Vacuum soft lithography to direct neuronal polarization†

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The ability to coat surfaces with pre-determined patterns of biomolecules by soft lithography has found use in areas ranging from fundamental biology to translational medicine, such as tissue engineering and diagnostics. However, existing surface patterning techniques (*e.g.*, microcontact printing and traditional lithography) are unable to pattern several biomolecules in a single step. Here we introduce a simple method to simultaneously pattern multiple biomolecules in complex two-dimensional configurations onto substrates with better than 2 μm resolution. This protocol, termed vacuum soft lithography, utilized below ambient pressures temporarily stored within a removable microfluidic template to expose specific regions of a substrate to multiple biochemical solutions. We demonstrate the utility of this vacuum soft lithography technique by fabricating a multi-component array that directs the adhesion, polarization, and neurite guidance of primary hippocampal neurons.

Nervous system formation involves migration of immature neurons to specific positions within the body. Once the immature cells find their positions, they continue to develop by sprouting several neurites that extend away from the cell body to probe the surrounding microenvironment. As maturation proceeds, one neurite develops into an axon, the conduit for sending electrical and chemical communications to other cells, while the remaining neurites develop into dendrites to accept incoming communications. This developmental process of neuronal polarization can be influenced by intracellular factors such as organelle positioning[1] as well as extracellular cues that initiate specific signaling mechanisms.[2] The growth cone at the tip of the axon guides the extending axon towards its intended target cell. This structure can respond to both biochemical[3] and biophysical[4] guidance cues that allow it to navigate a path to reach the appropriate target. A critical field of study within neurobiology is dedicated to understanding how these fundamental phenomena occur both within the context of a single cell and within the nervous system as a whole. As we learn more about the chemical and physical cues affecting neuronal growth and behavior, greater opportunities arise to use these cues to engineer living neural networks. These living neural networks can, in turn, be used to further study neural development and to investigate ways to prevent neurodegeneration or to repair damaged nervous tissue.[4,5]

Numerous methods have been used to control and manipulate the microenvironment of a neuron, including physical,[4] chemical,[3,6-8] electrical,[9] and optical[10] perturbations. Each of these methods offers the ability to affect and/or detect the activity of a cell, but none are yet capable of truly controlling the growth of a complex neural network. To achieve the directed growth of a neural network with pre-determined geometry, three separate modes of cell control must be exerted: (1) the cell body must be positioned at a discrete location, (2) the cell must polarize to extend an axon in a specific direction, and (3) the guidance of the axonal and dendritic processes must be extended along distinct and separate paths. While several groups have demonstrated proficiency in one or two modes, so far only Kam *et al.* have been able to accomplish all three by combining multiple microcontact printing steps, which requires difficult manual alignment of each individual pattern.[11] Other techniques have been unable to create patterns with this length-scale resolution, are limited in the size of the total patterned area, or are limited to a single patterned component.[12–17]

Here we demonstrate a biochemical patterning method that achieves all three modes of cell control by employing a new, one-step, vacuum soft lithography technique. Complex patterns of biomolecules are physically adsorbed onto glass slides using a removable polydimethylsiloxane (PDMS) microfluidic template. This simple method uses the gas permeability of PDMS to fill circuitous and dead-end microfluidic channels. Upon removal from vacuum, degassed PDMS templates store a negative pressure relative to atmosphere that can be used to pull solutions through closed microfluidic channels (Fig. 1). The method of using degassed PDMS molds to pull fluid through microfluidic channels without external pumping was first reported by Hosokawa *et al.*[18–20] Previously, this technique has been used only for mixing of reagents for microfluidic immunoassays. To the best of our knowledge, this is the first use of this technique to pattern surfaces with multiple bioactive molecules.

