Microcontact printing of axon guidance molecules for generation of graded patterns

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Microcontact printing (μCP) of proteins has been successfully used for patterning surfaces in various contexts. Here we describe a simple 'lift-off' method to print precise patterns of axon guidance molecules, which are used as substrate for growing chick retinal ganglion cell (RGC) axons. Briefly, the etched pattern of a silicon master is transferred to a protein-coated silicone cuboid (made from polydimethylsiloxane, PDMS), which is then used as a stamp on a glass coverslip. RGC explants are placed adjacent to the pattern and cultured overnight. Fluorescent labeling of the printed proteins allows the quantitative analysis of the interaction of axons and growth cones with single protein dots and of the overall outgrowth and guidance rate in variously designed patterns. Patterned substrates can be produced in 3–4 h and are stable for up to one week at 4 °C; the entire protocol can be completed in 3 d.

INTRODUCTION

Axon guidance molecules have been extensively studied *in vitro* in diffusible gradients[1–3]. Diffusible gradients, however, are difficult to maintain over a long time, their shape cannot arbitrarily be preassigned and they may not be suitable for studying membrane- or matrix-bound proteins.

There have been several attempts to produce substrate-bound gradients of axon guidance molecules.

Rosentreter and colleagues manufactured gradients of cell membrane fragments by sucking the material onto a filter[4]. This method, however, is not applicable for producing gradients of single, purified proteins.

Adams and colleagues produced gradients of a laminin peptide by photo-immobilization[5]. To do so, the peptide has to be modified with a photolinker and each gradient has to be written with a laser beam. The resulting gradients are continuous, but lack an accurate and reproducible geometry.

There are also reports about concentration gradients established in three-dimensional gels, which are comparatively stable for several hours[6,7]. To obtain durable and long-lasting graded distributions of surface-bound guidance molecules, μCP proved to be an useful technique.

μCP has been applied in several variants to generate patterned substrates for cell culture[8–15].

Here we describe a technique that generates stable and geometrically precise gradients of axon guidance molecules by μCP. In brief, a silicon master with a desired pattern comprising dots and lines with dimensions down to 0.3 μm is used as a 'printing plate'. Using two alternative methods, an unlimited number of reproducible substrates made with various proteins can be fabricated (**Fig. 1**). By the lift-off method, small protein-coated cuboids of PDMS elastomer are placed consecutively onto the master and a glass coverslip, thus transferring a protein print of the master to the glass surface. Alternatively, a reusable silicone elastomer cast may be made from the master. The cast is covered with protein solution ('printing ink'), dried and stamped onto a glass coverslip.

