Application of Polymer Microstructures with Controlled Surface Chemistries as a Platform for Creating and Interfacing with Synthetic Neural Networks

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***Abstract* - Hybrid silicon-polymer chips with microscale topography and contrasting surface chemistries were created using a novel combination of soft lithography techniques, and evaluated for their suitability as a platform to guide cell attachment, growth and differentiation. These capabilities are all necessary to synthesize organized neural networks *in vitro*. Neurons developed on these chips exhibit patterned growth and functional communication, evidenced by spontaneous and stimulated action potentials and intracellular calcium oscillations. Integration of planar patch-clamp technology into this platform to create a novel long-term interface is presented, with the formation of a high resistance (giga-ohm) electrical seal between the cultured cell membrane and the perimeter of a micron-sized orifice integrated into the substrate. This platform has potential as a tool to investigate mechanisms underlying neuro-genesis, synaptic transmission, and neuro-degeneration. It may also lead to the development of more sophisticated and functionally relevant bioassays and high throughput electrophysiological screening, thus speeding the drug discovery process.**

I. Introduction

Neural networks in the brain are formed by the concerted interaction of millions of neurons through fundamental units of connectivity called synapses. To form networks, neurons interact with the extracellular matrix, a highly functional bio-surface responsible for positioning, guiding, and connecting neuronal and non-neuronal cells. The fabrication of platforms that promote neurogenesis, guide neurite growth, and foster neuronal communication is an essential step towards the development of synthetic neural networks. Cues, such as surface chemistry (Castner & Ratner, 2002), topography (Wilkinson et al., 2002; Mahoney et al., 2005) and mechanical properties (Wong et al., 2004) can be manipulated to influence cell growth on a substrate. Patterned microstructures have been used as tools to position cells (Ratner & Bryant, 2004), and alterations to nano-scale surface topography, made by etching silicon wafers, have been used to guide growth through interactions with growth cone filopodia (Fan et al., 2002). Microcontact printing methods, such as lithographically applied polylysine-conjugated laminin patterns, have also been used to guide neuron attachment and axonal outgrowth (James et al., 2000).

Developing interfaces with synthetic cell networks will lead to a better understanding of synaptic transmission and plasticity. Towards this objective, neurons have been grown on electrode arrays using poly-L-lysine lines printed in close proximity to contact elements (Kam et al., 2001). Furthermore, noninvasive electrical stimulation and recording from a single somatosomatic synapse between large snail neurons strategically positioned on a semiconductor chip have been used to demonstrate synaptic potentiation (Kaul et al., 2004).

Understanding processes underlying synaptic transmission requires understanding the involvement of its most basic elements, ion channels. These dynamic protein nanomachines span the cell membrane and selectively regulate ion flow by changing structural conformation in response to either specific ligand activation or transmembrane voltage gradients. The use of patch-clamp technology to study ion channels has provided a wealth of information regarding function, and has recently been

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applied to study synaptic plasticity in micropatterned neuronal networks (Vogt, 2004). However, conventional patch-clamp methods are complex and labour-intensive, with notoriously low throughput. This technology has recently been advanced by incorporating the membrane-pipette interface into a planar glass microchip (Fertig et al., 2002). Since ion channels are potential targets for therapeutic intervention (Belusa et al., 2002), this new technology has value as a high throughput / high information content drug screening assay, and has already been commercialized for use with isolated cells in suspension (e.g. Aviva Biosciences). However, planar patch-clamp technology has not been applied to cells grown randomly in culture, much less to synthetic networks of neurons. Advances in this direction would be tremendously useful, since ion channel activity could be monitored simultaneously in multiple synaptically-connected cells in a well-defined circuit for extended durations. This would represent a far more sophisticated alternative to pharmacological screening that uses isolated cells in suspension. However, the challenge is not trivial. A highly resistive (gigohm) electrical seal must form between the cultured cell membrane and the circumference of a 2-5 µm orifice in the planar glass microchip, to permit detection of the picoamp currents that flow through ion channels. At the same time a substrate interface is needed that will position cells, encourage adhesion, and promote guided neural growth and connectivity.

