal polarization that act downstream of Sema3A (7, 13, 16).

2. Materials

2.1 Generation of silicon-based master molds

1. Silicon wafer (75 mm diameter, single-polished) with <100> orientation (University Wafer, South Boston, MA).
2. Chrome mask containing the desired micropatterns (Advanced Reproductions Corp. North Andover, MA).
3. Acetone (Sigma-Aldrich, Inc. St. Louis, MO).
4. Isopropyl Alcohol (Sigma-Aldrich).
5. S1811 positive photoresist (MicroChem Inc., Newton, MA).
6. SU-8 negative photoresist (MicroChem Inc.).
7. MF 312 developer (MicroChem Inc.).
8. Propylene glycol monomethyl ether acetate (PGMEA) (Dow Chemical Co., Midland, MI).
9. De-ionized (DI) water (we recommend using ultrapure water prepared by purifying deionized water to attain a sensitivity of 18 MΩ at 25°C).
10. Spin Coater - Brewer Science Cee 200CB (Brewer Science Inc., Rolla, MO).
11. Hot plates (VWR International, Radnor, PA).
12. Karl Suss MJB3 UV mask aligner (Suss MicroTec AG., Garching, Germany).
13. Deep reactive ion etcher (Oxford Instruments Plasmalab 100; Oxfordshire, UK).
14. Dry Vacuum pump (BOC Edwards, West Sussex, UK).
15. Convection Incubator (VWR International).

2.2 Preparation of PDMS micropatterns (Replica molding)

1. Polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Elastomer Kit) (Dow Chemical Co.,).
2. Sterile culture dishes (150mm, 100mm, 35mm) (Corning Inc., Corning, NY).
3. Bel-Art Vacuum chambers jar (Bel-Art Products, Wayne, NJ).
4. De-ionized (DI) water.
5. 70% Ethyl alcohol in DI water (Ethyl alcohol, Sigma-Aldrich).

6. Flat-head tweezers (6mm width) (Ted Pella, Inc., Redding, CA).
7. Convection Incubator (VWR International).
8. Branson Ultrasonic Bath (2.8L bath capacity, 40 kHz frequency) (VWR International).
9. Laminar tissue culture flow hood (The Baker Company, Sanford, ME).

2.3 Substrate Microfluidic patterning

1. Sterile Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4. To prepare 1 L PBS solution, add about 800 ml water to a 1-L glass beaker. Weigh 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4, and transfer to the glass beaker. Mix, and adjust pH to 7.4. Make up to 1 L with water. Filter sterilize and store PBS at room temperature.
2. Round 12mm diameter glass coverslips for neuronal culturing and pre-coating with poly-L-lysine (PLL), German Glass (Electron Microscopy Sciences, Hatfield, PA).
3. poly-L-lysine (PLL, Sigma-Aldrich). We recommend using 0.5 mg/ml of PLL prepared in water (*see* Note 1). To prepare 100 ml of PLL solution, add 80 ml water to a 0.5-L glass beaker. Weigh 50 mg PLL and transfer to the glass beaker. Mix, and make up to 100 ml with water. Filter sterilize and aliquot to 20 ml aliquots. Store PLL at −20°C.
4. Bovine Serum Albumin (BSA) (Sigma-Aldrich).
5. Stripe patterning solution contains: 50 μg/ml BSA (*see* Note 2), and 5 μg/ml fluorescently conjugated BSA (Invitrogen) (*see* Note 2), prepared in PBS. Fluorescently conjugated Bovine Serum Albumin (BSA), with the entire spectrum of emission wavelength, from the UV to the far infrared (Invitrogen Corporation, Carlsbad, CA) can be used (*see* Note 3). Add 0.5 or 0.05 μg/ml Recombinant human Semaphorin-3A/Fc chimera (R&D Systems, Inc. Minneapolis, MN). The final concentration of Sema3A used for stripe patterning needs to be determined experimentally (*see* Note 4). The same composition of stripe patterning solution can be used to pattern

[1]Procedures for PLL coating of glass-coverslips can vary and can be obtained from a variety of literature (13, 22, 23).
[2]The stripe-patterning solution contains Bovine Serum Albumin (BSA), because: *1)* it helps decrease the surface tension of the stripe-patterning solution to promote capillary-driven flow, and *2)* it might serve as a non-specific adhesive carrier to the protein of interest for enhanced adsorption to the glass coverslip. The concentration of non-fluorescent BSA will need to be determined experimentally for optimal adsorption. We found a concentration of 50–100 μg/ml BSA, to be optimal. For visualization of the stripes by fluorescence, fluorescently-conjugated BSA is added to the stripe-patterning solution, unless a fluorescently-conjugated version of the protein of interest is available (*see* Note 3).
[3]Fluorescently-conjugated BSA is available from several commercial vendors, with the entire spectrum of emission wavelength, from the UV to the far infrared, for compatibility and convenience with specific needs of immunohistochemistry.
[4]The final concentration of Sema3A used for stripe patterning needs to be determined experimentally. We found that a final concentration that is 10 to 100 times lower than the concentration used for bath application, is optimal, and can be determined based on the quality and uniformity of the stripes, and the effect on the neuronal culture. We recommend using 0.5 or 0.05 μg/ml Sema3A in the stripe patterning solution. The final concentration for the stripe patterning of other extracellular proteins as BDNF, NGF, or netrin-1 should also be determined experimentally (*see* Note 7).

other extracellular proteins, with suggested final concentrations: 0.5 ng/ml Recombinant human BDNF and NGF (PeproTech Inc., Rocky hill, NJ), 0.5 or 0.05 ng/ml Recombinant human netrin-1 (R&D Systems) (*see* Note 4).

6. De-ionized (DI) water.

2.4 Protein micro-contact printing

1. For efficient microstamping of the stripe patterning solution, a steady force needs to be applied on the PDMS to allow optimal contact between the PDMS and the glass coverslip. For optimal transfer of protein onto the coverslip, the force is applied on the PDMS using a "stamper" device that is composed of a steel flat-head machine screw (1/4″–20 × 1–1/2″; 17 g in weight; can be purchased at a hardware store). The stamper can be optimized by slightly varying its weight by adding 1/4″–20 Hex nuts, 1–2 gr each. To achieve a flat surface, glue a 12 mm glass coverslip to the flat-head of the screw (Fig. 4).
2. Texwipe TechniCloth Nonwoven Wipers (9×9 in.) (Thermo Fisher Scientific Inc. Waltham, MA).
3. Plasma Cleaner, PDC-001 (Harrick Plasma Ithaca, NY).

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Generation of silicon-based master molds for stripe patterning

The micropatterns that are used for protein stripe patterning by either microfluidics or micro-contact printing, are designed with a CAD (computer-aided design) program which is used to generate a chrome photomask. This photomask is used to produce the master mold by photolithography that will be used for PDMS replica molding (Fig. 1). We describe two methods for the generation of silicon master molds for stripe patterning. The first uses a subtractive process in which selective regions in a positive tone photoresist are removed after exposure to UV-light, leaving bare areas in the silicon substrate that are subsequently etched by reactive ion process (Fig. 1A). The second uses an additive process in which selective regions in a negative tone photoresist are cross-linked by UV-light and become permanently adhered to the silicon substrate, while the un-cross-linked regions of the photoresist are removed (Fig. 1B). Although specific microfabrication laboratories might prefer one method over the other, both methods are equivalent in micropattern resolution and have comparable master mold lifetime. These procedures need to be performed in a class 100/1000 clean room facility using specialized equipment for photolithography and deep reactive-ion etching. The procedures detailed here are standard protocols for the generation of the master molds, and should be carried out at the appropriate core facilities by trained professionals. Such core facilities can be found at several nano-fabrication centers in research institutions and universities.