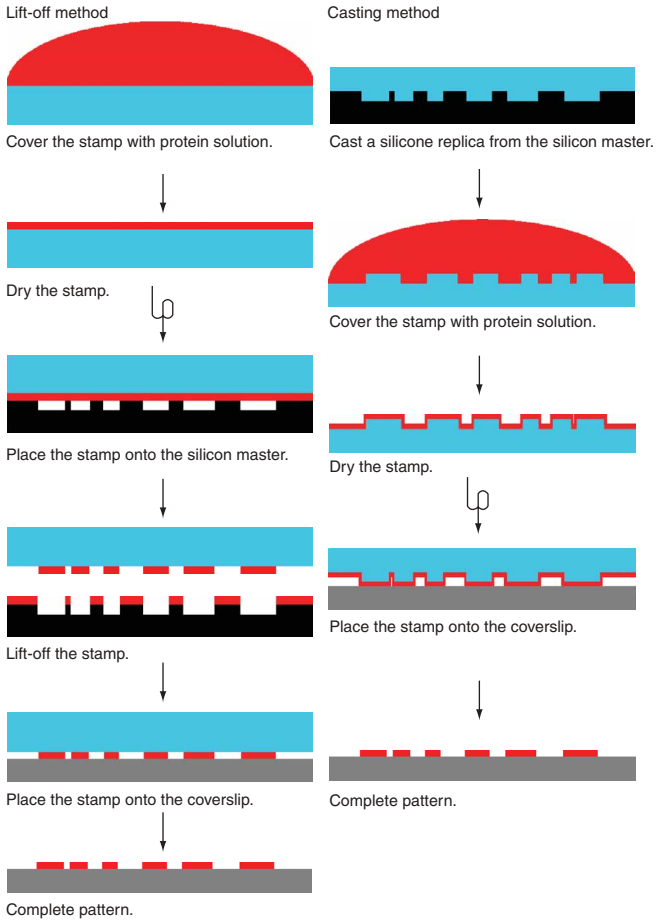

Figure 1 | Two different methods to generate a protein pattern (red) onto a coverslip (gray) from a silicon master (black) using a stamp (blue) made from PDMS elastomer.

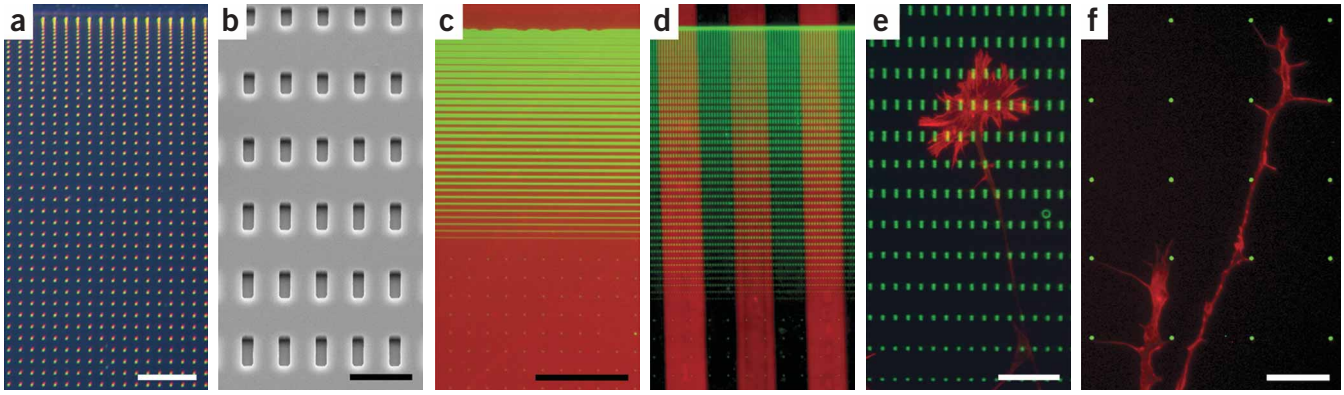

Figure 2 | Silicon masters and fluorescent protein patterns. (**a**) A silicon master with a graded pattern built up by dots seen with the light microscope. (**b**) Dots etched into a silicon master (approximately 650 nm deep) seen with the scanning electron microscope. (**c**,**d**) Graded protein patterns seen with the fluorescence microscope . The printed protein (ephrinA5) is shown in green, laminin covering the pattern is shown in red. In **d**, laminin was applied in stripes. (**e**,**f**) Growth cones navigating in protein patterns. The protein (ephrinA5) is shown in green, phalloidin-stained axonal actin in red. Scale bars: **a**,**c**,**d**, 100 μm; **b**, 5 μm; **e**,**f**, 15 μm.

The lift-off method offers a larger freedom of design in terms of pattern geometry and aspect ratio, because the structures are defined in the harder material (silicon) instead of the soft polymer. Furthermore, the lift-off method produces patterns that are less likely to be defective, but it requires the cleaning of the silicon master after each stamping process. Ideally, the master is cleaned in an oxygen plasma cleaner that ashes away all organic material, such as proteins.

