Neurite Outgrowth in Response to Micropatterned Molecular Cues and Three Dimensional Matrices

Celinda Gourd, Vivian Fong, and Diane Hoffman-Kim
Center for Biomedical Engineering and Department of Molecular Pharmacology, Physiology, and Biotechnology;
Brown University, Providence, RI 02912

Introduction:

Guidance by parallel stripes of substrate bound proteins can align a monolayer of cells in culture along the direction of these tracks. The ability of neurons to align to substrate cues is a basis for the design of conduits to assist in repairing damaged nerves. However, cells *in vivo* are confronted with a complex three dimensional environment in addition to these aligned substrates. The purpose of this study is to characterize the capacity of neurons to align to substrate bound cues when these are presented in conjunction with a more complex three dimensional environment.

Methods:

Microcontact printing was used to create two dimensional patterns of proteins on a glass coverslip. Soft lithography was used to fabricate silicon wafers with patterns of alternating 60 μm plateaus and 60 μm grooves from which polydimethylsiloxane (PDMS) microstamps were created. The stamps were coated with 10% sodium dodecyl sulfate (SDS) to facilitate protein release and coated with laminin (LN) or chondroitin sulfate proteoglycan (CSPG) guidnace cues and stamped onto plasma activated glass coverslips. A three dimensional matrix was added to the system by constructing a 3ml gel of bovine collagen I. In some cases, soluble LN or CSPG was mixed with the collagen to formulate matrices that are more permissive or inhibitory to neurite outgrowth. These solutions were used as a 1.5 mL base layer with an additional 1.5 mL unaltered collagen layer on top of which cells were plated. Dorsal root ganglia (DRG) explants were obtained from postnatal (P0-P4) rat pups. Cells were used either as whole explants or dissociated with trypsin and plated as a cell suspension. Cells or explants were either plated on a microstamped glass slide prior to collagen addition or were plated on pre-gelled collagen matrices before a microstamped glass slide was inverted onto the gel surface. DMEM, 10% fetal bovine serum, 50 ng/ml nerve growth factor, 4 nM L-glutamine, and penicillin (100 μg/ml)/streptomycin (100 μg/ml), were added over the glass slide and gel. These constructs were incubated at 37ºC and 5% CO_2 in a humidified environment. Samples were fixed with 2% paraformaldehyde/4% sucrose solution after 3 days. Images were obtained on a Nikon Eclipse TE2000-S microscope under phase contrast optics at 10X with a Hamamatsu Orca-ER camera and Orbit shutter controller (Improvision), outputting to OpenLab (Improvision). Neurites in focus along 2D-3D interface were measured using Adobe Photoshop CS2. Z-stacks were obtained in order to determine the length of the longest neurite extending into the matrix.

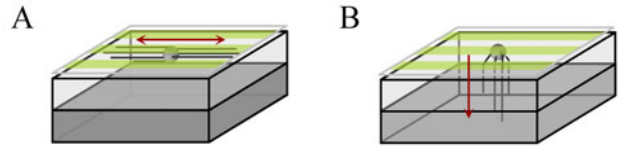

Figure 1: Schematic of DRG Explant Culture at the Interface of Molecular Cues and a 3D Matrix. (A) Growth in the direction of tracks at the interface (B) Neurites extending into the 3D matrix.

Results / Discussion:

DRGs plated on LN or CSPG microstamped tracks showed alignment of soma and neurites on LN tracks or in between the CSPG tracks as expected. The addition of neutralized liquid collagen showed no effect on cell morphology such as retraction of neurites as observed by time-lapse microscopy throughout the process of gelation (1 hour). Up to 4 days after the addition of the matrix, alignment to the molecular cues was undeterred, and no growth into the matrix was observed. In order to reduce the potential influence of gravity on the ability of neurons and neurites to enter the matrix, explants were cultured on top of a collagen matrix and then presented with overlaid, microstamped molecular cues. When presented with LN, the majority of neurons aligned in the direction of these tracks. Some neurites extended into the gel in contrast to experiments in which the matrix was added above the cells, but the number of extensions entering the matrix was lower than in control experiments where no molecular cue was present. When the explants were presented with CSPG tracks, some extension at the plane of the tracks was observed, but these extensions were shorter than those observed with LN tracks. Neurite outgrowth into the matrix was higher than from explants presented with LN or no molecular cue. Permissive (LN) and inhibitory (CSPG) cues were added to the matrix to investigate whether matrix composition affects neurite ingrowth. Neurite extensions from explants cultured on unaltered collagen were observed to be clustered in high density near the surface of the gel, especially when presented with LN tracks. When LN was mixed into the matrix, more downward extension into the gel was observed. In contrast, in collagen matrices mixed with CSPG, the amount of downward growth was decreased.

Conclusions: These results show that neurons integrate cues from their local environment which can be manipulated in precise manners using techniques such as microstamping and tailoring cues within a 3D matrix.

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