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Cell Placement and Guidance on Substrates for Neurochip Interfaces

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ABSTRACT: Interface devices such as integrated planar patch-clamp chips are being developed to study the electrophysiological activity of neuronal networks grown in vitro. The utility of such devices will be dependent upon the ability to align neurons with interface features on the chip by controlling neuronal placement and by guiding cell connectivity. In this paper, we present a strategy to accomplish this goal. Patterned chemical modification of SiN surfaces with poly-D-lysine transferred from PDMS stamps was used to promote adhesion and guidance of cryo-preserved primary rat cortical neurons. We demonstrate that these neurons can be positioned and grown over microhole features which will ultimately serve as patch-clamp interfaces on the chip.

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KEYWORDS: neurochip interface; cell placement; neurites guidance; proteins; patterning; PDMS stamp

Introduction

The brain processes information through complex signaling networks formed by the interaction of neurons through dynamic synaptic connections. Electrophysiological interrogation of these networks is critical to understanding this communication and to evaluating physiological changes in synaptic function and network activity. This is particularly important since dysfunction of synaptic connections is a common feature of neurodegenerative diseases. Spatially organized field potential recordings of electrical activity from synchronized populations of neurons in brain slices using multi-electrode arrays (MEAs), have provided a

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powerful tool for research in this area (Kristensen et al., 2001; Thiebaud et al., 1999). Similarly, MEAs have been used to record activity from brain cells grown randomly in culture (Gopal, 2003; Pancrazio et al., 2003). However, the multiplexed signals acquired from such preparations are complex and difficult to interpret. Therefore, developing tools to control cell placement, growth, and connectivity as well as aligning them with "structural features" of interfaces required for interrogation of electrophysiological function has great value. In the case of MEAs these structural features are microelectrode contacts.

An even higher resolution approach to monitoring electrophysiology is to directly measure the ion channel activity which regulates synaptic function by exciting or inhibiting electrical activity. This requires the use of the patch-clamp technique (Hamill et al., 1981). Unfortunately, patch-clamp is a laborious process requiring precise manipulation of pipettes on individual cells (Fig. 1a). Consequently, there is real demand for interrogation interfaces which increase throughput, while still providing high resolution of ion channel activity. Planar patch-clamp chips suitable for use with isolated cells in suspension are under development to address these demands (Dunlop et al., 2008; Fertig et al., 2002; Lau et al., 2006; Stett et al., 2003). They all feature a planar substrate with an integrated microhole in a thin membrane separating two reservoirs (Fig. 1b). Typically, a cell suspension is placed in the upper reservoir and negative pressure is used to attract a single cell over the microhole and to subsequently form a high-resistance electrical seal between the cell membrane and the rim of the microhole. Although this approach has improved throughput in pharmacological assessments of ion channel function, it is only suitable for use with isolated cells devoid of connectivity. Therefore, it is not suitable for

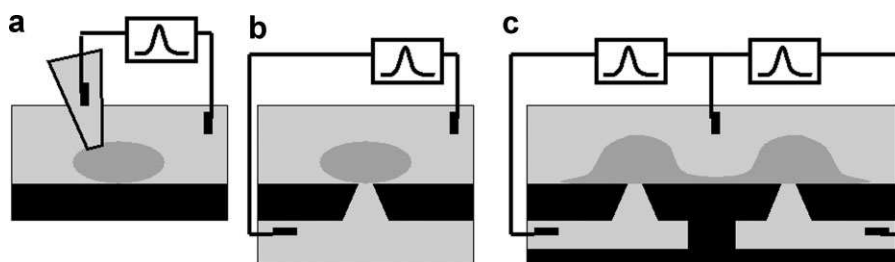**Figure 1.** **a**: Conventional glass pipette patch-clamp. **b**: Patch-clamp chip, separating the culture medium from the electrochemically conductive solution. **c**: Multiple probe patch-clamp chip with integrated subterranean microfluidics. The cells are cultured on-chip and have formed synaptic connections.

recording from more physiologically relevant brain cell networks.

