Substrate-Bound Protein Gradients for Cell Culture Fabricated by Microfluidic Networks and Microcontact Printing

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Substrate-Bound Protein Gradients for Cell Culture Fabricated by Microfluidic Networks and Microcontact Printing

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Graded distributions of proteins are pivotal for many signaling processes during development, such as morphogenesis, cell migration, and axon guidance. Here, we describe a technique to fabricate substrate-bound stepwise protein gradients by means of a microfluidic network etched into a silicon wafer with an array of parallel 14-micrometer–wide channels, which can be filled with a series of arbitrarily chosen protein solutions. In a subsequent microcontact printing step, the protein pattern is transferred onto a surface and is used as a substrate for cell culture. Cellular responses to a defined microscopic pattern of a protein, such as guided axonal outgrowth and directed migration, cell polarization, changes in morphology, and signaling, can be thus studied in a controlled in vitro environment.

Introduction

Ordered patterns and graded distributions of guidance factors and morphogens in a tissue are essential for many processes in cell and developmental biology, such as the establishment of cell and organ polarity and differentiation, the formation of compartment borders, cell migration, and axon guidance (*1*–*3*). During axon guidance, the specific connectivity in the nervous system is established mainly by specific distributions of different guidance factors. Graded distributions are especially suited for guiding this developmental step, because they encode positional and directional information for pathfinding axonal growth cones. The spatial distribution pattern of a guidance cue may thus be pivotal for its function (*4*, *5*). However, this aspect is often not taken into account when axon guidance factors are studied in vitro.

Axon guidance in a concentration gradient has been mainly investigated with so-called soluble or diffusible gradients generated by diffusion of guidance factor molecules that emanate from a pipette tip inserted into the medium (*6*, *7*). Diffusible gradients are difficult to maintain over a long time, their shapes cannot be fully arbitrarily designed, and they may not be suited for the study of membrane- or matrix-bound guidance cues such as ephrins. Growth cones typically respond to diffusible gradients with repulsive or attractive turning. Diffusible gradients thus have been used for the study of how a growth cone processes directional information and differentially activates signaling cascades close to or far from the gradient (*8*–*11*). The mechanisms by which a growth cone detects positional information and recognizes its target zone in a gradient, on the other hand, cannot be satisfactorily investigated with diffusible gradients.

The first substrate-bound in vitro gradients for the study of axon guidance were generated by sucking cell membrane fragments from tissue material onto a filter (*12*). Substrate- or matrix-bound gradients for cellular assays have been established by diffusion of growth factors in collagen gels (*13*), by laser-induced photo-immobilization of peptides onto a polystyrene surface (*14*), or by pressing a drop of protein solution onto an aldehyde-activated glass slide with a miniature squeegee (*15*). Well-defined and precise microscopic surface patterns have been successfully generated by microcontact printing (μCP) (*16*, *17*) and microfluidic networks (μFN) (*18*–*20*) and may be used for different cell culture assays.

Here, we describe the fabrication of stepwise concentration gradients consisting of an array of lanes in which increasing concentrations of protein are bound to the surface. This allows the analysis of the responses of cultured cells to custom-designed graded patterns of the substrate-bound protein. Stepwise gradients are prepared by combining a μFN etched into a silicon wafer with a subsequent μCP step. Because every "step" of the gradient correlates with a channel in the μFN, which is independently filled, an almost unlimited number of different graded patterns can be produced with a single, reusable μFN. Long gradients ranging over all channels are as feasible as an assembly of several short gradients, each ranging a shorter distance over a subset of channels. To fabricate a gradient of a single protein, a second inactive or neutral protein is used to generate a dilution series. Alternatively, the inactive protein can be exchanged for a second active component. In this way, countergradients or overlapping gradients of different proteins with independent concentration gradients can be generated.

We have studied the response of chick retinal ganglion cell (RGC) axons to stepwise gradients of the axon guidance molecule ephrinA5 (*21*). The printed gradient was overlaid with laminin stripes arranged in parallel to the direction of the gradient. The outgrowth-promoting laminin causes the RGC axons to grow on a more-or-less straight line into the gradient, allowing an analysis of how a history of differently encountered ephrin concentrations affects the response of the axon to the gradient.