The simplicity of this filling method is schematically outlined in Fig. 1A. First, a PDMS microfluidic chip that is reversibly adhered to a glass coverslip *via* conformal contact is subjected to vacuum. After removal of the vacuum, the channels quickly equilibrate back to atmospheric pressure while a vacuum is retained within the PDMS due to the slow diffusion of air molecules through the elastomer.[20] Drops of various solutions (1–5 μl) are placed on top of the inlet ports to seal off one end of the channels. The other ends of the channels are sealed either by placing a piece of tape across the outlet ports or by pipetting additional solution drops on top of the outlet ports. As the

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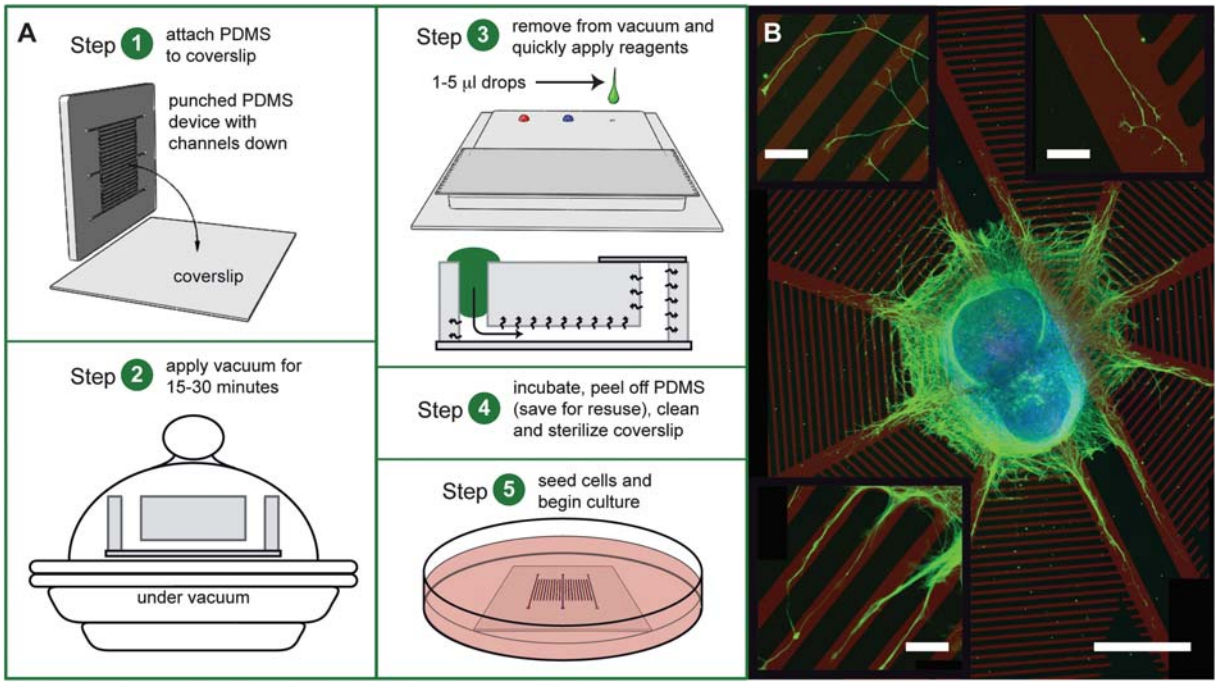

Fig. 1 (A) Sequence of vacuum soft lithography patterning. In step 3, internal PDMS pressure, which is below ambient, slowly recovers after device removal from vacuum and pulls the solution through the channel. Small arrows denote the movement of gas molecules. (B) Two clusters of primary chick dorsal root ganglion neurons adhere and extend neurites along a striped pattern of laminin. Cell nuclei are in blue (DAPI) and neurites are in green (β-tubulin). Inset scale bars are 50 μm; primary scale bar is 500 μm.

device equilibrates, air molecules are pulled into the PDMS from the channels, creating a negative pressure relative to ambient within the channels, which draws in the solutions (Fig. 1A and Video S1†). The solutions continue to proceed through the channels until all the channels are filled or the PDMS device has fully equilibrated back to atmospheric pressure.