We have proposed integrating multiple holes on a planar patch-clamp whereby the inside of the pipettes are replaced by subterranean microfluidic channels (Py et al., 2008). Because each hole has its dedicated microfluidic channel and associated measuring electrode, this chip can simultaneously and individually monitor the electrophysiological activity of several neurons engaged in synaptic connectivity, thereby combining the advantages of MEAs and patch-clamps (Fig. 1c). For this to occur, isolated cells must however be aligned with the microholes and allowed to mature in culture subsequently forming synaptic processes with neighboring cells. Controlling neuronal placement and guiding connectivity are the challenges addressed in this paper.

Our strategy consists of using surface functionalization to specifically promote cell adhesion on top of microholes. This can be achieved using an established stamping technique where a patterned PDMS stamp is used to transfer chemicals on to the substrate (Bani-Yaghoub et al., 2005; Charrier et al., 2006; Kumar et al., 1994; Offenhausser et al., 2007; Vogt et al., 2005; Wyart et al., 2002). In this study, we used poly-D-lysine (PDL), a commonly used cell attachment factor (Branch et al., 1998; Chang et al., 2003; Li and Folch, 2005). The bare surface of our chip, made of silicon nitride, is in contrast not conducive to neuronal adhesion. In the framework of developing high yield devices, we show that we are able to direct cell placement and growth on the chip substrate. Specifically, we demonstrate that neurons can be positioned and grown over microholes and guided to connect with neighboring neurons in a controlled fashion.

Materials and Methods

Patch-Clamp Chip

With conventional patch-clamp techniques, ion channel activity is measured by recording current flow across a localized patch of the cell's membrane while the voltage potential is held constant (clamped) (Fishman, 1973). In planar patch-clamp chips, current is typically measured between electrodes immersed on each side of the membrane in which the microhole is machined (Fertig et al., 2002). The membrane must have a high dielectric rigidity, and the small dimension of the hole requires the membrane to also be thin and mechanically strong enough that it can be suspended. For these reasons, we chose to work with silicon nitride (Mourzina et al., 2006) deposited by plasma-enhanced chemical vapor deposition, a standard semiconductor processing method. The fabrication process is similar to Pantoja et al. (2004) and will be reported elsewhere.

Fabrication of PDMS Stamps

A silicon-based master (prepared using traditional lithography techniques and SU8; MicroChem Corp., Newton, MA) photosensitive resin was used as a mold to replicate poly(dimethylsiloxane) (PDMS) stamp. Before replication, the native oxide was removed from the silicon surface by dipping the master for 1 min in 0.5% HF followed by quick rinsing in DI water and thorough blow drying with nitrogen. This procedure prevented later adhesion of the PDMS (Sylgard 184; Dow Corning, Midland, MI) to the substrate. The master was assembled in a Teflon enclosure. PDMS was prepared by weighing out 10:1 prepolymer curing agent. After thorough mixing, it was poured onto the master in the Teflon setup and left at room temperature for 1 h to allow air bubbles to escape. This method is slower than loading the sample in a vacuum chamber, but is not prone to the occasional bursts that this powerful section causes.

The PDMS was cured at 90°C for 2 h and carefully peeled off the master. To remove any remaining nonpolymerized PDMS from the stamp, the latter was washed in solvents following the procedure: reflux in hexane for 3 h at 60°C, soak in acetone for 1 h, soak in ethanol for 1 h, sonicate in 2:1 ethanol/water three times for 5 min, rinse in ethanol, blow dry with nitrogen, and dry in oven for 2 h at 65°C. Hexane, ethanol, acetone, and sodium dodecyl sulfate (SDS, 98.5%) were purchased from Sigma–Aldrich (Oakville, ON, Canada), sulfuric acid (H_2SO_4, 96%), and ammonium fluoride (NH_4F, 40%) from J.T. Baker (Phillipsburg, NJ).

Hydrofluoric acid (HF, 49%) was obtained from Arch Chemicals (Cheshire, CT) and hydrogen peroxide (H_2O_2, 30%) from Anachemia (Mississauga, ON, Canada). Cleaning and etching solutions were all clean-room grade. Deionized water (DI water, $18\,M\Omega$) was used for all experiments.