We present steps towards achieving this goal: 1) A methodology to fabricate microstructures with precise topographical features and contrasting surface chemistry characteristics. 2) An assessment of the efficacy of these substrates as a platform to develop functional synthetic neural networks. 3) A novel integrated patch-on-chip interface design to record ion channel activity in cultured neurons in a synthetic network.

II. Main Results

A. Microstructure Fabrication and Surface Chemistry Modification

The general protocol used for the rapid and efficient fabrication of the polymer microstructures and their subsequent chemical patterning is outlined in Figure 1 (Faid et al., 2005). A flexible stamp was made by replicating polydimethylsiloxane (PDMS), over a master mold following published procedures (Bensebaa et al., 2004). The microstructures on the master mold consisted of either 5, 10, 25, 50 or 100 µm wide recessed lines spaced by the same width or square pillars of 5, 10, 25, 50 or 100 µm spaced by the same dimensions. The replicated PDMS stamp (Fig. 2a,b**)** exhibited features complementary to those of the master SU8-silicon mold. The PDMS stamp was rendered hydrophilic by creating -OH groups on the surface in an air plasma reactor for 1 min at 2 x 10^{-1} mbar. The patterned PDMS-OH substrate was immersed overnight in 100 mM heptadeca-fluoro-1,2,2,2,-tetrahydro-decyl-triethoxysilane (HFS) solution in ethanol. The fluorosilane-modified patterned PDMS (PDMS-CF3) (Fig. 1b) was rinsed with ethanol after incubation and immersed in H_2O for polycondensation of the siloxane groups, and yielded highly hydrophobic substrates with a contact angle of 112.8°, indicating that the fluoro-silane derivative was covalently attached to the surface of the secondary PDMS mold.

The PDMS stamp was put in conformal contact (Fig. 1c,d) with an uncured PDMS film spin-coated at 2000 rpm to a thickness of 25-30 µm on a glass coverslip, and thermally cured at 90°C for 2 h. After curing, the two PDMS stamps were effortlessly separated due to fluorination of the first stamp. The features transferred to the polymer substrate were complementary and virtually identical to those of the first stamp (Fig. 2c,d). The imprinted PDMS was rendered hydrophilic in an air plasma reactor as shown. The imprinted PDMS-OH was stored in de-ionized water prior to further modification in order to preserve its hydrophilic character. A flat PDMS surface, obtained by thermal polymerization of a PDMS prepolymer in a polystyrene Petri-dish, was inked with HFS for 30 minutes, dried with nitrogen, and then put in conformal contact with the top of the imprinted PDMS-OH for 90 min (Fig. 1f,g) using a modified methodology (Li et al., 2001).

After removing the stamp, the imprinted PDMS now had a dual character: hydrophobic (fluorinated) on the upper surface and hydrophilic (silanols) inside the wells or channels (Fig. 1h).

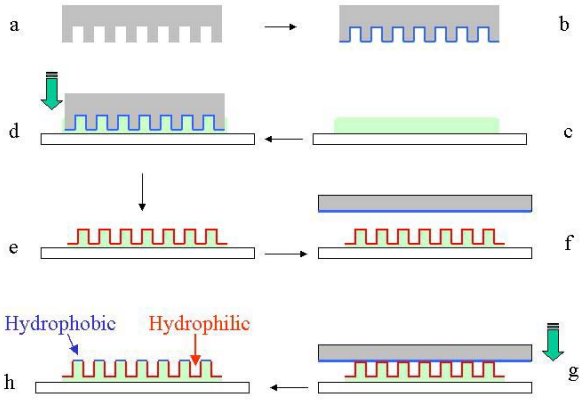

Figure 1. Fabrication and chemical patterning of PDMS microstructures. a) PDMS stamp with 10 µm deep channels hydrophilized by air plasma to introduce silanol groups on the surface. b) Chemisorption of a fluoro-siloxane derivative on the surface and curing in aqueous solution to form a highly hydrophobic surface. c) Spin coating of thin layers of uncured PDMS precursor on glass substrate. d) Imprinting of the microstructures by the fluorinated stamp and curing by heating. e) Hydrophilic microstructures created by air plasma. f) Chemical patterning of the PDMS microstructures through the introduction of hydrophobic functional groups. g) Transfer of the fluorinated siloxane and curing. h) PDMS microstructures with a dual hydrophobic-hydrophilic character.