3.1.1. Generation of silicon-based master molds for stripe patterning: Dry etched silicon master molds for stripe patterning (Subtractive Lithography)—

The procedures are summarized in Fig. 1A.

1. Cover the silicon wafer with acetone and isopropyl alcohol by placing 2 ml of each solution in the middle of the wafer, and spincoat at 575 × *g* for 30 seconds.
2. Using a transfer pipet, deposit 3–4 ml S1811 positive photoresist onto the silicon wafer, until 4/5 of the wafer area is covered, and spincoat at 1020 × *g* for 45 seconds.
3. Directly place wafer on a 110°C hotplate and prebake for 2 minutes.
4. Use a UV mask aligner to transfer micropatterns (50 μm wide stripes spaced 50 μm apart) from the chrome photomask onto the photoresist (*see* Note 5). Transfer the micropatterns to the photoresist by exposing the resist with UV light through a chrome mask containing the desired pattern. We routinely use a Karl Suss MJB3 Mask Aligner. Here in the positive photoresist, regions that are exposed to UV through the clear parts of the mask, become more soluble in the developer (**Method 3.1.1, Step 5**), whereas those regions covered by the chrome parts of the mask remain insoluble after development.
5. Place the wafer containing the exposed photoresist in a glass beaker containing a 1:1 (vol:vol) mixture of DI water and MF 312 developer, 10 ml each, and develop for 30 seconds.
6. Stop the development by rinsing with 20 ml DI water and drying with a gentle stream of nitrogen gas.
7. Perform a deep reactive-ion etch (DRIE) at a constant 25°C until the desired depth is achieved. We routinely etch the silicon microchannels to 3–5 μm depth (*see* Note 6 and Note 7).
8. Remove the remaining unexposed photoresist with 20 ml acetone, followed by DI water rinse (20 ml) and drying with a gentle stream of nitrogen gas.
9. For long-term storage, store the master molds (Fig. 1C) on-edge in a dust-free container in a cool and dry environment away from direct light. Handle the master molds only with gloves and flat-head tweezers.

[5]We routinely use 50 μm wide stripes spaced 50 μm apart. We found this pattern to be optimal for the examination and manipulation of early stages of cultured embryonic hippocampal neuronal development. The pattern can be designed according to the specific experimental needs.

[6]We routinely use 3–5 μm deep channels. We found this depth to be optimal for protein stripe patterning using microfluidics (**Method 3.3**), in which a protein layer is deposited on the glass coverslips following the flowing of the stripe patterning solution into the microchannels, and the subsequent drying of the stripe patterning solution in the microchannels. A depth greater than 3–5 μm might result in difficulty in drying, leading to leakage of the solution onto the gaps between the microchannels following dismounting of the PDMS from the glass coverslips (*see* Note 15) and thus defective stripe patterning.

[7]The depth of the microchannels would determine the amount as well as the uniformity in thickness of the protein layer that is deposited on the glass coverslip by microfluidics, and therefore needs to be determined experimentally. We found depths of 3–5 μm to be optimal for studying neuronal development (Fig. 2A) (7, 13, 16).

3.1.2. Generation of silicon-based master molds for stripe patterning: SU-8 master molds for stripe patterning (Additive Lithography)—The procedures are summarized in Fig. 1B.

1. Cover the silicon wafer with acetone and isopropyl alcohol by placing 2 ml of each solution in the middle of the wafer and spincoat at 575 × *g* for 30 seconds.
2. Using a transfer pipet, deposit 3–4 ml SU-8-2 negative photoresist onto the silicon wafer and spincoat at 1020 × *g* for 45 seconds, with a ramp of 16 × *g* for 5 sec.
3. Pre-bake (soft-bake) on a 65°C hotplate for 1 min, and then on a 95°C hotplate for 3 min.
4. Use a UV mask aligner to transfer the micropatterns (50 μm wide stripes spaced 50 μm apart) from a chrome photo mask onto the photoresist, by exposing with UV for about 10 sec. Transfer the micropatterns to the photoresist by exposing the resist with UV light through a chrome mask containing the desired pattern, using a Karl Suss MJB3 Mask Aligner. Here in the negative photoresist, regions that were exposed to UV through the clear parts of the mask will be insoluble in the developer, whereas those regions covered by the chrome parts of the mask become more soluble after development (**Method 3.1.2, Step 6**).
5. Directly place wafer on a 65°C hotplate and post-bake for 1 min, and then immediately transfer to a 95°C hotplate and post-bake for an additional 3 min.
6. Develop the pattern by immersing the wafer in 20 ml PGMEA (**Materials 2.1, Step 8**) for 5 to 8 min.
7. Rinse the wafer with 20 ml DI water and dry with a gentle stream of nitrogen.
8. For long-term storage see **Method 3.1.1, Step 9**.

3.2. Preparation of PDMS micropatterns (Replica molding)

1. PDMS preparation is done using the Sylgard 184 Silicone Elastomer Kit (*see* Note 8).

[8]Research using protein micropatterning with microfluidic devices and micro-contact printing is carried out using polymers, most notably poly(dimethylsiloxane) (PDMS) (9–11, 21, 24, 25). Substrate microfabrication using polymers is easy, cost-effective, and for the most part can be carried out under typical laboratory conditions. Microchannels or more complex micropatterns can be reproduced with high fidelity in PDMS by replica molding. Liquid PDMS prepolymer is poured on the silicon master (**Method 3.1**) and cured by low heat activation (**Method 3.2**). The micropatterns contained in the cured PDMS replica can be then used to pattern proteins or cells on varied substrates, including flat PDMS, glass, or silicon by microfluidics (**Method 3.3**) or micro contact printing (**Method 3.4**). PDMS has several advantages as a material for fabricating micropatterning devices for wide range of applications, including molecular, cellular and developmental biology, microelectronics, and drug development (9–11, 20, 21, 24, 25). PDMS is elastomeric, a property that allows micro-scale features to be reproduced in it with high resolution and to conform to surfaces. Unlike glass, silicon, or hard plastics, PDMS forms reversible (van der Waals contact) to smooth surfaces. PDMS devices are easily mountable and demountable, without causing damage to the polymer itself, to the mold or to the substrate used for micropatterning. PDMS is compatible with water, and most polar organic solvents, and can be easily sterilized for cell-culture applications. The PDMS

2. Pre-heat oven to 65°C degrees.

3. Place the master molds from **Method 3.1** in the middle of a 150 mm diameter polystyrene culture dish (Fig. 1C).

4. Prepare the PDMS in a 100 ml disposable plastic container by thoroughly mixing 35 ml Sylgard 184 prepolymer with 3.5 ml curing agent (mass ratio of 10:1) for 2 min, until robust bubbling appears throughout the mixture. The resulting ~40 ml solution is sufficient to cover the master mold in a 150 mm diameter culture dish (**Method 3.2, Step 3**).