Stamping Procedure

Prior to stamping, the Si/SiN chip was cleaned 20 min at $120°C$ in Piranha solution (1:2 H_2O_2/H_2SO_4), thoroughly rinsed in DI water, dipped 2 min in 2% HF to strip surface OH bonds, quickly rinsed in DI water, and blow dried with N_2. Although the stamping procedure also worked on oxidized Si/SiN chips, better results were obtained with nonoxidized surfaces.

PDMS stamps were mounted on glass slides. The stamps were sonicated 15 min in 70% ethanol, followed by 15 min in DI water, and 15 min in 10% SDS. Modification of the PDMS surface with SDS has been shown to enhance the transfer of poly-lysine to the substrate (Chang et al., 2003). The PDMS was quickly rinsed in DI water and blow dried with nitrogen. The stamp was then immersed for 30 min in a PDL saline solution [33 μg/mL PDL in phosphate-buffered saline (PBS)] then thoroughly blow dried. Stamps (surface area approximately 0.5 cm^2) were applied to the chip with a constant and homogeneous pressure of 500 g for 1 min and left another minute without pressure. This was sufficient to allow the transfer of the PDL from the stamp to the substrate. The chips were finally sterilized 2 min in 70% ethanol, quickly rinsed in DI water, and blow dried again with nitrogen.

For most samples, the stamping was done by hand. However, when precise localization of the chemical patterns was required, such as aligning the pattern with the microholes, a chip bonder (M9, Besi, Londonderry, NH) was used.

Neuronal Cell Culture

Cryopreserved rat cortical cells (QBM Cell Sciences, Ottawa, ON, Canada) were thawed in a $37°C$ water bath for 2.5 min. One milliliter of cell suspension, containing approximately 4×10^6 cells, was resuspended prior to transferring to a 15 mL sterile tube. To avoid osmotic shock, 9 mL of Neurobasal Medium (Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine (Sigma, St. Louis, MO), B-27 (Invitrogen), and 5% fetal bovine serum (Gemini, West Sacremento, CA) was added over a period of 2.5 min. Cells were then resuspended with a 10 mL pipette and inverted 2× to ensure complete mixing of cells and media prior to plating. One milliliter of cell suspension was added to each well of a 24 well plate (VWR Canlab, Mississauga, ON, Canada) containing either a patterned sample or a PDL coated glass coverslip (Bellco Glass, Vineland, NJ). Four hours post-plating media from all wells was removed and replaced with

1 mL of serum-free Neurobasal Medium. Media changes were performed biweekly by replacing half of the media with fresh Neurobasal Medium. No antimitotic drugs were added to reduce glial proliferation. Cell cultures were maintained at $37°C$ in a 5% CO_2 humidified incubator (NuAire, Inc., Plymouth, MN).

Immunofluorescence

Cultured cells plated onto patterned samples or coverslips were rinsed with PBS (140 mM NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.15 mM KH_2PO_40) then fixed for 30 min at room temperature in freshly prepared 4% paraformaldehyde. Cells were then washed 2× in PBS and permeabilized by incubation in 0.25% Triton-X for 10 min followed by two more washes in PBS. To reduce nonspecific binding of antibodies, blocking agent (Dako, Mississauga, ON, Canada) was added to cells for 1 h at room temperature. Mouse monoclonal Map2 antibody (1:200 dilution) and goat anti-rabbit GFAP antibody (1:800 dilution) (both antibodies from Sigma) were incubated with cells overnight at $4.0°C$. The next day cells were washed 2× with PBS and incubated in the following secondary antibodies: Alexa Fluor 568 goat anti-mouse (1:400) and Alexa Fluor 488 goat anti-rabbit (1:800) (both secondary antibodies from Invitrogen, Burlington, ON, Canada) for 1 h at room temperature. Cells were rinsed 2× with PBS. Patterned samples were inverted onto coverslips containing a drop of fluorescent mounting medium. Mounted samples were stored at $4.0°C$ until time of imaging.

Dye Preparation

Bath Medium (BM) was prepared as follows: 140 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM HEPES, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, and 15 mM glucose.

Calcein-AM (Invitrogen, Canada, Burlington, ON, C-3 100), was used as an indicator to access cell viability. A 5 mM stock solution was prepared in DMSO with vigorous vortexing. The stock solution was then diluted to a 40 μM sub-stock in BM. Cells were subsequently stained using Calcien-AM at 5 μM.