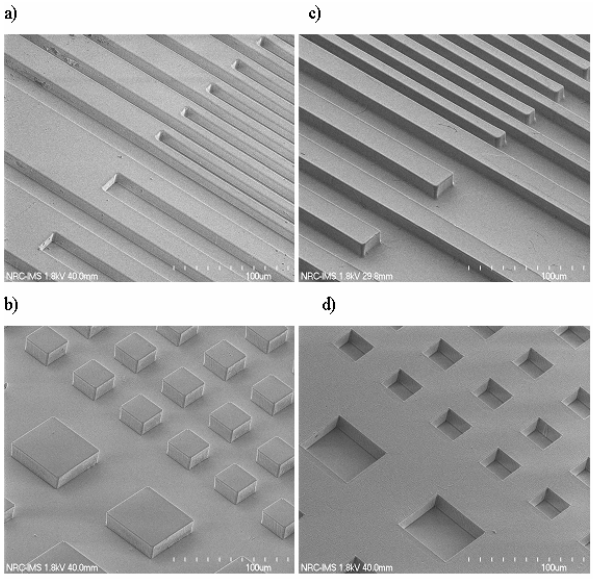

Figure 2. Scanning electron micrograph pictures of fluorinated PDMS stamps with a) channels and b) pillars used as secondary molds and c) and d) their respective replicated PDMS microstructured substrates.

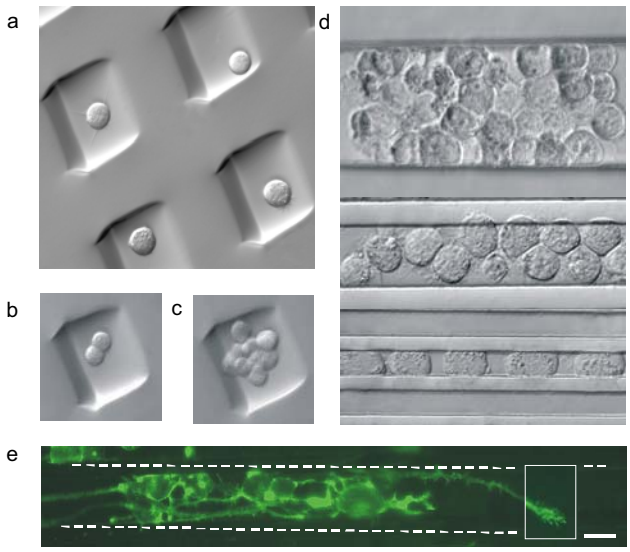

Figure 3. Microstructures and surface chemistry modifications effectively position N2a cells and guide proliferation. a) Hoffman contrast image showing that 50 µm square hydrophilic wells locate N2a cells and promote rapid attachment. b) Cells undergo division within 10 h and **c**) a colony has formed within 48 h. d) Similarly, N2a cells position and proliferate in hydrophilic channels 50, 25, and 10 µm wide (top to bottom). Channels narrower than the cell diameter alter cell shape and attenuate proliferation after a few divisions. e) F-actin immunostaining shows N2a cells extend processes along the edge of a 25 µm channel as they differentiate. Inset: deconvolved image of a growth cone guiding the neurite within the channel. Dashed lines represent the boundary of the channel.

B. Patterned PDMS Substrates Provide a Scaffold for Cell Positioning, Guidance, and Proliferation

The efficacy of these fabricated substrates as a platform to create simple neural networks was evaluated. N2a neuroblasts (ATCC, Manassas, VA) were cultured on the test substrates. Similarly, cortical neurons from embryonic day 13 or 17 (E13 or E17) mice were isolated (Tropepe et al., 1999) and plated accordingly.

Topographic features of the substrate effectively positioned N2a neuroblasts in squares or channels, (Fig. 3a,d) and the hydrophilic nature of these microstructures promoted selective cell attachment after plating within the boundary of the microstructure. A minimum ratio of 1.5:1 (channel width : cell diameter) was required for N2a cells to proliferate. Cells seeded in 10 µm channels displayed attenuated proliferation and oval morphology. Hence, varying channel width is a potentially useful tool to differentially control growth in a synthetic neuronal network. Histology using F-actin antibody showed neurites guided by their growth cone within the confines of the channel (Fig. 3e).