5. Use a stirring stick for mixing, as the mixture is too viscous for mixing by pipet or magnetic stirring. Remove the bubbles by degassing for 20 minutes.

6. Pour the unpolymerized PDMS over the master mold (Fig. 1C). Move the PDMS back and forth over the wafer using the stirring stick to allow full coverage. Pour the PDMS slowly to avoid the formation of new bubbles. If bubbles appear, set the mixture for 5 min before baking, to allow the bubbles to rise to the surface. It is essential to avoid bubbles in the PDMS microchannels because they will leave surface defects in the polymerized PDMS, which will interfere with the patterning. If bubbles persist on the mold and do not rise to the surface spontaneously, perform additional degassing for 10–20 min after pouring of the PDMS.

7. Place the master mold with PDMS in the oven, and cure at 65°C for 1 hour (*see* Note 9).

8. Remove the PDMS from the oven upon curing. Slowly release one edge of the cured PDMS using a scalpel blade. The rest of the PDMS can then be pulled gently from the master (*see* Note 10).

9. Using a clean razorblade, on a clean surface, cut the PDMS into rectangular shaped pieces (Fig. 1D), of about 8 mm in length and 2–3 mm in width. This size should fit onto a 12 mm diameter glass coverslip in a manner in which no part of the PDMS overhangs the edges of the coverslip (Fig. 2C). The orientation of the stripes will be parallel to the short edge of the PDMS rectangle (Fig. 2). This protocol describes preparation of stripe patterns for 12 mm diameter glass coverslips, pre-coated with poly-L-lysine (PLL), that are routinely used for neuronal

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Micropatterning devices can be used to pattern surfaces with proteins, cells, and other biomolecules using fluid flow or micro-contact printing. PDMS is nontoxic to proteins and cells, and mammalian cells can be cultured on it directly.

[9]Adjust the baking time to how your oven works and check on the PDMS samples continuously to be sure that bubbles in the PDMS are not rising if baking at too high a temperature. Alternatively, PDMS can dry on its own in about 24 hours at room temperature, and this might be a better solution to ensure that bubbles in the PDMS do not expand when baking at too high a temperature. If baking at 65°C, it is essential to keep the time to 1 hr. As PDMS polymerization is heat activated, the temperature and baking time will modulate the elasticity of the polymerized PDMS. Over-heating will result in stiff PDMS that will be difficult to pull from the wafer. Under-heating will result in partial polymerization and overly sticky PDMS in which the fidelity of the micropatterns will not be preserved.

[10]If the PDMS is cured correctly, and the PDMS release from the master mold is done neatly and slowly, the features of the micropatterns should be intact, and the master mold can be reused. If necessary, the master molds can be cleaned using acetone or isopropyl alcohol, using a lint free swab dipped in the acetone or isopropyl alcohol, by gentle wiping motions.

culturing (22, 23). The protocol can be adjusted to other size coverslips or material used as the culture substrate (*see* Note 1).

10. Clean and sterilize the PDMS micropatterns as follows: 1) Place the micropatterned PDMS pieces in a glass beaker and submerge in DI water. Rinse 3 times in DI water, 2) Replace the water with 70% ethanol and rinse 3 times in ethanol, 3) After the third ethanol wash, leave the ethanol in the container and place the glass beaker in a bath-sonicator. Sonicate in 70% ethanol for 15 min (**Materials 2.2, Step 8**). Cover the glass beaker while sonicating, 4) Replace the ethanol with DI water and rinse 3 times in DI water, and 5) Sonicate for 15 min in DI water. Cover the glass container while sonicating.

11. After the rinses and sonications, the PDMS pieces are sterile. Remove the glass beaker from the bath-sonicator, and place the covered container in a laminar tissue culture flow hood.

12. Discard the water and handle the PDMS pieces with sterile flat-head tweezers. Place the PDMS pieces in a sterile 100 mm culture dish, spaced approximately 5–7 mm apart, making sure the patterned side does not face down on the dish (Fig. 1D). Allow the PDMS to air-dry for 2 hours inside the laminar tissue culture flow hood. The PDMS is now ready for stripe-patterning, and can be stored in a sterile closed dish for up to 1–2 months (*see* Note 11).

3.3. Substrate microfluidic patterning

From **Method 3.2, Step 11** onward, carry out all procedures in a laminar tissue culture flow hood, unless otherwise specified.

1. Prepare PLL as in **Materials 2.3, Step 3**.

2. Using sterile tweezers, place four 12mm diameter glass coverslips in a 35 mm culture dish, making sure the coverslips are well spaced from one another (Fig. 2C). Add 2 ml of PLL, and incubate for 4 hr, ensuring the coverslips are submerged in the PLL solution. Following the coating with PLL, rinse the coverslips 3 times with sterile DI water, and allow them to air-dry in a laminar tissue culture flow hood (*see* Note 12).

3. Using sterile flat-head tweezers, pick-up a single PDMS piece. To identify the patterned side, using a sterile 20–200 µl tip, gently brush the edge of the tip lengthwise across the surface of the PDMS, slightly angled. The patterned side will vibrate significantly more (Fig. 2A,B).

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[11] Storage longer than 1–2 months of the PDMS should be avoided because: *1)* it might be difficult to maintain sterile and dust free conditions for long time periods, and *2)* in the polymerized PDMS there may be traces of un-polymerized monomers which over time may alter the elasticity of the polymer. As a result, old PDMS might not conform to the glass surface used for patterning as well as fresh PDMS.

[12] We recommend using filtered DI water to minimize the presence of particulate contaminants and to ensure optimal conditions for micropatterning.

4. Place a single PDMS piece on each glass coverslip intended for neuronal culturing, with the patterned side down on the coverslip (Fig. 2C).

5. Allow the PDMS first to conform naturally to the glass coverslip, while applying only minimal force with the edge of a tip or the flat-head tweezers. Application of excess force on the PDMS will result in flattening of the patterns against the coverslip causing distortion of the microchannels. The PDMS will reversibly seal on PLL-coated glass coverslip, and microchannels formed between the PDMS and the coverslip will be now used for flow patterning of the protein of interest.

6. Prepare 3–5 μl of the stripe patterning solution (with the appropriate protein present such as Sema3A) for each coverslip intended for patterning (**Materials 2.3, Step 5**) (*see* Note 7).

7. Overlay 3 μl of the stripe patterning solution on one side of the PDMS (the wide side so that the solution is aligned to flow in the channels) (Fig. 2C and Fig. 3A,B). The solution should naturally flow into the channels by capillary action, and the flow should be easily visible with the naked eye. If after 1–2 min the fluid has not filled all the channels, suction can be applied on the opposite side of the PDMS from the side of the solution application to aid the capillary flow (Fig. 3A,C). This will force the fluid to flow through the channels. Apply suction by using a sterile glass pipet directly connected to a vacuum outlet in the laminar flow hood, by gently applying the tip of the glass pipette on the PDMS. Make sure not to lift the PDMS from the coverslip while applying suction (Fig. 3A,C).