Tetramethylrhodamine conjugate of wheat germ agglutinin (WGA; Invitrogen, W-849) was used to stain cell membranes. A 1.0 mg/mL WGA conjugate stock solution was prepared in BM and used at 4 μg/mL in BM to stain cells.

Dye Loading

Each sample or chip was placed in an individual well of a 24-well plate. 350 μL of 5 μM calcein were placed in each well before incubation for 30 min at $37°C$ in the dark. Calcein was then replaced by the same volume of WGA conjugate, followed by incubation for 30 min before rinsing to

minimize nonspecific background fluorescence. Finally, 450 μL NBM was added to each well.

Cell Imaging

Fluorescence and reflection images of samples were obtained using an LSM-410 Zeiss (Thornwood, NY) confocal microscope equipped with a Krypton/Argon laser (Melles Griot, Carlsbad, CA) and an LSM Tech, Inc. (Etters, PA) objective inverter. For each dye, excitation wavelength and emission filter were appropriately selected and images of both dyes were collected sequentially. Calcein was excited with the 488 nm wavelength of the laser and an emission filter with a bandwidth of 515–540 nm was used. For the WGA conjugate, the excitation wavelength of 568 nm and a long-pass emission filter with a cut-off at 590 was used. Reflection images were collected using the 568 nm line of the laser with no filter in front of the photomultiplier tube (PMT). Reflection and fluorescence images of the same field were merged using Northern Eclipse software (Empix, Mississauga, ON, Canada).

Results and Discussion

Brain Cell Growth on Unpatterned Substrates

Figure 2a shows a typical fluorescence image of brain cells growing on a glass substrate coated with PDL. Cells were stained with calcein to confirm their viability, and WGA conjugate was used to identify cell membranes. Typical of growth on a flat homogeneous substrate, brain cells attached and grew randomly with no organized placement. The development of neuritic processes appeared randomly.

PDMS Stamping of Poly-D-Lysine Patterns

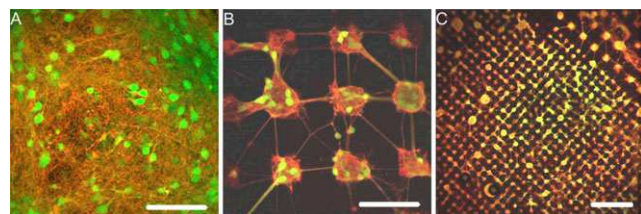Auger electron microscopy mapping (model PHI 700 Scanning Auger Nanoprobe) was used to chemically and

Figure 2. **a**: Brain cells adhered and grew randomly in culture on a glass cover slip uniformly coated with a layer of PDL. Fluorescent images were taken after 14 DIV. WGA–tetramethylrhodamine conjugate was used to stain cell membranes, which appear red. Calcein was used to label the cytosol of living cells which appears green. Scale bar: 100 μm. **b**: Brain cells 14 DIV cultured on a SiN surface stamped with a PDL pattern of 50 μm squares separated by 50 μm. Scale bar: 100 μm. **c**: As in (b) but at lower magnification, showing a larger field of the patterned substrate. Scale bar: 500 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]
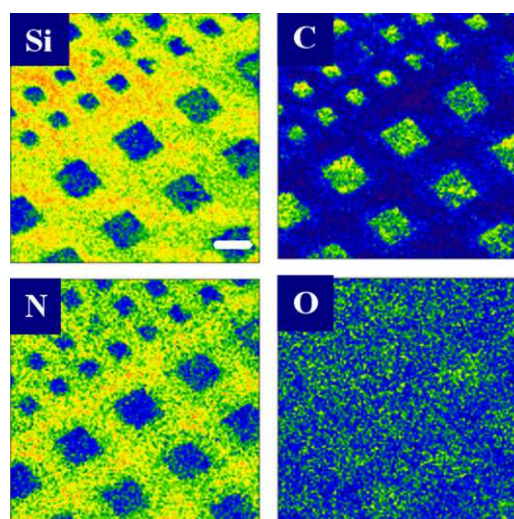