The casting method, on the other hand, may be preferable in labs not provided with an oxygen plasma cleaner. Multiple PDMS stamps can be cast from a single master and used simultaneously, facilitating the fast fabrication of many printed substrates.

Patterned substrates produced by either of the two above methods are biologically active for many proteins. After coating with laminin, embryonic chick retinal ganglion cell axons are grown overnight on the substrates and evaluated for specific responses to the pattern. Here we describe the culture of retinal explants on the microcontact printed substrates. However, other neural and non-neural cells may also be grown on the substrates, which could serve as a potent assay not only for axonal guidance but also for directed cell migration. Printed patterns in the submicron range produced by the method presented here might be also useful to study the function of synaptogenic molecules.

Gradients produced by μCP are discontinuous, that is, the spacing and size of the printed protein dots are varied in a way that the area covered by protein increases along the axis of the gradient (**Fig. 2**). Our results suggest that neuronal growth cones can integrate such discontinuous distributions of guidance molecules. In contrast to diffusible gradients in solution, gradients of membrane and/or matrix-bound guidance molecules are likely to be discontinuous *in vivo*[16]. Moreover, discontinuous patterns allow analysis of how the growth cone interacts with subcellular sized spots of guidance molecules.

We previously found that the fluorescence intensity of stamped labeled ephrinA5 increases roughly linearly with the protein concentration of the solution used to coat the PDMS stamps ('printing ink')[16]. The amount of protein transferred to the coverslip also depends on the incubation time of the printing ink on the stamp.

Laminin adsorbed from solution is used to fill in the spaces not yet covered in the printed pattern in order to act as a growth-permissive molecule. Fluorescence staining reveals that less laminin adheres to the surface already covered with the stamped protein (**Fig. 2c,d**). Control patterns printed with Fc (fragment constant of immunoglobulin) show, however, that the reduction of laminin coverage in the printed pattern does not affect axonal growth and guidance per se. Using special matrices to apply laminin in stripes overlaying the printed pattern provides a simple method to restrict axons to these stripes and to ensure straight outgrowth in a defined angle relative to the printed pattern (**Fig. 2d**).

Most proteins retain their biological activity during the stamping process[17], although they might undergo conformational changes[18]. There are exceptions, however, such as fibronectin (**Box 1**). Proteins that lose their biological activity when stamped via the method described here may still be successfully patterned by related methods such as using a gold surface by a technique employing self-assembled monolayers (SAM) of alkanthiolates[13,19,20]. These methods have the advantage that the protein is adsorbed to the substrate from a physiological solution. By contrast, areas not covered with protein after the first adsorption step are hydrophobic and prevent protein binding. Therefore, they cannot be filled with a second protein.

MATERIALS

REAGENTS

- Polydimethylsiloxane, PDMS (Elastosil RT 625A/B, Wacker Silicones, cat. no. 9101374869) or Sylgard 184 (Dow Corning, cat. no. 240-01673921)
- Ethanol > 99.8% p.a. (Roth, cat. no. 9065.4)
- Aceton > 99.5% p.a. (Roth, cat. no. 5025.2) **! CAUTION** Toxic.
- Millipore water (ddH_2O), sterile
- Nitrogen gas (with a pressure-adjustable valve)
- PBS: Dulbecco without Ca2+, Mg2+ (Biochrom AG, cat. no. L182-50)
- Embryonic chick retinal explants, E6–7
- Trichloro(octadecyl)silane, for casting only (Aldrich, cat. no. 104817) **! CAUTION** Toxic, explosive.
- n-Heptane > 99% p.a., for casting only (Roth, cat. no. T174.1) **! CAUTION** Toxic, flammable.
- Transparent adhesive tape, for casting only (Tesa)

BOX 1 | WHICH PROTEINS CAN BE STAMPED?

We successfully stamped several commercially available recombinant proteins as well as antibodies and a polyclonal serum at various concentrations, obtaining patterns that were bioactive for chick retinal ganglion axons.