To demonstrate the utility of vacuum soft lithography for applications in neuronal patterning, we first produced patterned stripes of laminin, a major glycoprotein basement membrane component known to stimulate neurite outgrowth, mixed with fluorescent bovine serum albumin for visualization of the patterned area. Laminin striped patterns have been shown previously to adhere neurons and guide neurite outgrowth.[4,5] The PDMS device was reversibly adhered to a glass coverslip *via* conformal contact and then filled with a laminin solution to allow physical adsorption of the laminin onto the exposed substrate regions. After patterning laminin, the PDMS mold was removed, the substrate was rinsed, and a cluster of chick dorsal root ganglion neurons was placed near the center of the pattern. Consistent with previous reports, we observed excellent adhesion of the cell clusters on the patterned laminin substrates and neurite extension along the laminin stripes over hundreds of microns (Fig. 1B). Therefore, this patterning technique is cyto-compatible and suitable to create single-component, striped protein patterns similar to those that have been previously reported using other patterning techniques.[4,5]

Next we demonstrated the use of this technique to simultaneously pattern multiple biomolecules on a single substrate with sub-cellular resolution. This technique is able to overcome two critical challenges inherent in the microfluidic patterning of bioactive molecules for potential neural circuit applications: (i) long channels with large aspect ratios and (ii) dead-end channels or features. The first issue is the extreme channel aspect ratios that are required in this device. In order to produce a multi-component pattern across a large experimental surface area, the length of the channel must be quite long (in this case, 390 mm) while the width and height are four to five orders of magnitude smaller (height 1 μm and width 5–30 μm). As a result, the fluidic resistance is extremely large, on the order of 10^{16} cP cm^{-3}, which greatly complicates channel filling using traditional positive pressure methods. The second limitation overcome with this technique is the ability to fill "dead-end" channels, which commonly go unfilled using other techniques. To address specific sites of each neuron with sub-cellular resolution, the multi-component pattern required the use of many dead end features (Fig. 2).

In addition to overcoming these difficulties, an added benefit of this technique is the very low sample volumes required. Because the solutions are applied directly to the inlets without the use of tubing or syringes, only a few microlitres are required per channel, saving wasted solution that would otherwise be present in the dead volume of the tubings. Many of the biomolecules useful for cell patterning can be expensive and/or difficult to obtain in large quantities; therefore, the ability to only use a few microlitres per chip is a great advantage. This method is simple, reproducible, fast, and cost saving for any patterning application.

We have used this technique to create large arrays (up to 2850 repeated elements on a single 22 mm × 30 mm coverslip) of three bioactive molecules embedded in an adsorbed polypeptide matrix with 2 μm resolution (Fig. 2 and S1, Video S1†). Primary rat hippocampal neurons plated onto these substrates aligned into ordered arrays, initiated axons at specified locations, and sprouted axons and dendrites that followed pre-determined paths (Fig. 3). Poly-L-lysine (PLL) is a positively charged polymer that is commonly used to non-specifically promote neuronal cell-body adhesion *via*

