Dual Micro Contact Printing of Proteins to Control Neuronal Adhesion and Outgrowth

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Introduction:

In vitro neuronal networks are inherently complex systems. Simplified networks could benefit applications such as cell-based biosensors, neuroelectronic circuits, and neurological implants while also addressing fundamental biological questions. Previously, we have employed geometric control of network formation by printing a protein grid of poly-D-lysine (PDL) and extracellular matrix gel (ECM) on a non-adhesive glass background. Although cells adhered to the pattern and formed various circuit types (linear connections, feedback loops, and branching/converging pathways), complex connectivity patterns still formed [1]. Here, we focused on creating more intricate patterns by; (i) dual-stamping of two different proteins [2], and (ii) nanostructured gradient patterns [3]. For dual-stamping, PDL "nodes" were printed to promote cell body adhesion, and aligned lines of laminin (LN) were printed to induce axon extension. After three days of neuron culture, neurons and protein patterns were immunofluorescently labeled and imaged. Line pattern type influenced neuron adhesion and axon extension, with a closer spaced pattern appearing optimal. Neurons also preferred lines of 1 μm width in comparison to gradients.

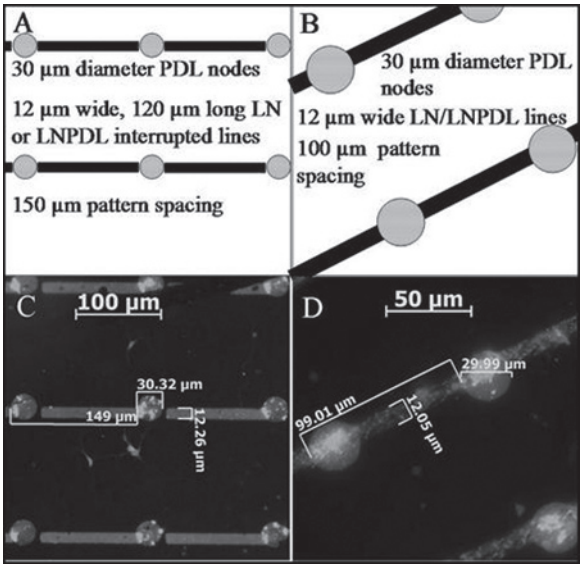

Figure 1: Dual-printed micropatterns, shown as: A. 150 µm schematic; B. 100 µm schematic; C. 150 µm printed pattern; D. 100 µm printed pattern.

Experimental Procedure:

Two stamp combinations were used for dual-printing (Figure 1). Stamps were created from a silicon master (dual-printing from standard photolithography, nanopattern from ebeam lithography) by baking polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) on top (60ºC, overnight). 1 cm^2 stamps were cut out, cleaned in ethanol, and dried (argon). Stamps were immersed in SDS (7.5%, 10 min), dried, rinsed (MilliQ H_2O), redried, and immersed in protein solutions for 20 minutes. For nodal stamps, a mixture of the fluorescent protein TRITC (10 μg/ml) and PDL (10 μg/ml) in Gey's Balanced Salt Solution (GBSS) was adsorbed. For lines, a LN solution (20 μg/ml) or LNPDL solution (20 μg/ml LN and 5 μg/ml PDL) was adsorbed. The stamps were then rinsed, dried, and stamped on a flamed glass substrate using a fine placer (20 min, 10g pressure) in quick succession to give aligned, dual-stamped substrates (Figure 1). Nanoprinting stamps were immersed in FITC-PDL (10 μg/ml) and ECM (10 μg/ml) mixture (Sigma). Stamped substrates were left overnight in petri dishes (pre-coated with PDL) containing Gentamycin (10 μg/ml). Primary embryonic rat cortical neurons were plated (50,000 cells/plate) and cultured for three days before fixing and immunofluorescently labeling for either Tau1 (axons, Chemicon), Map2 (dendrites, Chemicon) and LN (Abcam) or MAP2 and β-tubulin (Sigma). Imaging was completed with a Zeiss Apotome fluorescence microscope with AxioVisionRel [4].

Results:

For dual-patterned substrates, neuron cell bodies and neurites were typically restrained to the pattern for both protein mixtures on the 100 μm patterns (Figure 2). For the 150 μm patterns, those axons relatively restricted to the pattern were still noticeably less constrained by the lines than the 100 μm spaced patterns.

Cell body locations were counted (Figure 4A) as one measure of neuron adhesion to the protein pattern, ideally on PDL nodes. This cell body-to-node specific adhesion occurred for the majority of cells only on 100 μm line LN patterns with PDL nodes. For LNPDL patterns, more than half of the cells on the pattern adhered to the LNPDL lines, randomizing their position. This position is not ideal for applications such as in neuroelectronic circuits with electrode arrays. The majority of cells on the 150 μm patterns adhered to the

unstamped background. PDL contamination of the background due to stamp sagging or from pre-coated PDL petri dishes could be partly responsible.