8. While leaving the PDMS on the glass coverslip, let the solution dry in the channels for 6 hr in the laminar tissue culture flow hood.

9. Using sterile flat-head tweezers, gently remove the PDMS from the coverslip.

10. Rinse the patterned coverslips 3 times with sterile PBS.

11. The patterned coverslips are now ready for plating of dissociated neurons. The fluorescent stripes on the coverslips can be visualized by microscopy (Fig. 5) (*see* Note 13).

12. To review common problems with stripe patterning using microfluidics (**Method 3.3**), *see* Note 14, 15, 16, 17 and 18. Some of these problems will reinforce the basic principles of the procedures detailed here,

[13]We used the same protocol for stripe patterning of the membrane permeable fluorescent analogues of cAMP and cGMP, downstream effectors of Sema3A (7, 16). Membrane permeable fluorescent analogues of cAMP and cGMP are available from several commercial vendors, with large spectrum of emission wavelength, from the UV to the far infrared, for compatibility and convenience with specific needs of immunohistochemistry. In our studies we used: membrane permeable fluorescently conjugated analogues of cAMP and cGMP (F-cAMP/F-cGMP): Alexa Fluor-conjugated 8-[6-aminohexyl] aminoadenosine 3′,5′-cyclic monophosphate (F-cAMP) (Invitrogen Corporation, Carlsbad, CA); (8-[[2-[(fluoresceinylthioureido)amino]ethyl]thio] guanosine-3′,5′-cyclic monophosphate (8-Fluo-cGMP (F-cGMP) (BIOLOG). F-cAMP and F-cGMP were used at a final concentration of 2 nM for stripe patterning.

[14]The flow of the stripe patterning solution appeared to stop in the microchannels when using microfluidics, resulting in partially patterned protein stripe (Fig. 7A).

especially the importance of proper contact between the PDMS and the glass substrate, and the composition of the stripe patterning solution, to allow optimal flow and adsorption. Other problems pertain to the formulation of the protein of interest itself, specifically the proper concentration used for patterning and its water solubility, that will need to be determined experimentally.

3.4. Protein micro-contact printing (stamping)

The same PDMS preparation can be used for stripe patterning by inking the PDMS with protein solution and printing the pattern on a glass coverslip. The same stripe patterning solutions used for microfluidic stripe patterning can also be used for stripe micro-contact printing.

1. Prepare the PDMS and cut the rectangular PDMS pieces as in **Method 3.2**. While pouring and curing the PDMS, make sure the patterned surface is flat and well leveled. As optimal micro-contact printing requires application of steady force using a "stamper" device (**Materials 2.4, Step 1;** Fig. 4), an un-leveled patterned surface could result in tilting of the "stamper" and smearing of the stripe patterning solution inked on the PDMS, causing defects in stripe patterning. For micro-contact printing,

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Stop of flow might occur predominantly because of interfacial tension between the patterning solution and the PDMS or the glass substrate. Increasing the BSA concentration in the patterning solution will help decreasing the interfacial tension. However, if the BSA concentration is too high, the channels may become clogged causing partial microchannel filling. It is therefore crucial to optimize the BSA concentration in the stripe patterning solution to achieve optimal protein flow and adsorption. See **Materials 2.3, Step 5** for recommendation for optimal BSA concentration. The recommended BSA concentration may be diluted or increased by approximately a factor of two for optimal stripe patterning.

[15]The patterning solution leaked to the gaps between the channels, resulting in even rather than stripe-patterned restricted coating of the protein on the glass (Fig. 7B).

This might be a sign that the PDMS conformed to the glass poorly. Following few simple rules with the PLL-coating and subsequent washes of the glass coverslip, and cleaning of the PDMS, should ensure optimal PDMS-glass contact for stripe patterning. *a)* Make sure the glass coverslips are free of particulate contaminants before PLL coating. After PLL-coating, wash the glass coverslips thoroughly with DI water. Insufficient washes of the PLL might result in PLL aggregates that will prevent uniform conformation of the PDMS to the coverslip, resulting in leakage of the patterning solution outside the channels while flowing. It is recommended to use filtered DI water for PLL washes to minimize presence of particulate contaminants on the glass coverslip (*see* Note 12). *b)* Cleaning the PDMS thoroughly as in **Method 3.2, Step 10**, should ensure optimal contact between the PDMS and the glass coverslip. It is also recommended to avoid using PDMS older than 1–2 months (*see* Note 11) (also *see* Note 6).

[16]Poor adsorption of the protein to the glass (Fig. 7C). As the stripes are patterned together with fluorescently-conjugated BSA, the fluorescence intensity should be a good indication for the adsorption of the protein of interest to the glass. Weak fluorescence or stripes with poorly defined edges is usually an indication of inadequate drying (**Method 3.3, Step 8**). Doubling the drying time should resolve this issue.

A considerably more problematic issue might occur when a non-aqueous solvent (ethanol, isopropanol, or DMSO) is used to suspend the protein of interest, or when patterning non-protein molecules such as membrane permeable analogs of cAMP and cGMP, or inhibitors of these signaling pathways (16). In this case, the stripe patterning solution might adsorb more strongly to the PDMS rather than to the glass coverslip. The edges of the stripes will appear sharp and well defined, but the fluorescence intensity of the stripes will be weak, indicating poor adsorption to the glass coverslip. If indeed a non-aqueous solvent is essential for the protein resuspension, then multiple flowing of the patterning solution might resolve this issue. Make sure to *a)* keep the PDMS on the glass coverslip in between the sequential applications, and *b)* allow drying of the patterning solution after each application.

[17]Patchy and non-uniform stripes in continuously filled channels (Fig. 7D). This might be an additional problem when using a non-aqueous solvent to pattern proteins or other molecules (16). Because these molecules might be poorly adsorbed to the glass coverslip, while drying they might aggregate, resulting in a patchy pattern. Decreasing the concentration of the patterning protein or molecule will resolve this aggregation, but might result in a reduced overall fluorescence intensity, which will require multiple applications (*see* Note 16).

[18]When observed under the microscope immediately after flowing (**Method 3.3**) or micro-contact printing (**Method 3.4**), the stripes appear complete in length and uniform with strong fluorescence intensity. However, when placed in culture media, stripe intensity becomes considerably decreased. This might be an indication of insufficient drying and poor adsorption of the protein to the glass, resulting in its redissolution in the culture media. Although some protein redissolution is inevitable, increasing the drying time following flow or micro-contact printing should minimize this problem.

thicker PDMS is required than for microfluidic patterning. We found a thickness of 5 mm to be optimal. This ensures proper stability of the patterned surface and minimizes tilting of the stamper for optimal micro-contact printing. Pour the PDMS (as in **Method 3.2**) so that the thickness of the polymerized PDMS is about 5 mm.

2. Using clean flat-head tweezers, pick up the PDMS and identify the patterned side as in **Method 3.3, Step 3** (Fig. 2). Place the PDMS pieces in a clean 100 mm culture dish with the patterned side facing up.