Successfully printed proteins include: recombinant human ephrin-A5/Fc chimera (R&D systems, cat. no. 374-EA), recombinant mouse ephrin-A2/Fc chimera (R&D systems, cat. no. 603-A2), recombinant mouse ephrin-B1/Fc chimera (R&D systems, cat. no. 473-EB), recombinant mouse Eph-A3/Fc chimera (R&D systems, cat. no. 640-A3), laminin (Invitrogen, cat. no. 23017-015) and vitronectin (Sigma, cat. no. V 8379).

We found fibronectin (Sigma, cat. no. F 2006) to lose activity during the stamping process.

Protein concentrations ranged from 0.5–60 μg ml^{-1} in either 1× PBS or 1× PBS + 0.1% BSA. BSA carrier protein may stabilize the protein in solution. It should be noted, however, that BSA present in the stamping ink adsorbs to the stamp and thus reduces the adsorption of other proteins in the solution. To compare two different concentrations of the protein of interest, we took care to maintain a constant absolute protein concentration in the printing ink by adding accordingly inactive or unspecific protein such as Fc. Because molecular clustering is crucial for ephrin signaling, we clustered the recombinant ephrin protein with fluorescent Fc antibody (Alexa Fluor 594 goat anti-human IgG, Molecular Probes, cat. no. A11014) at a ratio of 1:3 for 30 min at room temperature under gentle agitation before stamping.

PDMS stamps are hydrophobic and provide a surface favored for protein adsorption. The stamp has usually no surface charge. Hence, the isoelectric point and the pH of the adsorption buffer are less critical.

Protein printing ink can be reused several times, but reuse may reduce the activity of the printed protein and/or the amount of protein transferred in the printing process.

EQUIPMENT

- Lithographically patterned silicon master having a surface relief structure (see EQUIPMENT SETUP)
- Oxygen plasma cleaner, e.g. plasma system 100E (PVA Technics Plasma AG) with a pressure of 0.8 torr and an RF (2.45 GHz) power of 200W
- Equipment for preparation and culturing of chick retinal explants
- Fluorescent microscope for evaluation of stained protein patterns and cells

REAGENT SETUP

Protein 'stamping ink' The desired protein dissolved with or without 0.1% w/v bovine serum albumin (BSA) carrier protein in 1× PBS (**Box 1**).
▲ CRITICAL For some proteins, it might be critical to make the stamping ink up on the day of the experiment to assure the protein stays active in solution. For details, see **Box 1**.
Laminin (natural, mouse) (Invitrogen, cat. no. 23017-015) Store at –80 °C in 1× PSB at 1 mg ml^{-1}, dilute before use in Hanks medium at 20 μg ml^{-1}
Preparation medium (stable at 4 °C for several months) Sterile Hanks medium without calcium/magnesium: 8.0 g l^{-1} NaCl, 0.4 g l^{-1} KCl, 60 mg l^{-1} NaH_2PO_4, 60 mg l^{-1} Na_2HPO_4 (2 H_2O), 0.35 g l^{-1} $NaHCO_3$, 1.0 g l^{-1} glucose, 7.76 g l^{-1} HEPES, 10 mg l^{-1} phenol red, adjust to pH 7.4.
Supplemented F12 medium (Gibco/Invitrogen, cat. no. 21700-026) For 500 ml, complete 435 ml F12 medium with 50 ml FCS, 10 ml chick serum, 5 ml 200 mM L-glutamine, 500 μl penicillin/streptomycin. Stable at 4 °C for several months.
Supplemented F12 medium with 0.4% methylcellulose For 500 ml, autoclave 2 g methylcellulose, add 325 ml sterile ddH_2O, stir for 3–4 d at 4 °C until the methyl cellulose is dissolved and add 110 ml of 4× concentrated sterile supplemented F12 medium. Centrifuge for 15 min at 500 r.p.m. and discharge the pellet. Stable at 4 °C for several months.
Fixative 4% paraformaldehyde + 0.1% glutaraldehyde in 1× PBS containing 0.3 mol l^{-1} sucrose ! CAUTION Toxic. Do not inhale and avoid skin contact.
Appropriate antibodies for staining the printed protein (optional) Staining agents for the retinal ganglion cell axons, e.g. Alexa Fluor 488 phalloidin (Molecular Probes, cat. no. A-12379)