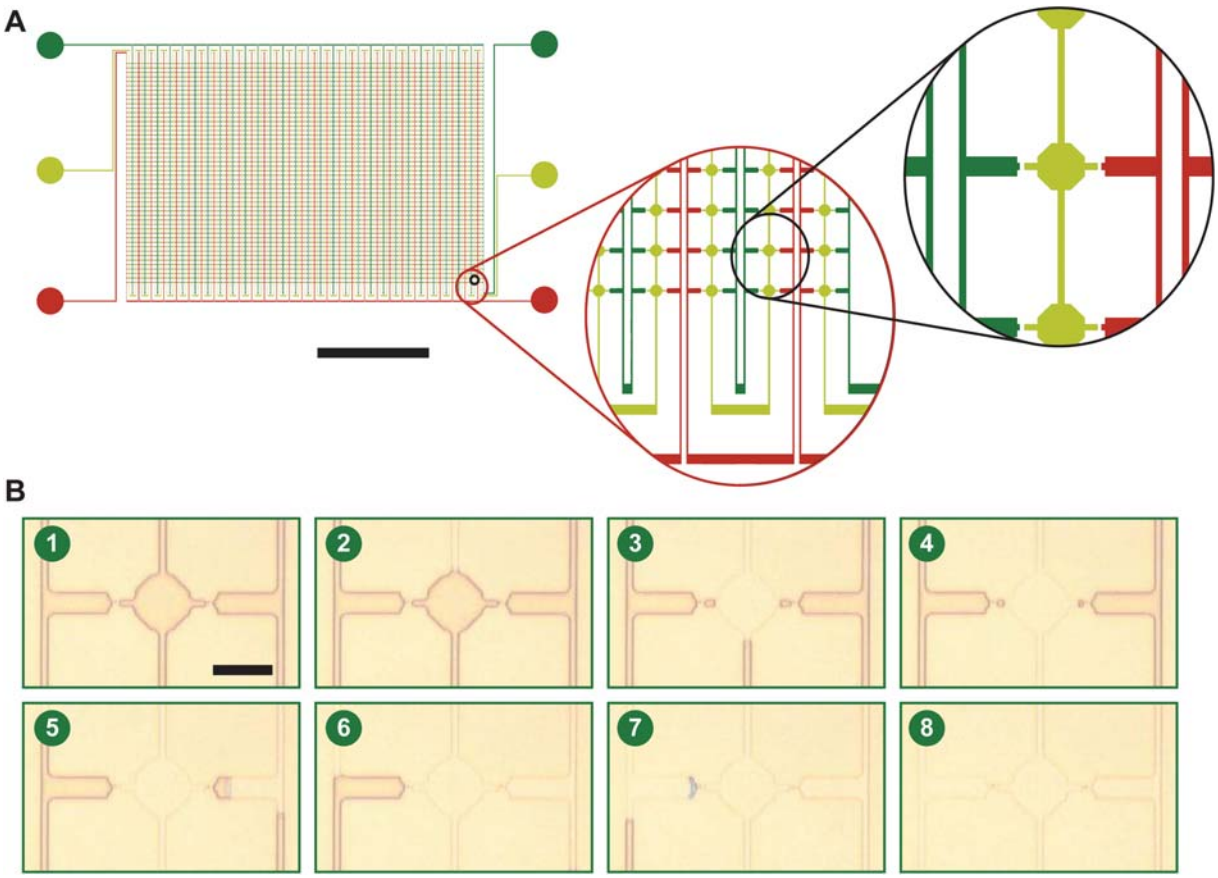

Fig. 2 Single-step, multi-component filling of 390 mm long channels. (A) Design of the microfluidic device. The design consists of three channels that serpentine back and forth to create an array of 2850 repeated elements. Scale bar is 3 mm. (B) Sequence of images from a video recording the filling of dead-end channels (Video S1†). The flow of solution is from top to bottom. Scale bar is 35 μm.

electrostatic interactions.[12,21] PLL patterns produced by soft lithographic micro-contact printing previously have been shown to adhere neurons and guide neurite outgrowth in a non-polarized manner.[12] Here, arrays of PLL "islands" were patterned to confine the adhesion of immature neuronal cell bodies to an array of precise locations that were "addressed" by additional biomolecule patterns to influence axon/dendrite specification, outgrowth, and guidance. Cyclic adenosine monophosphate (cAMP), a cytosolic second messenger that promotes axon initiation,[5] axon growth,[22] and axon guidance,[23] was chosen to induce polarization and axon path-finding. Similar to