Because the gradient assay can be performed with various other neuronal or nonneuronal cell types and with guidance cues other than ephrinA5, general instructions on how to fabricate customized patterned substrates for cell culture are described. Graded patterns control a vast number of signaling events; thus, this technique may aid in the design of meaningful in vitro experiments in the field of developmental and cell biology. The width of each protein-covered lane in the described gradients is smaller than the average dimensions of most cultured cell lines. Cells may therefore extend over several protein lanes and thus simultaneously contact different proteins or protein concentrations, which provides an interesting scenario to study signaling processes at a cellular and subcellular level.

Materials

Alexa Fluor 594 goat antibody to human immunoglobulin G (IgG) (R&D Systems, #A11014) for clustering

Bovine serum albumin (BSA)

Cells or cell explants and adequate cell culture media

Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+}, Mg^{2+} (Biochrom AG, #L182-50)

Ethanol > 99.8% p.a.

Glutaraldehyde, 25%

Glycerin

Human IgG, Fc Fragment, Plasma (Calbiochem, #401104)

Microloader tips

Mowiol 4-88 Reagent (Calbiochem, #475904)

Murine laminin (Invitrogen, #23017-015)

Nitrogen gas (with a pressure-adjustable valve)

n-Propyl gallate

Paraformaldehyde

Polystyrene Cell Culture Dishes (Nucleon Surface, Nunc, #150288)

Recombinant Human Ephrin-A5/Fc Chimera (R&D Systems, #374-EA)

Silicone Elastomer Sylgard 184, Base and Curing Agent (Dow Corning, #240-01673921)

Sucrose

Equipment

Binocular light microscope

Equipment for cell culture

Fluorescent microscope

Lithographically etched silicon master containing an appropriately designed μFN (see Instructions)

Oxygen plasma cleaner with a pressure of 0.8 torr and a RF (2.45 GHz) power of 200 W, for example, plasma system 100E (PVA Technics Plasma AG)

Peltier module

Silicone stripe matrices [presently distributed by the laboratory of M. Bastmeyer, University of Karlsruhe, Germany (bastmeyer@bio.uka.de)]

Thermo-hygrometer

Recipes

Recipe 1: Protein Solutions

Dissolve the protein of interest in sterile 1× PBS and store stock solutions at −80°C. Immediately before use, thaw stock solutions on ice and dilute them in 1× PBS to the desired final concentration.

Note: We prepare a stock solution of ephrinA5-Fc at 150 μg/ml.

Recipe 2: Blocking Solution

Dissolve BSA in sterile 1× PBS to give a final concentration of 5 μg/ml. Store at 4°C.

Recipe 3: Laminin Solution

Dissolve laminin in 1× PBS to give a final concentration of 1 mg/ml. Store at –80°C. Thaw on ice and dilute immediately before use in 1× PBS to give a final concentration of 20 μg/ml.

Recipe 4: Cell Fixative

To make 250 ml of cell fixative, dissolve 10 g of paraformaldehyde in 100 ml of hot (~60°C) double-distilled water (ddH_2O), stir for 10 min, and then add up to 500 μl of 1 N NaOH until the solution is clear. Filter the solution through filter paper. Add 25 ml of 10× PBS and 75 ml of ddH_2O. Mix and adjust the pH to 7.4 with 1 N HCl. Add 25 ml of 3.3 M sucrose and 25 ml of ddH_2O. Aliquot and store at –20°C. After thawing, the fixative can be stored for up to 1 week at 4°C. Before use, add glutaraldehyde to a final concentration of 0.1% (for example, add 5 μl of 25% glutaraldehyde to 1 ml of cell fixative).

Note: Paraformaldehyde and glutaraldehyde are toxic. Handle with care and do not inhale.

Recipe 5: Mounting Medium

Crush 50 g of Mowiol 4-88 Reagent with a pestle and mortar and dissolve in 200 ml of 1× PBS. Stir the solution overnight at 40°C. Add 100 ml of glycerin and stir for another 8 hours. Adjust the pH to 8.5 with 1 N NaOH and store 10-ml aliquots at –20°C. Before use, thaw an aliquot in a microwave (850 W for 30 to 60 s; take care that the solution does not boil over), add ~50 mg of *n*-propyl gallate, and centrifuge at 2000*g* for 10 min at 4°C.

Note: The mounting medium is stable at 4°C for several weeks.

