Neuronal Pathfinding on Dot Gradients of Mixed Permissive and Inhibitory Protein Cues

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Statement of Purpose: To further understand the complex environment found in neuron growth and regeneration, we developed substrates with combined inhibitory and permissive proteins. Previous work has shown that patterns of laminin (LN) and aggrecan (AG) were found to promote and inhibit neural process extension, respectively (Hodgkinson GN. Biomaterials. 2007;28:2590-2602). These patterns were effective at directing neurite extension and modifying their rates of growth (Hodgkinson GN. Biomaterials. 2008;29:4227-4235). Various methods for creating gradients for neuronal pathfinding have been tried, including step gradients and continuous gradients from diffusion. Since aligned astrocytes have been found to direct neuron growth while presenting various surface ligands, we developed a gradient of mixed cues (Biran R. Exp Neurology. 2003;184:141-152). Our approach is to create a concentration difference while maintaining regions of only a few molecules so that interactions between the cells and the substrate can be monitored. Microcontact printing has been an effective method for fabricating custom protein patterns on the micrometer scale (Hlady V. Mat.-wiss u. Werkstofftech. 2007;38:975-982). We were successful in using this technique to create dot gradient patterns and showed their applicability to neuronal cell studies. Cell directionality and outgrowth length are assessed using time-lapse microscopy and cell fixation followed by immunostaining.

Methods: Microcontact printing was used to deposit a protein monolayer onto sterile coverslips. Briefly, a design template was replicated in silicone rubber via standard microchip fabrication techniques and soft lithography. The rubber (polydimethylsiloxane, PDMS) was coated with protein solution which adsorbed to the surface. It then was brought into contact with the clean glass surface and adsorption again allowed protein to be transferred from the stamp to the surface. The dot gradients (50 µm wide, 900 µm long) were designed by randomly distributing micrometer-sized patches from 0 to 100% coverage along the gradient's longer axis. Individual gradients had 100 µm separations between them. To create mixed signal substrates, adsorption or printing with a flat stamp of another protein was used prior to gradient printing. The proteins used were LN and AG which were fluorescently labeled when used as the gradient-forming compound. Dorsal root ganglia (DRG) and astrocytes were cultured on the patterned surfaces in serum free media. Cells were fixed at different time points and immunostained for fluorescent imaging. Live cells were allowed to attach and then imaged repeatedly with transmitted light for time-lapse recordings. Coverslips covered with selected proteins with no gradient patterns were used as controls.

Results: Gradient patterns were confirmed with fluorescence microscopy. When compared with gradient template, some features were lost during lithography and fabrication steps of the PDMS stamps. This was especially apparent on the extreme ends of the gradient. The gradient created is still useful, but has a steeper change than the original design. Astrocytes responded to the patterns by avoiding high concentration AG regions and preferring LN. DRG has similar response, and extended neurites into regions of low AG. We have yet to correlate surface concentration with outgrowth lengths. Cells preferentially followed the gradient over purely inhibitory regions as shown in the following figures. DRGs on only permissive substrates had longer outgrowth at a given time than their counterparts cultured on gradients.

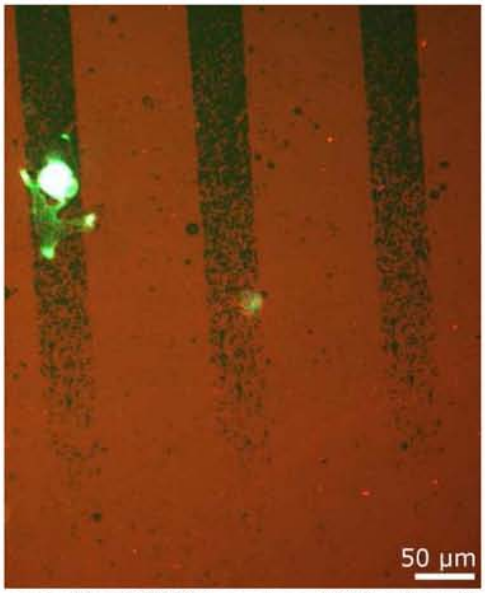

Figure 1. Fixed DRG (green) on AG (red) gradient.

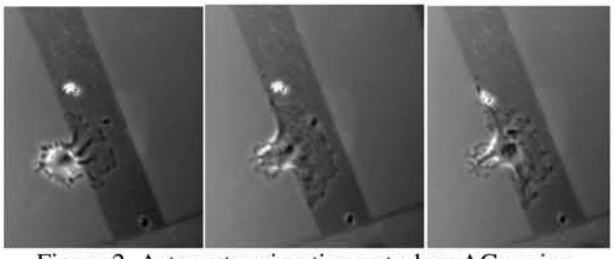
Figure 2. Astrocyte migrating onto low AG region (representative images at 6, 12, and 18 hours left to right).

Conclusions: We were able to create protein patterns with micrometer-scale features in a random gradient. The patterns were active in eliciting cellular responses from both DRGs and astrocytes. AG surfaces were inhibitory while LN was permissive and cells would migrate or retract structures located on AG. Quantitative values for outgrowth length versus concentration will be obtained.

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