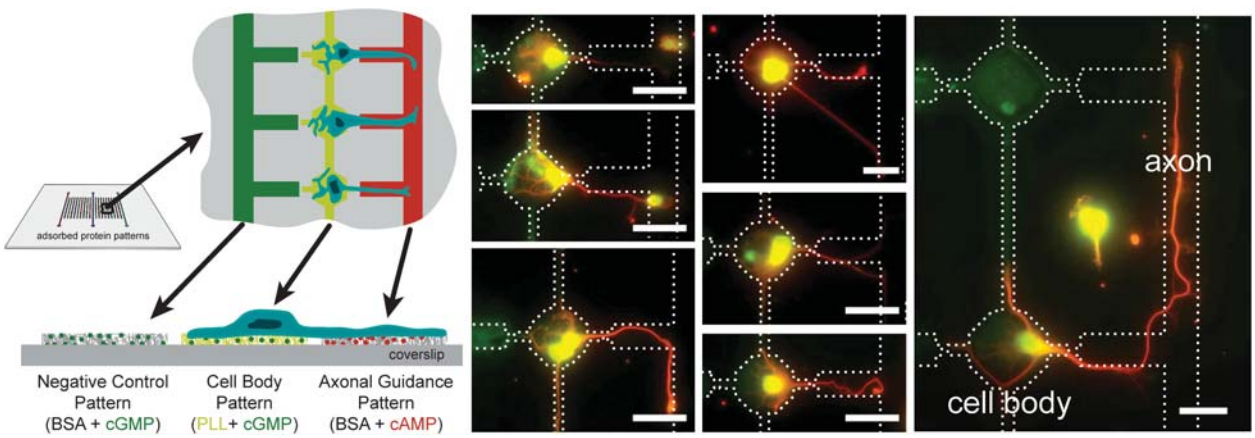

Fig. 3 Multi-component patterns with sub-cellular resolution direct cell body placement, neuronal polarization, and axon guidance of hippocampal neurons. (Left) Schematic of patterning scheme showing the relative locations of the negative control pattern, cell body pattern, and axonal guidance pattern. (Right) Guidance patterns are outlined with dotted lines (MP-cGMP, green). Confocal microscopy of several neurons growing on the multi-component patterns shows cell bodies (DAPI, yellow) localize on the cell body pattern. Neurites (β-tubulin, red), contacting the axonal guidance pattern uptake MP-cAMP, develop into axons, and elongate to follow the guidance pattern. In contrast, the other neurites remain on the cell body pattern. All scale bars are 30 μm.

cAMP, cyclic guanosine monophosphate (cGMP) is also a small second messenger molecule found in the cytoplasm of cells. Because cAMP and cGMP often display antagonistic influences on cell behavior, cGMP was chosen as a negative control molecule that is reported to suppress axon formation.[24] To activate an asymmetric cAMP or cGMP signal transduction cascade with sub-cellular resolution, membrane permeable, fluorescently tagged analogues of these molecules, MP-cAMP and MP-cGMP, were used.

Since MP-cAMP and MP-cGMP are small molecules, they do not readily physically adsorb to the surface of glass. Previous work has shown that bovine serum albumin (BSA), known for adsorbing to many surfaces, can entrap the two molecules on a glass surface without preventing their cellular uptake.[5,24] Importantly, it has been shown that patterned BSA alone cannot guide neuronal polarization or neurite outgrowth.[5] Thus mixtures of BSA with either MP-cAMP or MP-cGMP were chosen for creation of axonal-guidance patterns and negative-control patterns, respectively (Fig. 3). In addition, MP-cGMP was mixed with the PLL solution to discourage axon growth on the adhesive cell body patterns. Unpatterned areas remained as bare glass, a substrate unconducive to primary hippocampal neuron attachment. After biomolecule adsorption and removal of the PDMS devices, the deposited patterns were found to be quite robust and were not observed to desorb even after multiple washes. Fluorescent patterns retained high fidelity after 5 days in cell culture followed by more than 15 washing steps during cell fixation and immunocytochemistry (Fig. 3). The residual fluorescence of the rinsed solution from each wash step was found to be negligible.

Immature hippocampal neurons were dissected from embryonic rats (stage 18) and immediately seeded randomly onto the substrates. Neurons were allowed to grow three days before fixation and immunocytochemical staining to enable visualization. By tuning the cell seeding density, we discovered an optimal balance between filling as many PLL/MP-cGMP islands as possible with a cell body (which is enhanced at higher cell densities) and preventing cell adhesion to other regions of the pattern (which is best achieved at lower cell densities). At an optimized seeding density of 200 cells per mm^2, 78% of the PLL/MP-cGMP islands were occupied with a cell body. At this seeding density, cells were rarely observed to adhere on the plain glass (4% of all seeded cells) or BSA/MP-cGMP regions (11%), a significant percentage did adhere on the BSA/MP-cAMP regions (22%), and the majority of cells adhered to the intended PLL/MP-cGMP islands (63%) ($n = 178$ cells).