Instructions

Design and Fabrication of the μFN

We recommend manufacturing custom-designed μFNs in cooperation with an adequately equipped physics or materials science laboratory. Because this Protocol focuses on the biological applications of μFNs, we will not give detailed instructions for photolithography. However, the following guidelines are provided for the design and fabrication of the μFN by the photolithography lab (Fig. 1). An optimal μFN contains an array of parallel microchannels, each connected to filling and venting ports that are accessible for filling with a microloader tip. Exemplary μFNs (Fig. 1, A and B) consist of 16 or 32 channels, each 14 μm wide and separated from each other by a 6-μm-wide barrier. These channels run parallel over a distance of 2 mm in the middle of the μFN and diverge on the outside in order to open out into

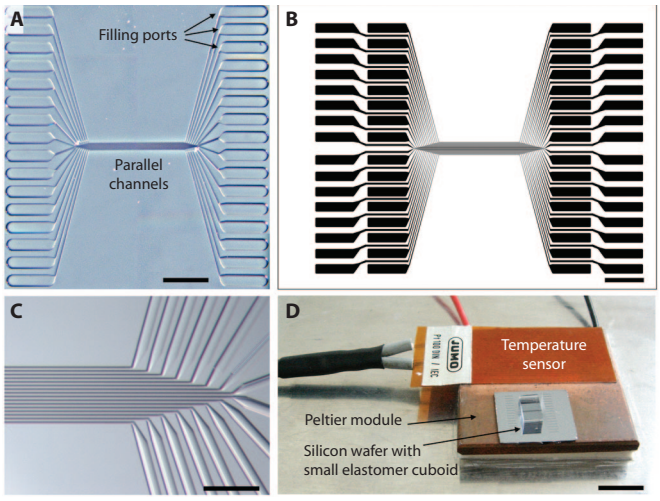

Fig. 1. Examples of μFNs designed for the generation of stepwise gradients of substrate-bound guidance molecules. (**A**) A μFN etched with 16 parallel channels connected to rows of filling ports. (**B**) Scaled schematic drawing of a μFN with 32 parallel channels with the filling ports arranged in intercalating rows. Etched areas are shown in black. (**C**) Detailed view of filling channels opening out into the central array of parallel channels. (**D**) Setup for filling the μFN: a silicon wafer containing a μFN sealed with a small elastomer cuboid on a Peltier module. Scale bars: (A and B) 2 mm, (C) 300 nm, (D) 1 cm. [Figure modified after (*21*) with permission of Springer Science and Business Media]

arrays of filling or venting ports (Fig. 1C). The μFN is created by photolithography on a silicon (Si{100}) master. The parallel channels have a depth of 50 μm; the filling and venting ports are 300 μm deep. Briefly, masters are made by exposing a photoresist on the silicon surface through a mask featuring the elements of the μFN and subsequently etching away the uncovered SiO_2 with hydrofluoric acid (*21*).

Preparation of Silicone Elastomer Stamps

The silicone elastomer cuboids that are used as stamps are prepared from Sylgard 184 base and curing agent. The general procedure is described below. Additional details can be found in the manufacturer's instructions (http://pibeta.web.psi.ch/handbook/suppliers/sylgard.pdf). We recommend first fabricating large silicone elastomer disks in big Petri dishes and then cutting them into custom-sized small cuboids.

1. Thoroughly mix the silicone elastomer Sylgard 184 base with the curing agent in a 10:1 weight ratio.
2. Pour the mixture into clean, dust-free Petri dishes to a height of approximately 5 to 7 mm. For example, pour between 65 and 75 ml of silicone elastomer into a 145-mm-diameter dish.
3. Remove any small air bubbles by placing the dishes containing the silicone elastomer in a vacuum chamber for 3 min.
4. Polymerize the silicone elastomer in an oven at 60°C overnight.

   Note: The side of the silicone elastomer disk that is in contact with the bottom of the dish, which is dust-free and flat, is used for protein transfer and is referred to as the "stamp side."
5. Trim the polymerized elastomer disk on a clean glass surface in custom-sized cuboids: One size should slightly exceed the dimensions of whole μFN on the silicon master; a second, smaller size should just cover the parallel channel system of the μFN (Fig. 2A).

   Note: For cutting, use a long, clean blade to obtain clean cutting edges. Avoid contamination with dust particles.
6. Store the cuboids with the plane "stamp side" down in a new clean Petri dish.

   Note: The cuboids can be stored for several months at room temperature in a dust-free environment.