3. Plasma oxidize the PDMS in the 100 mm culture dish, as follows (*see* Note 19). Remove the lid of the culture dish that contains the PDMS with the patterned side facing up. Place the dish in a Harrick Plasma cleaner, seal the door tightly and apply vacuum. Allow the pressure to stabilize for 1 min, and turn on the plasma at high power for 2 min. Turn off the power and turn off the vacuum pump. Slowly leak air into the chamber until the door can be opened (*see* Note 20).

4. Using clean flat- head tweezers, carefully remove the PDMS from the culture dish and place them pattern-side up in a clean and dry glass beaker. Gently press down the PDMS to the bottom of the beaker, so they are firmly attached to the bottom.

5. Add 70% ethanol to the glass beaker until the PDMS pieces are fully submerged. Cover the glass beaker. Place the glass beaker in a bath-sonicator, and perform the sonications and subsequent washes as in **Method 3.2**, **Step 10**. Ensure the PDMS are submerged in DI water, so that they do not regain hydrophobicity (*see* Note 20).

6. After the rinses and sonications, the PDMS pieces are sterile. Remove the glass container from the bath-sonicator, and keeping the container covered, place it in a laminar tissue culture flow hood. Quickly discard the water, and handle the PDMS pieces with sterile flat-head tweezers. Place the PDMS on a sterile (previously autoclaved) cotton-wipe to quickly dry the PDMS.

7. Sterilize the stampers (**Materials 2.4, Step 1**) by autoclaving and store in a sterile container in the laminar tissue culture flow hood (Fig. 4B, C).

8. Place the PDMS pattern facing up in a sterile 100 mm culture dish.

9. Prepare 50 μl stripe patterning solution for each coverslip intended for patterning, as in **Materials 2.3, Step 5**. Overlay 50 μl of stripe patterning

[19]The surface of PDMS is hydrophobic as it contains repeating units of O-Si$(CH_3)_2$ groups. The PDMS surface can be made hydrophilic by exposing it to oxygen or air plasma that disrupts methyl groups (Si–CH_3) and introduces silanol (Si–OH) groups. Plasma-oxidized PDMS can be easily wetted by aqueous solutions, as the protein printing solutions used here. We found that Plasma-oxidized PDMS is optimal for glass coverslip micro-contact printing. As microfluidic patterning (**Method 3.3**) requires a reversible seal between the PDMS and the glass coverslip, Plasma-oxidized PDMS is not to be used for this application.

[20]After a while, on standing, the hydrophilic, oxidized PDMS surface will become hydrophobic again, as the surface reconstructs and as non-crosslinked components of the prepolymer rise to the surface. Plasma treated PDMS can be kept hydrophilic by keeping the PDMS well submerged in water.

solution on the patterned side of the PDMS. Spread the drop evenly across the entire surface of the PDMS with a pipet tip. Incubate for 10 min to allow inking of the PDMS (Fig. 4A).

10. Remove most of the solution from the PDMS via pipetting or gentle aspiration.

11. Dry the PDMS with a gentle stream of nitrogen gas for about 1 min. For optimal micro-contact printing, the PDMS inking needs to occur only on the ridges in the micropattern (Figs. 2A and 4A). The drying of the inked PDMS ensures that there is minimal solution present in the microchannels, and this avoids smearing of the pattern upon contact-printing on the glass coverslip (*see* Note 15).

12. Using sterile flat-head tweezers, flip the PDMS over a PLL-coated glass coverslip pattern facing down on the coverslip (Fig. 4).

13. Gently place the sterile stamper on top of the PDMS and leave for 5–10 min to allow printing of the stripe patterning solution on the glass coverslip (Fig. 4C, D). The time of the micro-contact printing should be optimized experimentally.

14. Remove the stamper and gently and gradually lift the PDMS from the glass coverslip using sterile flat-head tweezers.

15. Allow the micro-contact printed glass coverslip to dry for 6 hr in the laminar tissue culture flow hood covered from light.

16. Rinse the patterned coverslips 3 times with sterile PBS. The micro-contact printed stripe-patterned coverslips are now ready for plating of dissociated neurons. The fluorescent stripes on the coverslips can be visualized by microscopy.

17. To review common problems with stripe patterning using micro-contact printing (**Method 3.4**), *see* Note 15, 16, 17 and 18. (See also **Method 3.3, Step 12**).

3.5. Sequential substrate micro-contact printing and microfluidic patterning for generating alternating stripe patterns of two distinct proteins (‘print then flow’)

Using a sequential micro-contact printing followed by microfluidic patterning, we now describe stripe patterning with two alternating stripes of two different proteins, each patterned together with fluorescently-conjugated BSA at different emission wavelengths (Fig. 6).

1. Prepare the stripe patterning solution for the two proteins of interest in PBS, together with fluorescently-conjugated BSA at two different emission wavelengths, as in **Materials 2.3, Step 5**.

2. Stripe by micro-contact printing the first protein on the glass coverslip, as in **Method 3.4**.

3. Allow to incubate for 30 min, while keeping the PDMS on the glass coverslip.

4. Following the micro-contact printing, flow the second protein solution through the PDMS microchannels as in **Method 3.3**, while keeping the PDMS on the glass coverslip. The first protein is patterned by micro-contact printing utilizing inking of the ridges in the PDMS micropattern (Fig. 2A). Then, the second protein is patterned using the channels in the PDMS micropattern (Fig. 2A), allowing alternate stripe patterning of two proteins on the same glass coverslip.

5. Allow to dry for 6 hr, and then lift the PDMS from the glass coverslip, and proceed as in **Method 3.3, Step 9.**

6. Two alternating fluorescent stripes can be visualized by microscopy throughout the glass coverslip (Fig. 6). Following washes as in **Method 3.3, Step 10**, the patterned coverslips are now ready for plating of dissociated neurons.

7. Quantification of the deposited protein on the stripes, can be done directly using ellipsometry (8), or indirectly by fluorescence measurements. For neuronal culturing protocols and analysis of axon/dendrite polarization on stripes, see the following studies (7, 13, 14, 16, 22, 23).

Acknowledgments

This work was supported by a grant from NIH, NS084111 (M. Shelly), and by grants from the Deutsche Forschungsgemeinschaft, FZ 111 (S. Pautot). Research was carried out in part at the Center for Functional Nanomaterials, Brookhaven National Laboratory, which is supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under Contract No. DE-SC0012704.