Figure 3. Scanning electron microscopy mapping of a SiN chip surface after transfer of PDL from PDMS stamps with 50 and 100 μm squares. The C_{KVV}, Si_{LVV}, N_{KVV}, and O_{KVV} auger transitions were measured separately. Images are $700 \times 700 \mu m^2$. Scale bar: 100 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

spatially characterize the surface of the stamped chip. The result is shown in Figure 3 for the C_{KVV}, Si_{LVV}, N_{KVV}, and O_{KVV} auger transitions. In this figure, PDL was transferred from a patterned PDMS stamp with 50 and 100 μm square features. For each of the images, a brighter color (increasing from blue to yellow) indicates an increase of the considered atomic species. In contrast with the rest of the sample (SiN), the patterned areas show strong decreases of the Si and N species, while they are enriched in O and particularly C species. This indicated good transfer of the PDL to the SiN surface. Such samples were typical of substrates used for brain cell adhesion.

Brain Cell Growth On Poly-D-Lysine Patterns

Brain cells were plated on PDL patterned substrates and cultured for several weeks. Figure 2b,c shows fluorescence images of cultures after 14 DIV. Cultures were co-labeled with viability and membrane dyes, which appear as green and red, respectively. Cell growth was confined to stamped areas (50 μm squares, with 100 μm spacing), indicating their preference for attachment and growth on PDL-treated surfaces over untreated SiN.

Effects of Pattern Dimensions on Cell Growth and Connectivity

Cryopreserved rat cortical neurons have a cell body diameter of 10–12 μm. Consequently, the size of the PDL-patterned areas will influence the number of neurons that will adhere and grow on individual squares. For neurochip interface
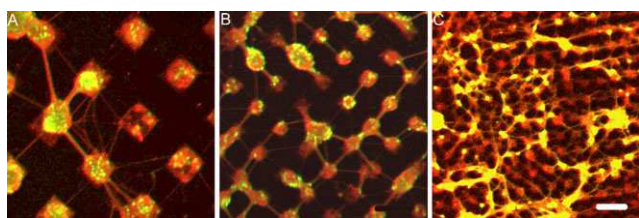
Figure 4. Fluorescence images of neurons grown 14 DIV on patterned substrates with: (**a**) 100 μm, (**b**) 50 μm, and (**c**) 25 μm PDL squares. WGA–tetramethylrhodamine conjugate was used to stain cell membranes, which appear red. Calcein was used to label the cytosol of living cells which appears green. Scale bar: 100 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

devices to work effectively, cells must be aligned with sensing features on the chip. In the case of a patch-clamp chip, cells must grow over microhole features in the substrate. In practice, this is not easily achievable. To find the best conditions to promote such growth, PDMS stamps were prepared with 25, 50, and 100 μm squares. Cell growth at 14 DIV on PDL patterns created using these stamps are shown in Figure 4a–c. The same concentration of brain cells was plated on each patterned sample.

For the 100 μm patterns (Fig. 4a), PDL squares were covered with 10 ± 3 cells ($n = 25$). In cases where the number of neurons per PDL area exceeded 12, large three-dimensional aggregates formed, covering most of the patterned area. For 50 μm patterns (Fig. 4b), 3 ± 2 cells ($n = 60$) adhered to the PDL areas and had isolated cells that could be identified. Finally, for the 25 μm squares, 1 ± 1 cells ($n = 60$) were observed on each area, while about 50% of the squares were vacant.

Based on these observations, we selected the 50 μm patterns to investigate further. Immunostaining was used to confirm the identity of neurons (MAP2-red) and astrocytes (GFAP-green) growing on the PDL-patterned SiN (Fig. 5). By 14 DIV processes extended between small groups of neurons isolated on a single square, to proximal populations of neurons on neighboring squares, such that 3 ± 1 processes extended from each neuronal subpopulation ($n = 25$).