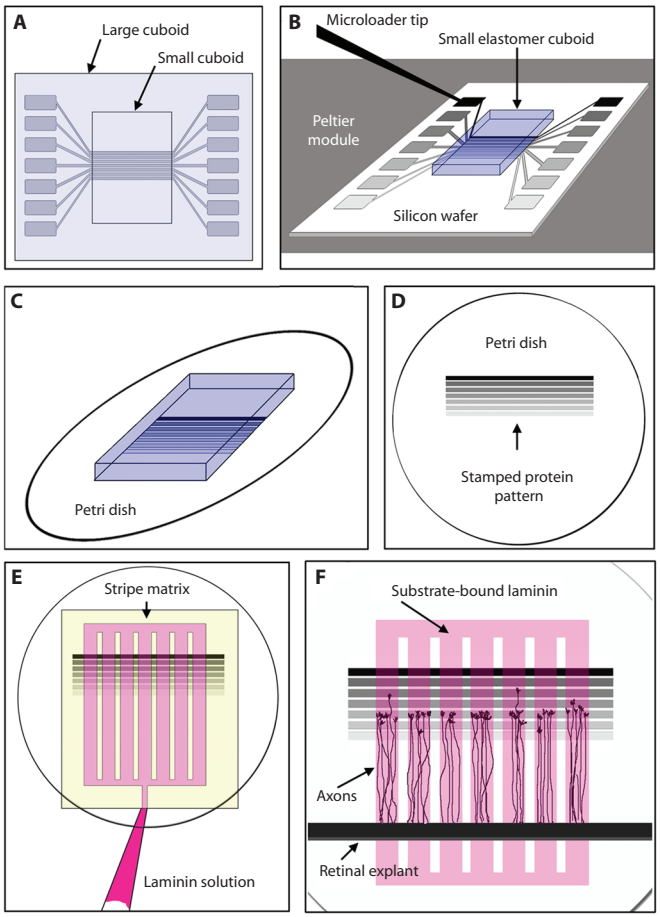

Fig. 2. Schematic illustrating the creation of a stepwise gradient overlaid with laminin stripes. (**A**) The elastomer cuboids of two sizes are shown relative to the μFN. (**B**) The μFN is filled with a microloader tip while the smaller cuboid is in place. (**C**) The adsorbed protein is transferred onto a polystyrene Petri dish to create a μCP. (**D**) A stamped stepwise protein gradient is shown from the top. (**E**) Laminin stripes (pink) are applied with a silicone stripe matrix. (**F**) A diagram of RGC axons growing on the patterned substrate is shown.

Filling of the μFN and μCP

A graded protein pattern is transferred onto the even silicone elastomer cuboid surface by placing a cuboid over the μFN channel system etched into the silicon wafer. The μFN channels are then filled with protein solution. During incubation of the protein solution in the μFN, protein adheres to the cuboid surface and the protein-decorated silicone elastomer cuboid can subsequently be used as a stamp in a μCP step (Fig. 2).

Protein solutions are prepared as serial dilutions of the protein that will form the gradient and an inactivated protein or neutral protein, such as the Fc portion of an antibody or BSA. This ensures that the total protein concentration in each sample is constant. A number of different proteins, including proteins of the extracellular matrix (such as vitronectin), antibodies, and polyclonal serum, retain their activity during the stamping process. Appropriate protein concentrations to elicit a cellular response must be empirically determined. Below, we describe the details for preparing ephrinA5 gradients using a fusion protein of ephrinA5 and the Fc portion of an

antibody (ephrinA5-Fc), as an example. RGC axons stopped at lanes filled with ephrinA5 concentrations of 40 to 60 μg/ml; therefore, we used a range that exceeded the concentrations that produced the cellular response. In addition, we cluster the chimeric protein with a fluorescent antibody before use in order to potentiate the activity of ephrinA5 and to mark it with a fluorescent label (*21*).

If it is desirable to visualize the printed protein gradient, which is necessary if verification of the transfer process is desired, then the gradient solutions should be prepared with fluorescently labeled antibodies or with fluorescently tagged proteins. We found that clustering ephrinA5-Fc with up to a 10-fold higher concentration (in μg) of fluorescently labeled antibody against the Fc portion did not interfere with the biological activity of the printed protein. Direct labeling of the protein solution results in fluorescent patterns, which can be observed without a subsequent staining step. This is especially advantageous if live cells are observed on the pattern. Alternatively, an unlabeled pattern can be immunostained and analyzed after fixation.