Neural progenitors from E13 mouse cortex were also cultured on microchannel-patterned PDMS substrates. Neurons developed within the confines of the hydrophilic channels and displayed organized parallel architecture similar to that seen in brain substructures (Fig. 4). Unlike N2a cells, they were not hindered in the 10 µm wide channels (Fig. 4c). Remarkably, in contrast to neurons, astrocytes were not influenced by topological or chemical patterning features and grew randomly (Fig. 4d).

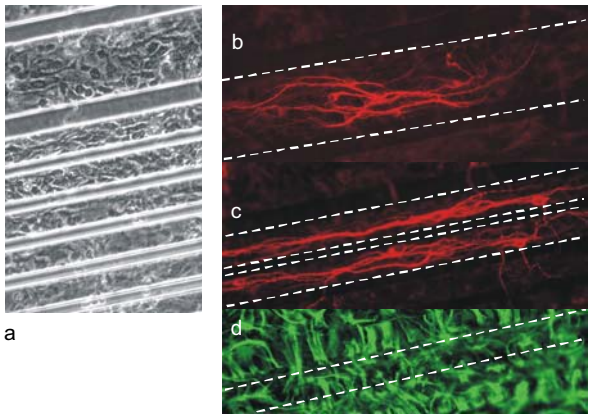

Figure 4. Microchannels and surface chemistry modifications effectively position cultured E13 neurons and guide growth. a) Hoffman contrast image showing neurons grown on 50, 25, and 10 µm wide hydrophilic channels (top to bottom). b) MAP-2 staining showing neurons (red) growing in 50 µm, c) or 25 µm wide channels. Dashed lines represent the boundary of the channel. d) GFAP-positive astrocytes (green) are not guided on the same patterned substrate.

C. Assessment of Functionality of Neurons Grown on Patterned Substrates

The excitability and connectivity of cells grown on PDMS substrates with 25 µm wide hydrophilic grooves was examined using N2a cells and E17 cortical rat neurons (Fig. 5a). Intracellular calcium was monitored using Fluo-3 and Fura-red in combination with ratiometric fluorimetry (Fig. 5b). Brief trains (3 s, 10 Hz) of current were applied at 30 s intervals using a bipolar tungsten electrode placed at one end of a PDMS groove. This stimulation paradigm was designed to induce multiple action potentials, sufficient to produce large, prolonged calcium oscillations, detectable with low-speed imaging. Calcium oscillations were recorded in E17 cortical neurons cells propagating along the grooves, demonstrating excitability and functional connectivity. Representative results from 1 of 3 experiments are shown in Figure 5b. Cells in adjacent channels, distal to the stimulating electrode, were unresponsive, indicating directional propagation of the signal. In similar experiments, N2a cells were unresponsive (n=5, data not shown).

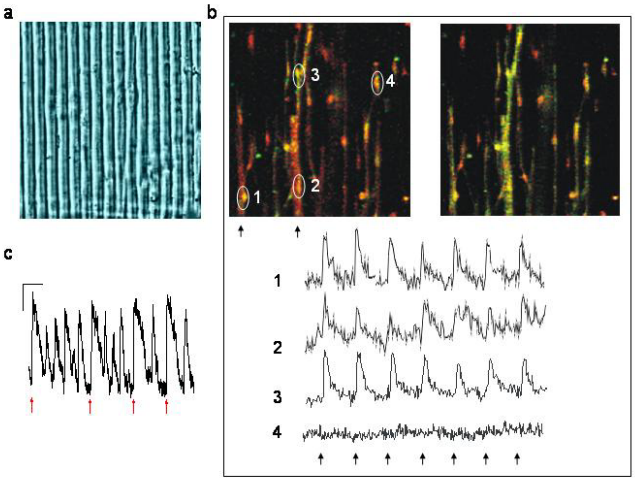

Figure 5. Simple synthetic neural networks display excitability and connectivity on patterned PDMS substrates. a) Phase contrast image of a PDMS substrate, showing 25 µm wide hydrophilic channels to guide neural growth. Oriented E17 neurons can be observed in these channels. b) Fluorescence images taken 20 s apart show neurons loaded with calcium-sensitive dyes. A stimulating electrode was positioned at one end of the PDMS channels (arrows). Traces 1-4 show relative changes in intracellular calcium concentration at numbered regions of interest identified in the top left image. Stimulation was applied at 30 s intervals (indicated by arrows below traces) to induce calcium oscillations. c) Voltage oscillations recorded from an E17 neuron, using whole-cell patch-clamp. Spontaneous, cyclical waves, composed of multiple action potentials, were observed. It was possible to briefly synchronize this activity using current stimulation (indicated by arrows) through the patch pipette. Scale bar: 10 mV, 1 s.