Alignment of Neurons With Substrate Microhole Feature

Ultimately, monitoring of ion channel activity from neuronal networks on patch-clamp chips will require that isolated cells be aligned over microholes and allowed to mature in culture. To accomplish this alignment an aligner bonder was used to align the patterned area with microholes features on the chip substrate. A PDMS stamp with 50 μm squares, separated by 50 μm was placed over two 3 μm diameter holes, 100 μm apart and a force of 500 g was applied to the stamp for 60 s. Figure 6a,b shows images of neurons growing on this region of the substrate after 14 DIV.
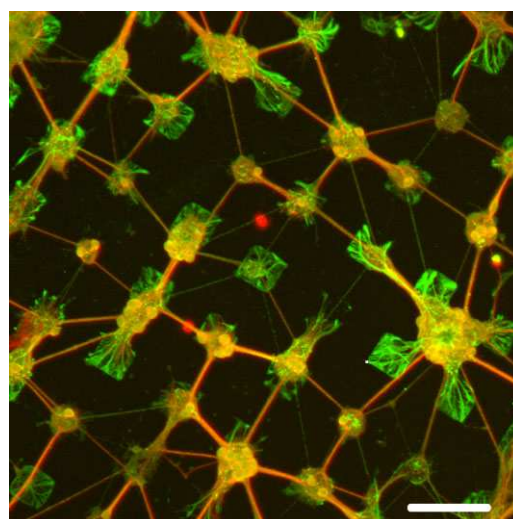

Figure 5. Immunofluorescence staining of cultured brain cells (14 DIV) on SiN surfaces stamped with a PDL pattern with 50 μm squares repeating with 50 μm of separation. Neurons stained red with MAP2, while astrocytes stained green with GFAP. Note the neuritic processes that extend between small groups of neurons isolated on a single square, to proximal populations of neurons on neighboring squares. Scale bar: 100 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

The image is a superimposition of reflection and fluorescence images. Neuronal cells are growing over the patterned motif. A slight shift of the pattern has occurred while stamping on the lower microhole region, probably due to deformation of the PDMS stamp while applying pressure. However, the upper microhole region is covered with cells and a neuron is growing directly over the microhole.
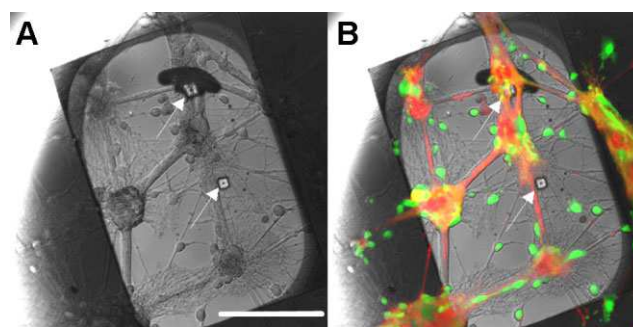

Figure 6. Silicon nitride chip with two microholes (100 μm apart, 3 μm diameter), stamped using PDMS squares of 50×50 μm², 50 μm apart inked in PDL. The microfluidic channels would underneath the surface of the silicon nitride and are not visible here. The stamp was aligned with the microholes using an aligner-bonder and stamped with a 500 g force for 1 min. After 14 DIV, cells bodies were labeled with Calcein-AM (green) and membranes with WGA conjugate (red). **a**: Reflection image: The microholes are indicated by arrows. **b**: Superimposition of the images obtained in reflection mode and fluorescence. A neuron is positioned over the upper microhole. Scale bar: 100 μm. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Conclusions

Surface functionalization was used to grow isolated cryo-preserved neurons into a patterned network. PDL was transferred to a silicon nitride surface with a PDMS stamp to promote cell adhesion. We demonstrate the utility of such a procedure to control the growth of neurons on a surface conducive to the fabrication of neurochips to interrogate electrical activity. The number of neurons per patterned area can be adjusted by controlling the dimensions of the patterns. With 25 µm squares, only one or two neurons will adhere making them easy to identify. We also show that processes develop between neurons located on different squares even in the absence of guidance lines between squares. A distance of 100 µm separating the squares was found to be suitable to control and direct the formation of neurite processes between neighbors. Finally, we show that neurons can be cultured directly over microhole features on the SiN chip substrate, and that these neurons extend processes to neighbors. The ability to culture neurons over these microholes is critical for the subsequent development of planar patch-clamp neurochips that interrogate network function. Patterned or guided neuronal growth aligned with stimulating and recording interfaces, such as planar patch-clamp or MEAs, will enhance the study of synaptic plasticity by establishing simple defined networks where pre- and postsynaptic communication can be controlled, studied and applied to advance computational neuroscience.

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