1. Cluster 150 μg/ml ephrinA5-Fc with Alexa Fluor 594–conjugated goat antibody to human IgG at a ratio of 1:3 for 30 min at room temperature with gentle agitation. Cluster 150 μg/ml Fc under the same conditions to use as the "diluent, inactive" protein.
2. Prepare serial dilutions of the clustered ephrinA5-Fc solution and the clustered Fc solutions with ephrinA5 concentrations ranging from 10 to 150 μg/ml (Recipe 1). Keep the solutions on ice.
3. Place the silicon wafer with the etched μFN onto a small Peltier module (Fig. 1D), which is mounted on a binocular light microscope.
4. Place the larger-sized elastomer cuboid onto the clean, etched μFN so that it adheres tightly to the silicon surface.

   Note: This reduces the hydrophilicity of the silicon surface surrounding the etched μFN structures, thus preventing leakage of the protein solution later filled into the μFN.
5. Remove the large elastomer cuboid with clean forceps and place the smaller-sized cuboid onto the etched channel system, tightly sealing and separating the channels from each other (Fig. 2B).
6. Mark the position of the channels (seen through the transparent cuboid) with a permanent marker on the cuboid surface.
7. Place the silicon wafer onto a Peltier module adjusted to ~5°C above the dew point to prevent desiccation or condensation during the filling of the μFN.

   Note: The dew point is calculated from air temperature and relative humidity (for a fast and simple calculation, see http://www.pages.drexel.edu/~brooksdr/DRB_web_page/WaterVapor/dewpoint.htm*). If condensation occurs, raise the temperature. However, if the temperature is too high, then the protein solutions will dry out.*
8. Cut a microloader tip with a sharp blade to a size and shape that will fit into the filling ports.
9. Starting with the most dilute solution, fill the μFN with the protein solutions that will form the gradient by adding 1 to 2 μl of protein solution with a cut microloader tip and gently touching the filling port of the first channel. Monitor the filling of the μFN using the binocular microscope.

   Note: The liquid will flow into the channel by capillary force and appear in the connected port on the other side of the μFN (Fig. 2B). Avoid overloading the filling ports to ensure that the protein solutions in the neighboring ports do not mix.
10. Empty the tip by touching it against a piece of a clean Whatman filter paper. The tip can be reused for the next solution.
11. Proceed until all channels of the μFN are filled.
12. After filling, incubate the μFN for 10 to 20 min at room temperature to allow adsorption of the protein in the channels on the elastomer stamp.
13. While holding the silicon wafer with clean forceps, briefly rinse the filling ports of the μFN with sterile ddH_2O with a washing bottle.
14. Dry the filling port area with a stream of N_2.

    Note: Take care that the elastomer cuboid does not fall off during the washing and drying process.
15. Place the silicon wafer onto the Peltier module.
16. Wash the μFN for 3 min with Blocking Solution (Recipe 2) by adding 300 μl of Blocking Solution by pipette on one side of the elastomer cuboid and waiting until the channels are flushed.
17. Pipette 200 μl of Blocking Solution (Recipe 2) on the other side of the elastomer cuboid.
18. Slowly remove the elastomer cuboid with forceps from the silicon wafer, leaving the wafer in place. While doing so, let the Blocking Solution flow between the cuboid and silicon surface.

19. Rinse the cuboid with 5 ml of ddH_2O.
20. Thoroughly dry the cuboid under a stream of N_2.

Creating μCP Surfaces

In general, μCP can be performed onto polystyrene, for example, a cell culture dish, as well as onto glass surfaces.

Option 1: Stamping a cell culture dish

For some applications, it is easier to create the gradient pattern on the bottom of a cell culture dish. It is crucial that the cuboid with the adsorbed proteins adhere tightly to the dish during the stamping process. Adhesion may be perturbed by dust particles on the cuboid or the dish. Therefore, we recommend working in a laminar flow hood.

1. Place the elastomer cuboid "stamp side" down onto a polystyrene cell culture dish, pressing gently with forceps, if necessary, to ensure a tight seal. The cuboid should tightly adhere, thus transferring the adsorbed protein onto the dish surface (Fig. 2, C and D).
2. Turn over the dish with the adhering cuboid and copy the position marks on the cuboid onto the bottom of the dish.
3. Carefully remove the cuboid and discard it.
4. Verify transfer by visualizing the protein pattern with a fluorescent microscope.