The cell bodies of neurons that contacted the PLL/MP-cGMP cell-body islands were observed to adhere to the substrate, to adopt a flattened morphology, and to sprout an average of 3.11 neurites per cell within 12–24 hours on the substrate. In contrast, neurons that landed on bare glass were unable to begin the maturation process and remained non-adhesive and rounded, with an average of 0.57 neurites per cell (Fig. 3, far right panel). Cells on BSA/MP-cAMP and BSA/MP-cGMP regions behaved similarly and tended to adopt a bi-polar morphology, sprouting averages of 2.22 and 2.18 neurites per cell, respectively. Once the immature neurons settled on the patterned array of PLL/MP-cGMP islands, the neurites began the maturation process. During this time a subset of neurites may come into contact with the BSA/MP-cAMP and/or BSA/MP-cGMP patterns. Several modifications of the multi-component design were evaluated to identify the pattern that produced the most consistent neuronal polarization and neurite guidance. The BSA/MP-cAMP and BSA/MP-cGMP patterns were located about 2 μm away from each PLL/MP-cGMP island, which requires a motile growth cone to momentarily contact the non-adhesive, plain glass substrate. We observed that small protuberances originating from the cell-body pattern and leading toward the guidance patterns were necessary to orient the neurites in the correct direction. Without these orientation projections, neurites (axons and dendrites) preferred to remain on the highly adhesive PLL/MP-cGMP cell-body pattern and rarely explored the lower-adhesive BSA/MP-cAMP or BSA/MP-cGMP patterns (Fig. S2†).

With the orientation projections added to the design, 60.4% of the cells positioned on PLL/MP-cGMP islands extended axons along the intended BSA/MP-cAMP pattern, while 31.3% extended axons that remained on the more adhesive PLL/MP-cGMP region. Axons were rarely observed on the BSA/MP-cGMP pattern (8.3%) and were never observed on plain glass. As immature neurites contact the axonal-guidance or negative control patterns, MP-cAMP or MP-cGMP, respectively, are readily taken up into the cytosol to induce asymmetric signaling events. A single immature neurite that experiences increased MP-cAMP levels initiates an asymmetric signal transduction process involving protein kinase A (PKA), LKB1, and STRAD, which ultimately results in neurite elongation, axon specification, and neuronal polarization.[3] Following this neuronal polarization, the other neurites mature into shorter dendritic processes, which may involve long-range acting inhibitors that are initiated by the axonal growth cone and potentially suppress dendrite growth.[25] All neurites observed to contact the axonal guidance pattern continued to extend along the BSA/cAMP pattern for a minimum of 30 μm and often for much longer distances (Fig. 3), demonstrating successful axon guidance.

In summary, this is the first demonstration of a vacuum soft lithography technique that can be used to easily produce complex patterns of multiple biomolecules with sub-cellular resolution in a single step. The resulting patterns demonstrated the ability to array primary hippocampal neurons at particular locations, to orient the polarization of the neurons, and to guide the polarized neurites. All three of these functions are necessary requirements towards achieving the larger goal of creating living neuronal networks with pre-determined connectivity. Previously, a variety of neuronal patterning strategies have been developed for a wide range of applications in neurobiology. For example, Jeon *et al.* designed a microfluidic device that precisely separates neuronal cell bodies and axons into two separate compartments for further biochemical analysis.[26] While this device performs very well at its intended function, it is not well suited to creating neuronal networks, since the axons are restricted from contacting other neuronal components and forming synaptic connections. Similarly, other patterning strategies aimed at elucidating axonal guidance mechanisms have contributed greatly to our understanding of neuronal polarization; however, they cannot be applied directly to forming precise neuronal networks because they do not specify the location of the cell body.[3–5,24,27] Two groups recently have reported two distinct strategies to create arrays of individual, polarized neurons: multi-step, micro-contact printing of multi-component patterns[11] and the use of single-component patterns with geometric constraints.[17] The work presented here takes inspiration from both of these strategies by combining the enhanced cellular control of a multi-component pattern with the ease of single-step processing.

Due to the speed, flexibility, simplicity, and low cost of this patterning process, this technique should prove useful for a multitude

of applications involving biomedical diagnostics and the formation of multi-cellular systems for studies of cell–cell communication. This technique should be compatible for patterning of any substrate that can form a reversible seal *via* conformal contact with the microfluidic mold. Future work will focus on optimization of the polarized neuronal arrays for integration with planar microelectrode arrays (pMEAs), which are commonly used to record and stimulate networks of neurons. Previous work by others using micro-manipulation[28] and single-component patterning[12] over pMEAs to create non-polarized neuronal arrays suggests that high density placement of single polarized neuronal cell bodies over individual microelectrodes will enable the development of several new experimental protocols for the study of living neuronal networks.

Materials and methods details are available in the ESI†.

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