Membrane potential oscillations were recorded in E17 cortical neurons using whole-cell patch-clamp. Spontaneous, cyclical waves of membrane depolarization, composed of multiple action potentials, were observed, suggesting hyperexcitability, possibly due to a high degree of connectivity in a region at the periphery of the microchannels. Given this hyperexcitability, it was not possible to accurately measure membrane resting potential, but the most polarized potentials recorded were between -43 and -58 mV. It was possible to briefly synchronize this activity using current stimulation, delivered through the patch pipette. Representative results from 1 of 4 experiments are shown in Figure 5c. In similar experiments, using N2a cells, the membrane resting potential was -23 ± 3 mV and the neuroblastoma cells were unresponsive to stimulation (n=4, data not shown).

D. Patch-on-Chip Interface with Cultured Neurons in a Synthetic Neural Network

Planar patch-clamp technology has not been applied to cells grown randomly in culture, much less to synthetic networks of neurons. Towards this goal we have designed a novel integrated patch-clamp interface to monitor ion channel activity in neurons synaptically connected in a patterned network (Mealing, 2004), which is schematically represented in Figure 6. This platform will permit the extended and non-invasive recording of single ion channel activity in the "cell-attached configuration". Rupturing the membrane spanning the orifice, using a mechanical or voltage pulse, or by microfluidic application of a pore-forming antibiotic, permits whole-cell recording of populations of ion channels. In addition, a host of fluorescent probes could potentially be introduced into the cytoplasm and monitored simultaneously, using integrated fibre optic sensing. Developmental work is ongoing.

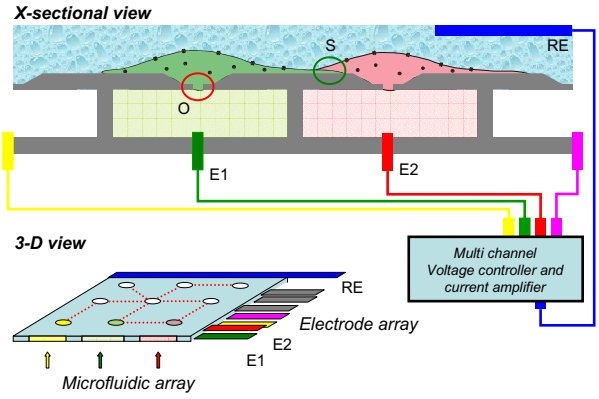

Figure 6. Schematic drawing of novel planar patch-clamp interface for neurons in a synthetic network grown on a patterned substrate. Neurons are positioned using locating wells (similar to those shown in Figure 3a) over 2-5 µm diameter orifices (O) which individually communicate with a specific subterranean fluidic channel housing an electrode. A high resistance seal between the cell membrane and the perimeter of the orifice ensures detection of current flow through ion channels in the membrane patch covering the orifice. Neurite growth is directed towards neighbours using guidance pathways (dotted lines), similar to the patterned channels shown in Figure 5a. Electrodes in the subterranean microfluidic array are connected to a multichannel voltage controller/current amplifier and referenced to an electrode in the upper perfusion chamber.

Advances in integrating patch-clamp interfaces with neural networks will ultimately enable researchers to simultaneously monitor ion channel function in multiple, synaptically-connected cells in a well-defined circuit for extended durations. This will provide a powerful research tool to investigate synaptic function and network signalling. Furthermore, from a pharmacological screening perspective, it presents an attractive alternative to fluorescence intensity plate reader assays, or to electrophysiological assays using isolated cells in suspension.