Note: This step is optional and will only work if the protein pattern is created with a fluorescent label.

Option 2: Stamping glass coverslips

For some applications, it is desirable to print graded patterns onto glass coverslips. Because adhesion may be perturbed by dust particles on the cuboid or the coverslips, we recommend working in a laminar flow hood and cleaning the coverslip using the following procedures.

1. Arrange the coverslips in a rack and incubate them in a glass container for 10 min in a 1:1 solution of ethanol and ddH_2O in an ultrasonic water bath in order to remove small dust particles.
2. Wash the glass coverslips for 6 hours in a 1:1 solution of ethanol and acetone.
3. Rinse three times for 15 min each in 100% ethanol.
4. Allow the coverslips to dry in a clean, dust-free place.
5. Before printing protein patterns, bake the coverslips in the glass container for 6 hours at 220°C.

Note: Baking is thought to increase the hydrophilicity of the glass surface and can be critical for successful printing.

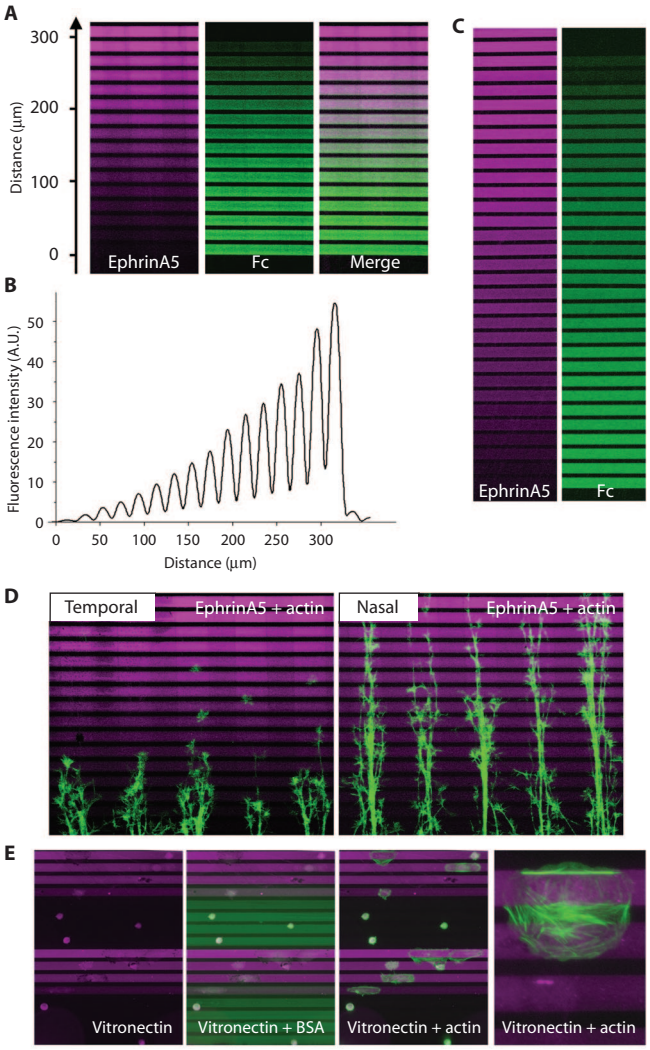

Fig. 3. Examples of gradients fabricated with a μFN. (**A**) The 16 channels of the μFN were filled with solutions of antibody-clustered ephrinA5-Fc linearly increasing in concentration from 10 to 150 μg/ml (purple). The ephrinA5-Fc solutions were diluted in antibody-clustered Fc protein (green). (**B**) Fluorescence intensity of the antibody-clustered ephrinA5 gradient shown in (A), measured by a rolling average over 7 μm. The fluorescence is approximately directly proportional to the concentration of ephrinA5. (**C**) A gradient produced with a μFN containing 32 channels, which were filled with ephrinA5 solutions ranging in concentration from 2 to 32 μg/ml. (**D**) Chick RGC axons (stained for actin, in green) growing on ephrinA5 gradients (purple) with overlaid laminin stripes. Temporal axons stop in the gradient, nasal axons advance up to the end of the gradient. Between the laminin tracks occupied by the axons, dark shadings can be distinguished where the silicone stripe matrix has removed some of the substrate-bound ephrinA5. (**E**) A pattern of two adjacent gradients built by lanes covered with vitronectin (purple) and BSA (green). BRL cells (stained for actin, in green) preferentially spread on the lanes with high vitronectin concentrations. Width of all shown μFN lanes is 14 μm.