EQUIPMENT SETUP

Design and fabrication of silicon masters Silicon masters can be fabricated using lithography. Standard photolithography can be employed for feature sizes of the relief pattern larger than 1–2 μm. In contrast, electron beam lithography can be used to create structures in the 300-nm regime. A minimum of ~50 nm is conceivable using harder PDMS variants for the casting method[21]. Stamps for the casting method should have geometric patterns that do not collapse when printed, i.e. they should have an aspect ratio (ratio of structure height to width) not exceeding five[22]. Furthermore, they should have a reasonable fill factor that ensures the stability of structures on contact. Our masters were made using a positive-tone resist (PMMA) and a lift-off process for chromium (25 nm) as a mask for the subsequent reactive ion etching[23]. We etched the structures 650 nm deep into the silicon surface (**Fig. 2b**). As an alternative to etching, masters with the developed resist pattern can also be used; but these should not be cleaned in an oxygen plasma nor treated with solvents dissolving the patterned resist.

PROCEDURE

Preparation of cover slips

1| Glass coverslips should be cleaned carefully to avoid interference during the printing process. For a thorough cleaning, wash coverslips for 10 min in 1:1 ethanol:ddH_2O in an ultrasonic water bath. Incubate coverslips for 6 h in 1:1 ethanol:acetone and wash 3× 15 min in 100% ethanol. Dry the coverslips in a clean place. Patterns may also be stamped onto sterile plastic petri dishes. Because the surface properties of petri dishes vary with manufacturers and/or lots, it is highly recommended to first test their applicability for μCP before using them as a matter of routine.
■ PAUSE POINT Clean coverslips can be stored several months in dust-tight containers and in an oil-free environment.

2| Before printing protein patterns on the clean coverslips, bake them for 6 h at 220 °C. Baking might increase the hydrophilicity of the glass surface and can be critical for optimal protein transfer.

3| Patterns can be made using either the lift-off technique (option A) or by casting an inverse PDMS mold from the silicon master, the casting technique (option B).

(A) Lift-off technique

(i) Mix the silicone PDMS components according to the manufacturers instructions and pour the mixture approximately 5–7 mm high (corresponds to 65–75 ml PDMS mass) in clean, dust-free big petri dishes (145 mm diameter). Polymerize the PDMS in an oven at 60 °C over night (>8 h) according to the manufacturers instructions.
▲ CRITICAL STEP The hardness and tensile strength of the polymerized PDMS varies depending on the ratio of its components, its crosslinking and the polymerization conditions. Stamps should neither be brittle nor too soft[18].
■ PAUSE POINT Polymerized PDMS disks can be stored several months in the petri dishes before cutting them into custom-sized stamp cuboids.
? TROUBLESHOOTING

(ii) Cut the polymerized PDMS stamp-side-up on a clean glass surface in small cuboids: they should be slightly bigger than the dimensions of the pattern on the silicon master. Use a scalpel with a long, clean blade to obtain clean cutting edges. Preferably, the cutting should be done on a dust-free sterile bench. Place the cuboids with the stamp side down in a new clean petri dish. They should tightly adhere to the dish bottom.
▲ CRITICAL STEP The perfectly plane side of the PDMS disk facing the bottom of the petri dish (stamp side) is later coated with protein and placed on the silicon master, so it should be carefully protected from contamination.
■ PAUSE POINT Stamps can be stored this way for several months in a dust-free place.
? TROUBLESHOOTING

(iii) Prepare the protein stamping ink solution with your desired protein (**Box 1**). Proteins should be dissolved in sterile PBS and can be labeled before the printing process with fluorescent antibodies.
? TROUBLESHOOTING
▲ CRITICAL STEP Work at a sterile bench to avoid contamination with dust and dirt particles.