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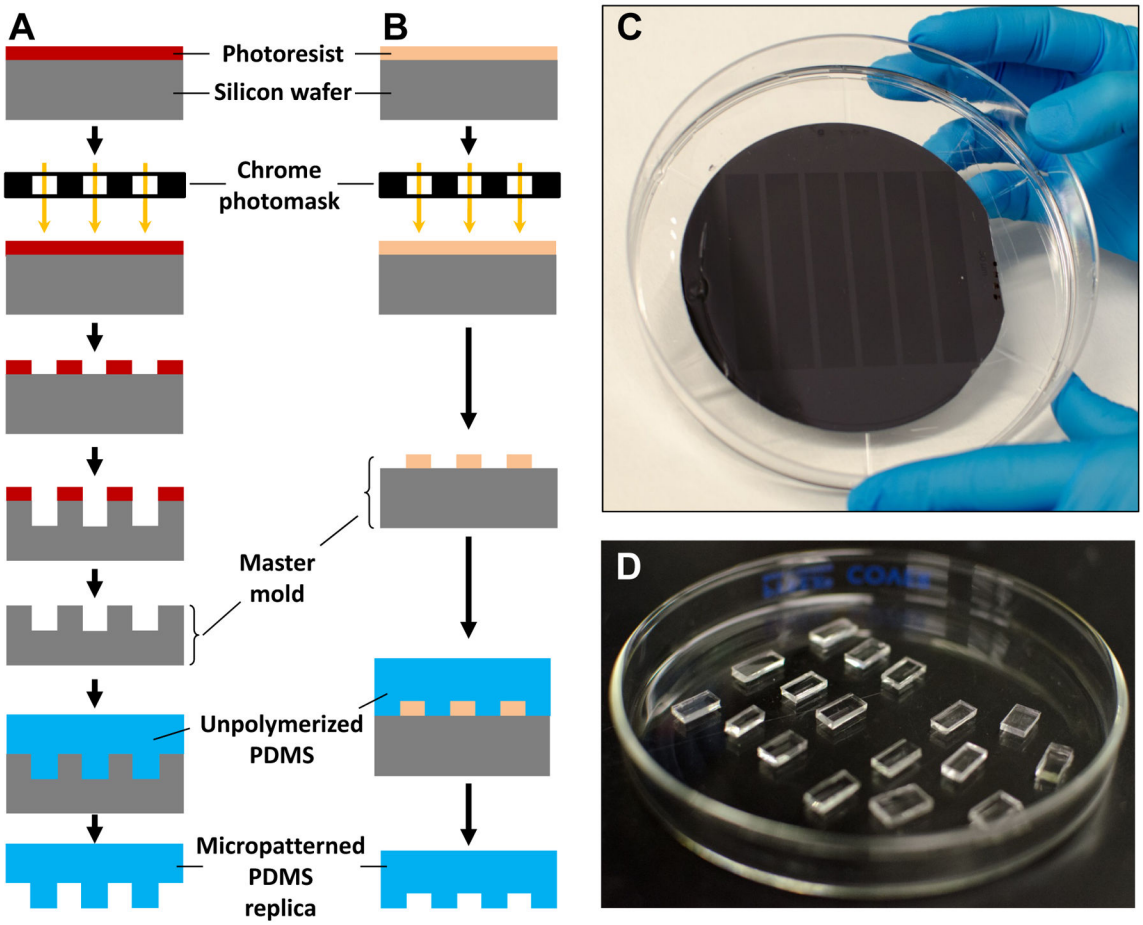

Figure 1. Generation of silicon-based master molds for stripe patterning

(A) Schematic depiction of the generation of silicon-based master mold using dry etching (Subtractive Lithography). Following the deposition of S1811 positive photoresist onto the silicon wafer, a UV mask aligner is used to transfer stripe micropatterns (50 μm wide stripes spaced 50 μm apart) from the chrome photomask onto the photoresist. A deep reactive-ion etch (DRIE) is then performed to achieve the desired depth of microchannels (3–5 μm deep channels). The remaining unexposed photoresist is removed, to generate the master mold for PDMS replica.

(B) Schematic depiction of the generation of silicon-based master mold using SU-8 Additive Lithography. Following the deposition of SU-8-2 negative photoresist onto the silicon wafer, a UV mask aligner is used to transfer stripe micropatterns (50 μm wide stripes spaced 50 μm apart) from the chrome photomask onto the photoresist, to generate the master mold for PDMS replica. Following the generation of the master molds (A, B), unpolymerized PDMS is poured over the master mold, and cured by baking, to generate the micropatterned PDMS replica.

(C) Illustration photo of a silicon-based master mold placed in a 150 mm dish prior to pouring and curing of unpolymerized PDMS.

(D) Illustration photo of rectangular shaped PDMS replica pieces of about 8mm in length and 2–3mm in width, ready for protein stripe micropatterning on 12 mm diameter glass coverslips.

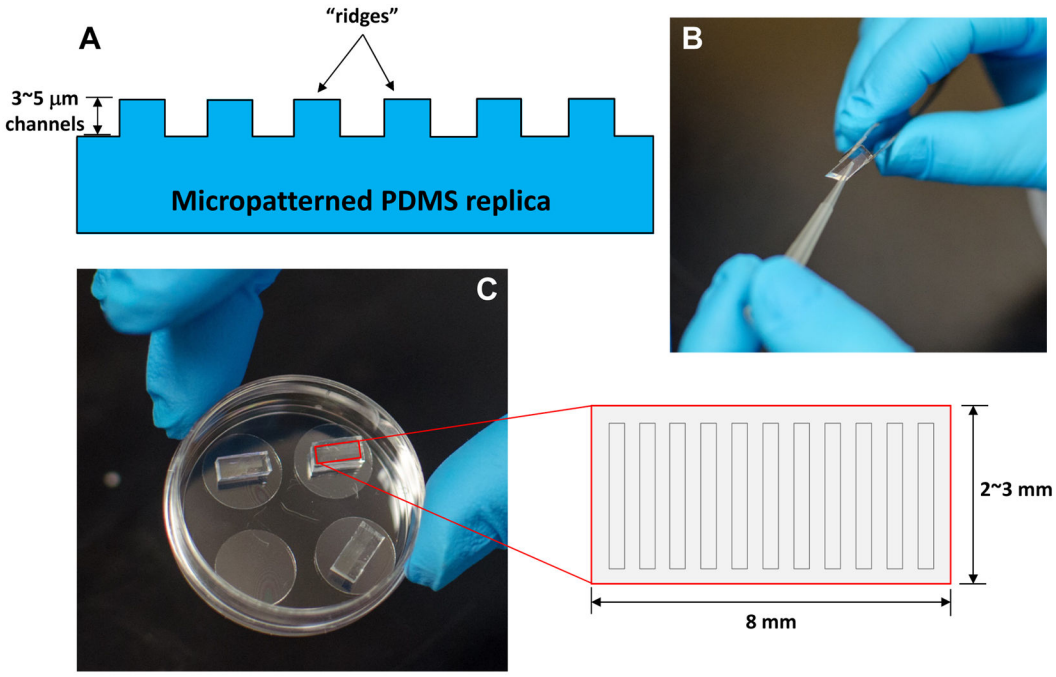

Figure 2. Preparation of PDMS micropatterns (Replica molding) for stripe micropatterning

(A) Schematic depiction of PDMS replica micropatterned with 50 µm wide stripes spaced 50 µm apart, with microchannels' 3–5 µm deep. We found this micropattern design to be optimal for the examination and manipulation of early stages of cultured rodent hippocampal neuronal development. Patterning by micro-contact printing is performed utilizing inking of the ridges in the PDMS. Microfluidic stripe patterning is performed by flowing the stripe patterning solution into the microchannels of the PDMS replica.

(B) Illustration photo for identifying the patterned side in the PDMS replica. Using a sterile 20–200 µl tip, the edge of the tip is brushed gently lengthwise across the surface of the PDMS, slightly angled. The patterned side will vibrate significantly more.