6. Place the elastomer cuboid "stamp side" down onto a cleaned, baked glass coverslip, pressing gently with forceps, if necessary, to ensure a tight seal.
7. Turn over the coverslip with the adhering cuboid and copy the position marks on the cuboid onto the bottom of the coverslip.
8. Carefully remove the cuboid and discard it.
9. Verify transfer by visualizing the protein pattern with a fluorescent microscope.

   Note: This step is optional and will only work if the protein pattern is created with a fluorescent label.

Coating the Printed Pattern with Laminin (Optional)

Option 1: Homogeneous coating with laminin

1. Coat the stamped protein pattern with 150 to 200 μl of Laminin Solution (Recipe 3) and incubate for 30 min at 37°C in a tissue culture incubator.
2. Wash with 500 μl of PBS two times for 2 min each.

   Note: Take care that the laminin-coated area does not dry out and avoid touching the area of the printed pattern with the pipette tip.
3. Cover the coated area with cell culture medium and incubate for 10 min at 37°C in the tissue culture incubator.

   Note: Culture medium will vary with the cells used for the assay.

Option 2: Application of laminin stripes

Another option is to create a striped pattern of laminin by using a silicone elastomer stripe matrix available upon request from the Bastmeyer lab (Fig. 2E) (*22*, *23*).

1. Place a silicone stripe matrix containing an array of small channels onto the Petri dish over the pattern (Fig. 2 E).
2. Press firmly to ensure that it adheres tightly to the dish surface.
3. Inject 200 to 300 μl of Laminin Solution (Recipe 3) into the filling channel of the matrix so that all channels are filled with solution and do not contain any air bubbles. Incubate for 30 min at 37°C in a tissue culture incubator.
4. Wash the channels of the matrix with 500 μl of PBS.
5. Remove the matrix with forceps.
6. Cover the coated area with cell culture medium and incubate for 10 min at 37°C in a tissue culture incubator.

Cell Culture, Fixation, and Staining

1. Remove the cell culture medium from the μFN, add the desired cells at an approximate density of 10^5 cells/ml, and allow cells to grow for the desired time.

   Note: The culture medium and growth conditions will vary with the cells and assay.
2. Fix the cultures with 500 μl of pre-warmed (37°C) Cell Fixative (Recipe 4) for 30 min at room temperature.

   Note: If fluorescently labeled antibodies were used for clustering or labeling the printed protein, then protect the cultures from light.
3. Wash with 1 ml of PBS three times for 5 min each.

   Note: At this stage, if desired, cells can be stained for actin with phalloidin or for other proteins with the appropriate antibodies or reagents.
4. To mount, pipette 100 to 200 μl of Mounting Medium (Recipe 5) with a cut 1-ml pipette tip onto the area of interest and place a clean glass coverslip on top.
5. Evaluate the cells with a fluorescent microscope.

Cleaning the Silicon Wafer

1. Hold the silicon wafer with clean forceps and rinse with 50% ethyl alcohol (EtOH) in ddH_2O and then with 100% EtOH with a focused stream from a washing bottle.
2. Dry the wafer in a stream of N_2.

   Note: Check the wafer for inorganic contaminants, such as small elastomer debris, with a light microscope before placing it into the O_2-plasma cleaner. Silicone elastomer particles may be immobilized in the plasma and clog the μFN structures. Remove remaining particles by repeated washing and drying steps.
3. Clean the wafer for 1 min in an O_2-plasma cleaner (pressure 0.8 torr, load coil power 200 W).

Troubleshooting

Problems Filling the μFN

Successful filling of the μFN requires steady hands, patience, and some practice. The filling ports are filled by capillary action using a microloader tip that has been cut with a sharp blade to a convenient size and shape to fit into the filling ports. To ensure optimal fluid flow, the μFN has to be perfectly clean. Therefore, if the solutions do not flow well, check that the wafer is clean and repeat the cleaning process if necessary. The temperature of the Peltier module is also critical as a sufficiently low temperature is required to prevent the protein solution from drying out, yet filling the μFN is also disturbed by condensation water, which appears on the silicon surface when the temperature of the Peltier element drops too low. Thus, adjusting the temperature may aid in filling.