(iv) Place the desired number of PDMS cuboids with the stamp side up in a fresh petri dish. Carefully check the PDMS surface under a binocular microscope for contaminations and irregularities and discharge any dirty cuboids. Pipette a drop of protein stamping ink onto the PDMS cuboid to cover the entire stamp surface with solution. For a cuboid surface of 1 cm $\times$1 cm, approximately 250 µl of printing ink is needed. Because the PDMS stamps are of hydrophobic nature (contact angle $\theta_a > 115^\circ$), wetting the surface initially seems a bit cumbersome. By the adsorption of the protein, the stamp surface turns hydrophilic. Note that the volume needed to cover the stamp surface increases more than linearly with the surface area dimensions.

(v) Incubate the inked stamp for 30 min–2 h (optimal incubation times may vary with the protein concentration, see **Box 1**) at 37 °C in a humid chamber or a cell culture incubator.

(vi) Pick up the cuboid with clean forceps and discard the coating solution. Alternatively, the coating solution can be recovered and reused (**Box 1**). If you collect the coating solution for reuse, be careful not to touch the stamp surface with the pipette tip. While holding it with the forceps, wash the cuboid twice for 5 s each by sequentially submerging it into two small beakers filled with approximately 100 ml sterile ddH_2O. Dry the stamp surface under a stream of nitrogen.

(vii) Carefully place the protein-covered surface of the PDMS cuboid onto the clean silicon master. Take care that the etched pattern of the master is completely covered by the stamp and that the edges of the stamp do not lie within the pattern. Do not press the stamp onto the master; it should adhere tightly by itself. In order to know approximately where the protein pattern will be located on the coverslip, view the pattern of silicon master under the microscope while the cuboid is placed on it and mark the cuboid surface with a pen approximately where the pattern can be seen.
▲ CRITICAL STEP Handle the silicon master with care! For details, see **Box 2**.

(viii) Remove the cuboid carefully from the silicon master and place it with the stamp side down onto a coverslip. The cuboid should adhere tightly to the glass surface by itself. Flip over the cuboid–coverslip unit, so the coverslip is on top. Mark the coverslip where the pen marks on the cuboid can be seen. Remove the cuboid and discharge it.
▲ CRITICAL STEP Perform Steps vi–viii quickly and without letting the stamp sit drying for an unnecessarily long period.
■ PAUSE POINT Dry protein patterns stamped onto coverslips can be stored at 4 °C protected from condensation for up to one week. Protect from light if a fluorescent protein was stamped.
? TROUBLESHOOTING

(ix) Seize the master with clean forceps without touching the etched pattern and wash it consecutively with 1:1 ethanol: ddH_2O and 100% ethanol with a washing bottle. Dry the master under a stream of nitrogen.

(x) Clean the master in an oxygen plasma for 5–10 s in order to remove organic contaminants. Before applying the plasma, carefully check the master for PDMS contamination. PDMS is vitrified in the oxygen plasma and then cannot be removed from the master afterwards. The master is now ready for reuse in Step 3Avii.

(B) Casting technique

(i) In order to use silicon (typically wafers having a native silicon oxide on it) as a master to mold PDMS from, the master has to be rendered hydrophobic by silanizing it: submerge the master in a 1 mM trichloro(octadecyl)silane solution in heptane (4.1 µl trichloro(octadecyl)silane in 10 ml heptane) for 10 min.
! CAUTION Trichloro(octadecyl)silane has to be stored and handled under a inert gas (such as nitrogen) atmosphere. It is explosive when mixed with water or oxidizing substances. Avoid skin contact and wear gloves.