(C) Illustration photo and schematic depiction of PDMS replica cut into rectangular shaped pieces of about 8 mm in length and 2–3 mm in width. This size is optimal to fit onto the center of a 12 mm diameter glass coverslip, pre-coated with poly-L-lysine (PLL), that is routinely used for neuronal culturing. This dimension of the PDMS rectangular shaped piece is optimal and ensures that when positioned in the center of the glass coverslip, the entire piece is at least 2–3 mm away from the edges of the coverslip, for optimal coating. The orientation of the stripes is parallel to the short edge of the PDMS rectangle.

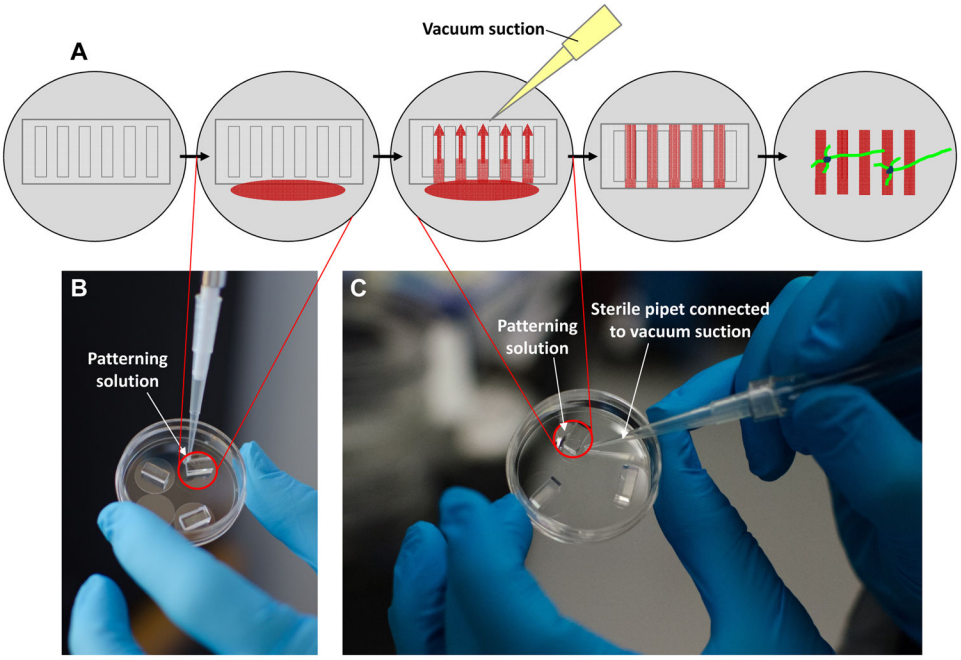

Figure 3. Substrate microfluidic stripe patterning

(A) Schematic depiction of stripe micropatterning of glass coverslips using microfluidics. A single PDMS piece is reversibly sealed on a PLL-coated 12 mm diameter glass coverslip, with the patterned side down on the coverslip. The stripe patterning solution is overlayed on one side of the PDMS, the wide side so that the solution is aligned to flow in the channels. The solution should naturally flow into the channels by capillary action, which is visible to the naked eye. After 1–2 min, if the fluid has not filled all the channels, suction can be applied on the opposite side of the PDMS from the side of the solution application to force the fluid through the channels. Following drying of the stripe patterning solution, the PDMS is removed from the coverslip. The fluorescent stripes on the coverslips can be visualized by microscopy, and the patterned coverslips are now ready for plating of dissociated neurons.

(B) Illustration photo for stripe patterning solution application on one side of the PDMS, the wide side so that the solution is aligned to flow in the channels. The solution should naturally flow into the channels by capillary action.

(C) Illustration photo for application of suction to force the fluid through the channels. Suction is applied by using a sterile glass pipet directly connected to a vacuum outlet in the laminar flow hood, by gently applying the tip of the glass pipette on the opposite side of the PDMS from the side of the solution application.

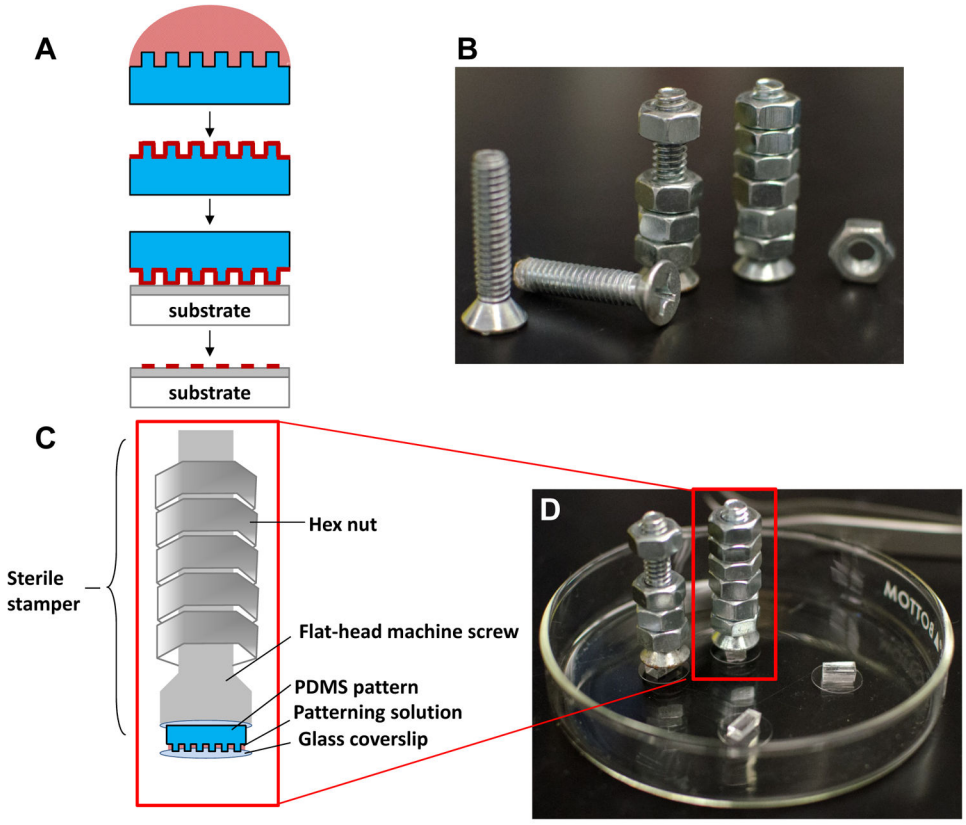

Figure 4. Substrate micro-contact printing

(A) Schematic depiction of stripe micropatterning of glass coverslips using micro-contact printing. Following plasma oxidization of the PDMS replica, stripe patterning solution is overlayed on the patterned side of the PDMS, and spread evenly across the entire surface of the PDMS to allow inking of the PDMS. After incubation and drying of the protein patterning solution on the PDMS, the PDMS is placed on a PLL-coated glass coverslip, pattern facing down on the coverslip. For optimal printing of the glass coverslip, a "stamper" (see B,C) is placed on the PDMS. Upon removal of the PDMS and subsequent drying, the micro-contact printed stripe-patterned coverslips could be visualized by microscopy, and the coverslips are ready for plating of dissociated neurons.