Undetectable Protein on the Dish Surface

If the protein used to make the gradient is fluorescently labeled, then it is possible to verify that the protein has adsorbed onto the elastomer surface of the small cuboid used for sealing the μFN by inspection of the cuboid under a microscope. Note that successfully transferred protein can only be detected if it is appropriately labeled. For complete transfer of the protein pattern from the elastomer cuboid onto the Petri dish surface, it is crucial that the cuboid adhere tightly to the dish during the stamping process. It might be necessary to gently press it onto the dish with the help of forceps. Adhesion may be perturbed by dust particles on the cuboid or the dish.

Faulty or Blurred Printed Protein Patterns

To obtain a precise pattern, it is crucial to avoid any contamination of the μFN and the elastomer stamp with dust or dirt particles. Work as cleanly as possible, preferably in a laminar flow hood. If the elastomer cuboid is not carefully dried before stamping, smeared patterns may result. During stamping, take care that the cuboid is not distorted by excessive squeezing or pressing. Patterns may be also destroyed if the cuboid is unwittingly touched with a pipette tip.

Related Techniques

Substrate-bound gradients can also be fabricated by μCP alone [(*24*), described in detail in (*25*)]. For this, a graded pattern of stripes or dots, which vary in size and spacing, is designed and photolithographically etched into a silicon wafer. The etched wafer serves as printing plate and is used to transfer the pattern onto an elastomer cuboid homogeneously covered with an adsorbed protein layer. The resulting gradient is discontinuous on a microscale: Concentration differences arise from the geometry of the protein-covered areas.

Notes and Remarks

Examples of fluorescently stained gradients and the corresponding countergradients of inactive protein used for dilution are shown in Fig. 3. The quality of the produced gradient can be checked by measurement of the fluorescence intensities of the protein of interest: The protein concentrations of the solutions used for filling of the μFN are roughly proportional to the

fluorescence intensity (Fig. 3B). Combined images of RGC axons and gradients demonstrate the specific activity of ephrinA5, which provides a stop signal for temporal, but not nasal, growth cones (Fig. 3D). A pattern created by two subsequent gradients of the extracellular matrix protein vitronectin (diluted with BSA) is also shown (Fig. 3E). Buffalo rat liver (BRL) cells cultured for 90 min on the vitronectin gradients preferentially spread and adhere on high vibronectin concentrations. At higher magnification, a cell can be seen extending over neighboring lanes of the gradient.

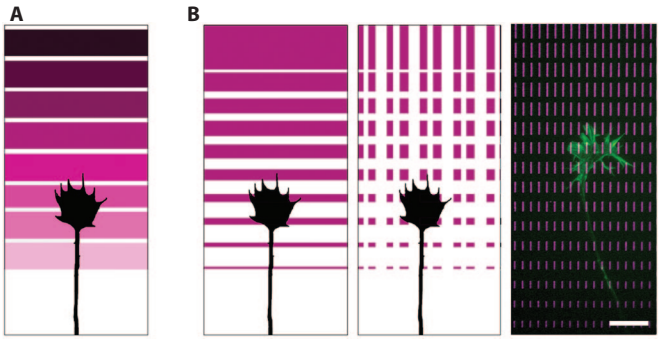

Fig. 4. Stepwise gradients produced with a μFN in comparison with patterns produced by μCP alone. (**A**) Schematic drawing of a μFN-based stepwise gradient encountered by an axonal growth cone (black). (**B**) Schematic drawings of μCP patterns consisting of stripes and dots, respectively, and an example of an actin-stained growth cone (green) in a μCP ephrinA5 pattern (purple). Scale bar: 15 μm.

Figure 4 illustrates the differences in design between stepwise gradients produced with a μFN (described in the present protocol) and graded patterns produced by a single μCP step (*25*). A given μFN can be used to produce various gradients and nongraded patterns, because every channel is independently filled with a protein solution of a given concentration. Alternatively, lanes covered with different proteins can be printed next to each other without mixing. In contrast, with a single μCP step using an etched silicon printing plate, a single protein solution of a fixed concentration and composition is used to print the whole pattern (Fig. 4B). To obtain different geometries, differently etched silicon wafers must be used. Patterns produced with a μFN thus resemble an array of cells, which have different protein expression patterns, whereas patterns fabricated in a single μCP step mimic a tissue with a variable density of cells that all express the same concentration of protein.

References and Notes

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