(ii) Dry the master under a stream of nitrogen. Test the hydrophobicity of the master by pipetting a drop of ddH_2O onto the silicon surface if the contact angle is large and the drop rolls off, the silanization was successful.
(iii) Pour ~50 µl of liquid, well-mixed PDMS prepolymer over the master. Avoid air bubbles! Place a small piece of clean glass on top of the PDMS, fix it with metal weights and polymerize the mold at 60 °C overnight. It might be advisable to de-gas the PDMS prepolymer using an exsiccator before molding it.
(iv) Separate the silicone cast from the master. You may attach a small grab handle to the cast to facilitate the stamping process.
(v) Cover the stamp with protein printing ink and incubate at 37 °C for 30 min–2 h.
(vi) Decant the printing ink, rinse the stamp with ddH_2O and dry the stamp under a stream of nitrogen. Quickly proceed to the next step.
(vii) Carefully place the stamp onto a glass coverslip. If desired, mark the position of the pattern on the backside of the coverslip with a thin pen using a binocular microscope.
(viii) Remove the stamp. Stamps can be cleaned and reused.

■ **PAUSE POINT** Dry protein patterns stamped onto coverslips can be stored at 4 °C protected from condensation for up to one week. Protect from light if a fluorescent protein was stamped.

? TROUBLESHOOTING

(ix) Clean the stamp by putting a small piece of transparent adhesive tape over the stamping surface and peel it off to remove dust and dirt particles. Wash the stamp with ethanol. Persistent contaminations can be removed by placing the stamp for 10 min in ethanol in an ultrasonic waterbath. Alternatively, wash the stamps in 1% SDS solution and rinse them with ddH_2O.

Cell culture on printed patterns

4| Cover the printed protein pattern with ~150 µl laminin solution (20 µg ml^{-1} in Hanks medium) and incubate at 37 °C in a cell culture incubator for 30 min. Alternatively laminin can be applied in stripes overlaying the printed pattern (**Fig. 2d**). To do this, place a PDMS matrix containing an array of small channels (described in ref. 24; matrices can be purchased from M.B.) over the pattern and press it carefully onto the coverslip. Inject 100–200 µl laminin solution into the matrix and incubate at 37 °C for 30 min. Before removing the matrix, flush the channels with 300 µl Hanks medium.

5| Remove the laminin solution and wash the coverslip three times for 1 min in Hanks medium. Cover the pattern with ~200 µl supplemented F12 medium without methylcellulose at 37 °C for at least 10 min.

■ **PAUSE POINT** Covered patterns can be stored at 37 °C for several hours, allowing the preparation of chick retinal explants in the mean time.

6| Remove the medium, place 1–2 explants onto the coverslip, place two small metal weights on the filter stripes holding the explants and add ~250 µl supplemented F12 medium with 0.4% methyl cellulose. Incubate overnight in a cell culture incubator.

Fixation and staining procedures

7| Remove the medium and fix the cultures in ~300 µl fixative at room temperature (~20 °C) for 30 min.

! CAUTION Avoid skin contact with the fixative and wear gloves.

8| Wash three times for 15 min in 1× PBS and continue with individual protocols to stain the cells and the printed protein pattern.

? TROUBLESHOOTING

9| Evaluate under a fluorescent microscope.

● **TIMING**

Steps 1–2: Coverslip preparation, ~1 h + 6 h incubation + several hours drying; 6 h baking just before the printing process.
Step 3, option A: Lift-off technique: PDMS stamp preparation, ~1 h + overnight polymerization + 30 min cutting; stamping of protein patterns, ~3–4 h, depending on incubation time; cleaning of the silicon master, ~15 min.
Step 3, option B: Casting technique: Casting PDMS stamps from silicon masters, ~30 min + overnight polymerization; stamping of protein patterns, ~2–3 h, depending on incubation time; cleaning of the stamp, 10–30 min.
Steps 4–6: Cell culture on printed patterns, ~2 h + overnight growth of the retinal axons.
Steps 7–9: Fixation and staining, ~1 h for fixation.