(B) Illustration photo of steel flat-head machine screws (1/4″–20 × 1–1/2″; 17 g in weight), used to generate the "stamper" for the micro-contact printing. The "stamper" can be optimized by slightly varying its weight by adding 1/4″–20 Hex nuts, 1–2 gr each. To achieve a flat surface, a 12 mm glass coverslip is glued to the flat-head of the screw.

(C) Schematic depiction of the "stamper" for optimal micro-contact printing. Upon placing the stripe patterning solution inked PDMS on the glass-coverslip, for optimal printing of the glass coverslip, a "stamper" is placed on the PDMS. The stamper is composed of steel flat-head machine screws optimized by slightly varying their weight by adding 1/4″-20 Hex nuts (see B).

(D) Illustration photo of a "stamper" placed on stripe patterning solution inked PDMS on the glass-coverslip, for optimal micro-contact printing of the glass coverslip.

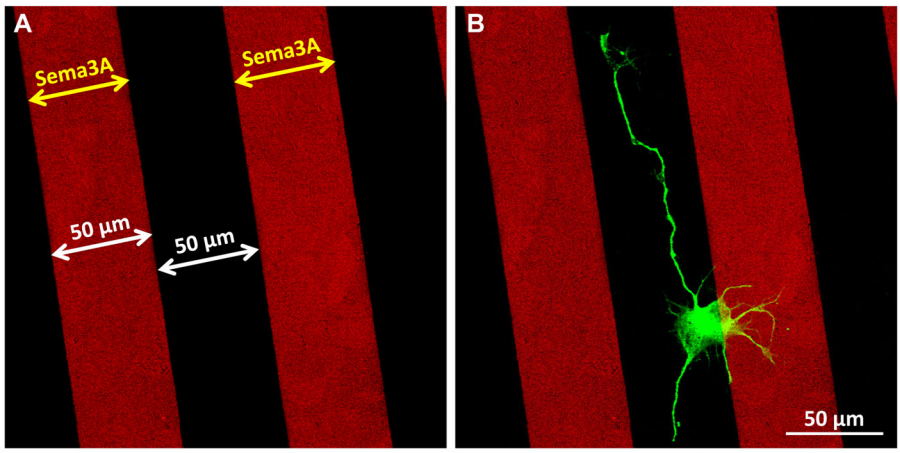

Figure 5. Substrate stripe-patterning of Sema3A

(A,B) Images of glass-substrates coated with stripes (red) of Sema3A, 50 µm wide stripes spaced 50 µm apart, generated by microfluidic flow of Sema3A patterning solution (A,B). Images taken at 60 hr after plating of dissociated rat E18 embryonic hippocampal neurons and immunostaining (at 60 hr) for Tuj-1, a neuron-specific class III β-tubulin (green) (B). Note the preferential axon initiation and guidance on an *off-stripe* region, and preferential dendrite initiation and guidance on the Sema3A stripe (B). Scale is 50 µm.

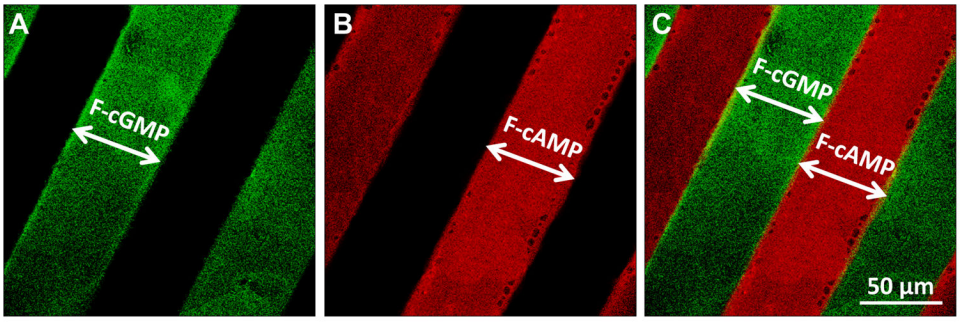

Figure 6. Sequential micro-contact printing and microfluidic patterning for generating alternating stripes of membrane permeable fluorescent analogues of cAMP and cGMP

(A–C) Images of glass-substrates coated with stripes of F-cGMP (green) (A) and F-cAMP (red) (B), 50 µm wide stripes spaced 50 µm apart, generated by micro-contact printing of F-cGMP (A), followed by microfludic flow of F-cAMP (B). Two alternating fluorescent stripes can be visualized by microscopy throughout the glass coverslip (C). Scale is 50 µm.

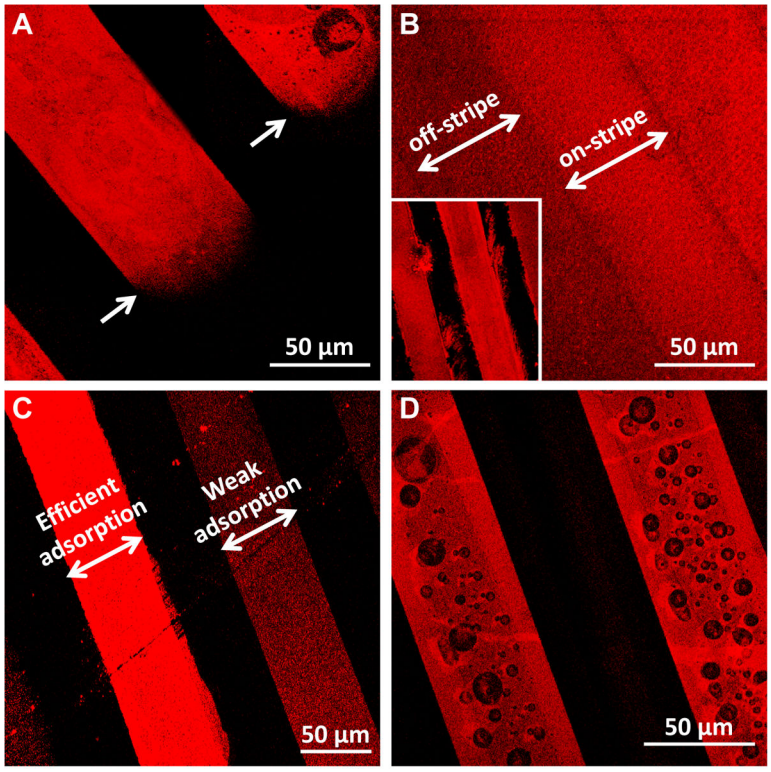

Figure 7. Common problems with stripe patterning using microfluidics and micro-contact printing

Images of glass-substrates coated with stripes (red) of Sema3A, 50 µm wide stripes spaced 50 µm apart, illustrating common problems with stripe patterning using microfluidics and micro-contact printing (A–D).

(A) The flow of the stripe patterning solution appeared to stop in the microchannels (arrows) when using microfluidics, resulting in a partially patterned protein stripe. Scale is 50 µm.

(B) The patterning solution leaked to the gaps between the channels, resulting in even rather than stripe-patterned restricted coating of the protein on the glass. Inset, (left, bottom) depicts a less severe leakage of the patterning solution away from the channels. Scale is 50 µm.

(C) Poor adsorption of the protein to the glass, as visualized by weak fluorescence intensity. Scale is 50 µm.

(D) Patchy and non-uniform stripes in continuously filled channels. Scale is 50 µm.