III. Conclusions

A potentially cost-effective method to fabricate microstructured polymer substrates having wells, channels, and orifices with contrasting hydrophilic and hydrophobic surfaces is described. We show that these substrates are effective tools to position neural cells, selectively promote their attachment and proliferation, and guide their connectivity with neighbours. Excitability and functional connectivity is demonstrated in cortical neurons grown on these microstructured substrates, using electrophysiological and ion imaging endpoints. Hence, we have produced a basic synthetic neural network capable of synaptic communication. Finally, a novel design for an integrated patch-clamp interface with this network is presented and its potential applications are discussed.

References

1. R. Belusa,. J. Eriksson, J. Ohisson, BioSeeker Group Research Report, “Ion channels as therapeutic targets for multiple diseases,” www.bioseeker.com, (Nov 2002)

2. F. Bensebaa, P. L’Ecuyer, K. Faid, C. Py, T. Tague, R.S. Jackson, Appl. Surf. Sci., (in press)

3. D. Castner, B. Ratner, “Biomedical surface science: Foundations to frontiers,” Surf. Sci. vol. 500, pp. 28-60 (2002)

4. K. Faid, R. Voicu, M. Bani-Yaghoub, R. Tremblay, G. Mealing, C. Py, R. Barjovanu, “Rapid fabrication and chemical patterning of polymer microstructures and their applications,” Biomed. Microdev. (in press)

5. F. Fan,. S. Cui, Q.Hou, L.Xu, Chen I. Lee, “Culture of neural cells on silicon wafers with nanoscale surface topography,” J. Neurosci. Met. vol. 120, pp. 17-23 (2002)

6. N. Fertig, R. Blick, J. Behrends, “Whole cell patch clamp recording performed on a planar glass chip,” Biophys. J. vol. 82, pp. 2831-2 (2002)

7. C. James, R. Davis, M. Meyer, A. Turner, S. Turner, G. Withers, L. Kam, G. Banker, H. Craighead, M. Isaacson, J. Turner, W. Shain, “Aligned microcontact printing of micrometer-scale poly-L-lysine structures for controlled growth of cultured neurons on planar microelectrode arrays,” IEEE Trans. Biomed. Eng. vol. 47, pp. 17-21 (2000)

8. L. Kam, W. Shain, J. Turner, R. Bizios, “Axonal outgrowth of hippocampal neurons on micro-scale networks of polylysine-conjugated laminin,” Biomat. vol. 22, pp. 1049-54 (2001)

9. R. Kaul, N. Syed, P. Fromherz, “Neuron-semiconductor chip with chemical synapse between identified neurons,” Phys. Rev. Lett. vol. 92(3), 038102-1 (2004)

10. Y. Li, T. Pfohl, J. Kim, M. Yasa, Z. Wen, M. Kim, C. Safinya, "Selective surface modification in silicon microfluidic channels for micromanipulation of biological macromolecules," Biomed. Microdev. vol. 3, pp. 239, (2001)

11. M. Mahoney, R. Chen, J. Tan, W. Saltzman, “The influence of microchannels on neurite growth & architecture,” Biomat. vol. 269(7), pp. 771-8 (2005)

12. G. Mealing, U.S. Provisional Patent #601,568,220, Synthetic cell network/chip interface (2004)

13. B. Ratner, S. Bryant, “Biomaterials: where we have been and where we are going,” Annu. Rev. Biomed. Eng. vol. 6, pp. 41-75 (2004)

14. V. Tropepe, M. Sibilia, B. Ciruna, J. Rossant, E. Wagner, D. Van der Kooy, “Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon,” Dev. Biol. vol. 208, pp. 166-88 (1999)

15. A. Vogt, G. Wrobel, W. Meyer, W. Knoll, A. Offenhausser, “Synaptic plasticity in micropatterned neural networks”, Biomat. (in press)

16. C. Wilkinson, M. Riehle, M. Wood, J. Gallagher, A. Curtis, “The use of materials patterned on a nano- and micro-metric scale in cellular engineering,” Mat. Sci. Eng. vol. 19, pp. 263-269 (2002)

17. J. Wong, J. Leach, X. Brown, “Balance of chemistry, topography, and mechanics at the cell-biomaterial interface”, Surf. Sci. vol. 570, pp. 119-133 (2004)