? TROUBLESHOOTING

See **Table 1** for troubleshooting guidance.

TABLE 1 | Troubleshooting table

Problem	Possible reason	Solution
No protein transfer can be detected.	The protein concentration of the printing ink is too low.	Use higher protein concentrations in the printing ink, increase the incubation time. Try also to stain the stamp after printing to see if protein has adsorbed to it.
	The protein has been transferred, but not correctly stained.	Check the quality and concentration of the antibody used for staining the transferred protein.
	The protein did not transfer.	Proceed quickly with the macroscopically dried stamp before printing. After blowing the stamp dry with nitrogen gas, the stamp should be used immediately.
	The stamp did not adhere to the master or the coverslip during the transfer process.	Check if the stamp adheres to the master and the coverslip. You may press it down slightly and carefully with forceps, but do not exert too much pressure.
The printed pattern is defective and blurred.	The transfer was disturbed by contaminations of the silicone cuboid or the silicon master.	Take care to work as cleanly as possible. See also **Box 2**: Guidelines for silicon master handling.
	The stamp touched the master and/or the coverslip more than once.	Take care to place the stamp onto the master and the coverslip precisely. Do not let it relapse while you remove it.
	The silicone cuboid was distorted.	Don't squeeze the cuboid while holding it with the forceps.
		Don't press it onto the master or the coverslip surface.
		Check the hardness of the silicone and change eventually the polymerization conditions.
	The stamp was too wet.	Dry the stamp thoroughly before using it under a stream of nitrogen.
	The pattern on the master was designed in a disadvantageous way.	Avoid large etched areas, where the silicone stamp may sag and touch inadvertently the master.
		Check the hardness of the silicone and change, if necessary, the polymerization conditions.
The printed protein is not active.	The protein was not stable in solution.	Check if the soluble protein in the printing ink solution is still active. Addition of carrier protein may improve the stability in solution.
	The dry pattern was stored too long.	Use the pattern for cell culture immediately after the stamping. Submerge it in the solution of the cell culture medium. Storage of the pattern might not be possible for all proteins.
	The protein might be not suitable for the dry lift-off μCP technique.	Try to print linker proteins like antibodies, streptavidin, protein A/G or the like; perhaps use other μCP techniques (see Introduction).

ANTICIPATED RESULTS

The printed protein pattern should be a clear and precise inverted copy of the pattern etched into the silicon master. Lines and dots down to 0.3 μm can be reproducibly transferred onto the coverslip surface. Stained patterns and axons can be photographed under the microscope and combined in one picture as shown in **Figure 2e,f**. Ideally, the patterns specifically

BOX 2 | GUIDELINES FOR SILICON MASTER HANDLING

Cleanliness is key to a perfect surface. Although you do not need a cleanroom for working with silicon master, try to avoid contamination with dust and dirt particles. Masters that appear clean and dust-free under the binocular microscope are in general sufficiently clean for μCP.

Avoid touching the etched surface on the master.

Handle the masters with clean forceps without scratching.

Plasma cleaning removes organic contamination, but it might immobilize inorganic particles such as silicone debris on the master surface. Check the master for inorganic contaminations each time before cleaning it in the oxygen plasma. They might be removed by a focused stream of ddH_2O from a wash bottle.

Silicon masters are brittle and break easily, particularly those that carry deeply etched features on the surface.

influence the outgrowth and guidance of the growth cones that are in contact with them. The reaction of the axons may vary depending on both the geometry of the pattern and the concentration of the protein printing ink, allowing a comprehensive functional analysis of the printed guidance molecule. Axonal outgrowth can be easily quantified by measuring the fluorescence intensity of phalloidin-stained axons in a field of interest.

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