Axon locations were also recorded as a meter of line guidance (Figure 4B). 150 μm lines had more axons unconstrained or only slightly constrained by the lines. The axons on 100 μm lines of LN were guided best by these lines, which also correlated with typical axon length [3]. Studies [2] have suggested that a pattern in which neurons cross from a different substrate onto LN gives the best restriction to LN. Perhaps an interrupted line pattern, without overlap of LN and PDL, would give better guidance by LN.

For nanopatterned gradient substrates, neurons were fluorescently-labeled to monitor all neurite projections along the patterns (Figure 3). Preliminary experiments completed here showed that neurites preferred 1 μm line widths over all other gradients or lines provided by the pattern.

Conclusions:

Both the cell body adherence and line guidance were significantly better for the 100 μm spaced patterns than for 150 μm lines. LNPDL randomized the cell body location across the protein pattern, as cells would adhere equally to the PDL printed as a line or node. Of the few options tested, the 100 μm spaced pattern with LN lines would seem best for limiting locations of cell body attachment.

Future Work:

Future work could involve basic stamp redesign to optimize neuron growth and differentiation on these patterns, such as making narrower lines to prevent cell body adhesion, larger nodes to contain all dendrites, and shorter pattern spacings such as 80 μm [3] to match typical axon length. Other adhesion proteins, such as L1, NCAM, or tenascin C could also be substituted.

Acknowledgements:

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References:

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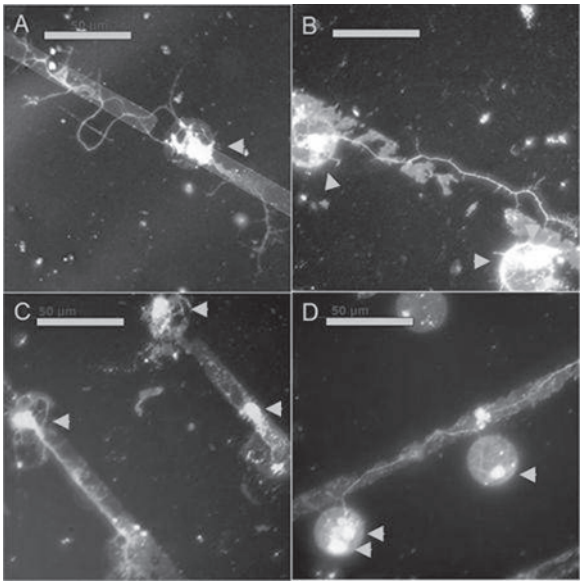

Figure 2, top: Neurons cultured on: A. 150 µm LN interrupted lines; B. 150 µm LNPDL interrupted lines; C. 100 µm LN lines; D. 100 µm LNPDL lines. 50 µm scale bar.

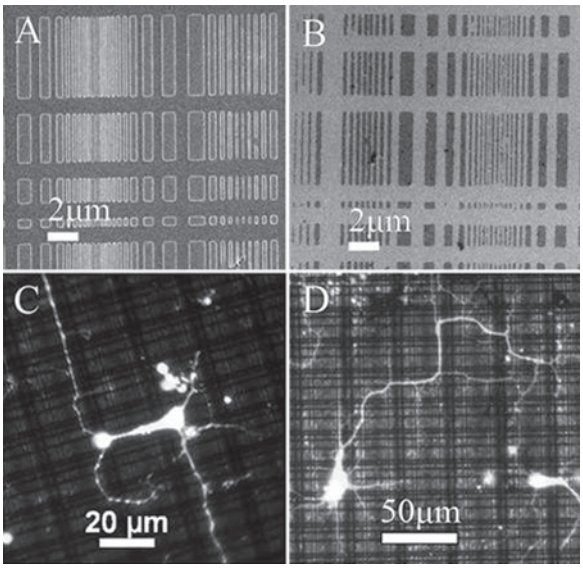

Figure 3, middle: Preliminary nanocontact printing of axon guidance protein gradient. A. Stamp SEM image. B. printed PDL-ECM SEM. C. and D. Neurons growing on nanopatterned PDL-ECM.

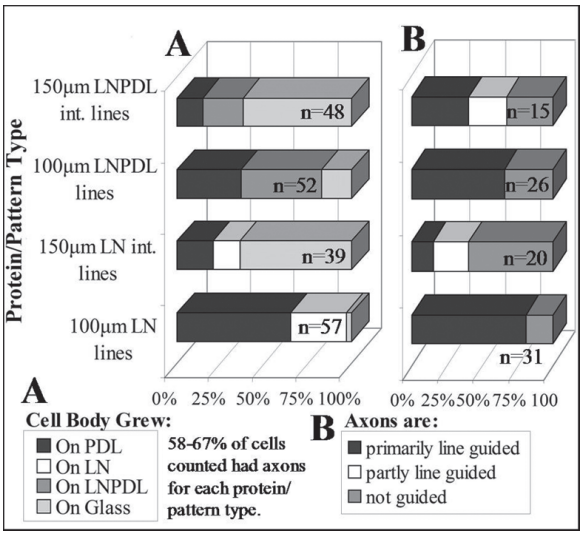

Figure 4, bottom: Quantification of Pattern Resilience A. Cell body-to-PDL node location; B. LN and LNPDL-to-axon location.