Characterization of Embryonic Rat Cortical Cells Grown on Microcontact Printed Protein Patterns

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Abstract:

Microcontact printing (μCP) provides a simple, reproducible method of creating a defined pattern on various substrates for the purpose of studying neuronal cell growth and polarity (direction of axon and dendrite growth relative to the cell body). A protein mixture was stamped onto substrates (generally silanized glass) in a specific pattern. Cells are plated and allowed to grow for one, two, or three days *in vitro* (DIV). The growth of the axon and dendrites are controlled by the pattern and were visualized by immunostaining. Single-cell polymerase chain reaction (PCR) was used to analyze changes in gene regulation caused by the protein pattern.

Methods:

Microcontact Printing. Stamp master molds were created using standard photolithographic methods described elsewhere [1]. Polyolefin plastomer (POP) stamps were created by heating a hot embossing method. POP was placed on the master mold, heated to 90°C, and weighted with 900 grams.

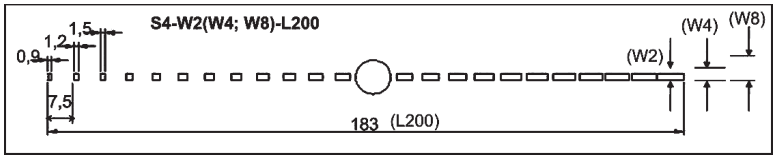

Figure 1: Schematic representation of the micrograd ient pattern. Units are in micrometers. The label stands for slope of 0.04 (0.03/7.5), width of 2 µm, and length of 200 µm. Positive direction is towards larger squares.

Stamps were washed with ethanol for 10 minutes and soaked in a protein solution of 1:100 (v/v) FITC-labeled poly-L-lysine (FITC-PLL, green flourescent) and 1:200 laminin (v/v) in Gey's Balanced Salt Solution (GBSS) for 20 minutes. Stamps were dried by nitrogen stream, and manually held against the substrate for two minutes. Figure 1 shows a diagram of the pattern used.

Substrates were homogeneously coated by covering the entire surface with the above solution for one hour and washed three times with GBSS.

Silanization. Glass coverslips (25 mm diameter) or microelectronic arrays (MEAs) were silanized using (3-glycidoxypropyl) trimethoxysilane (3-GPS). Substrates were cleaned in a plasma generator at 180W for three minutes using oxygen plasma. Substrates were then placed in a desicator with 70 μl of 3-GPS at 45 mbar for one hour. Silanized substrates were kept in a dry, oxygen-free environment until use.

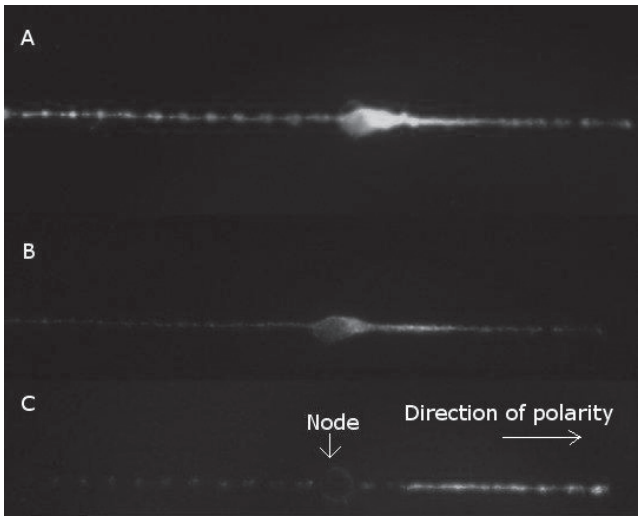

Figure 2: Representative micrograph of a neuron growing on a micrograd ient pattern. A) MAP 2 proteins, B) Tau-1 protiens, and C) unoccupied pattern.

Cell Culture. Embryonic rat cortical neurons were plated on patterned or homogeneously coated substrates at 16,000 or 50,000 cells per substrate, respectively. Cells were cultured in supplemented neurobasal media (Invitrogen™), which was changed 3-5 hours after initial plating and every third day. Cells were allowed to grow for one to three (DIV) for PCR and three DIV for immunostaining.

Immunostaining. Cells at three DIV were fixed with paraformaldehyde and then blocked in a bovine serum albumin and goat serum solution. Neurons were stained with Anti-Tau-1 (axons) and anti-MAP-2 (dendrites) antibodies that were visualized by secondary antibodies bound to Alexa-488 (green) and Alexa-568 (orange), respectively.

Polymerase Chain Reaction (PCR). For bulk PCR, messenger RNA (mRNA) was isolated from cells grown for one, two, or three DIV and reverse transcribed into complementary DNA (cDNA). This was done off of homogeneously coated substrates. For single-cell PCR, cells grown for one, two, or three DIV were placed in a patch clamp solution. Individual cells were then sucked off the substrate (homogeneously coated or microgradient patterns), cells were lysed, and mRNA was reverse transcribed to cDNA. cDNA was then cleaned from other cellular debris by centrifugation. PCR was then performed, on both methods of collection, using a Roche LightCycler. Melting point data was used to determine specificity of products and absolute quantification was used to determine the crossing point. The relative expression of desired product to the housekeeping gene can then be determined.

Results and Discussion:

Microcontact Printing, Cell Culture, and Immunostaining. Microcontact printing allowed for creation of reproducible protein patterns on various substrates. Figure 2 shows a representative micrograph of a gradient on silanized glass with plated neurons. Silanized glass was chosen over silicon oxide, hydrophilized glass, or polystyrene. Under qualitative analysis, silanized glass guided the cell on the gradients most effectively. The neuron grows on the node while the axon grows in the positive direction and the dendrites in the negative direction. This matches our predictions and results of others [1].

PCR. Using the bulk PCR method, GAPDH was chosen as the housekeeping gene due to constant expression at DIV one, two, or three. Establishing this housekeeping gene then allows for comparison of relative amounts of gene expression. To determine changes in gene expression, single-cell PCR was used as it, in principle, prevents contamination from the solution or other cell types. Unfortunately, a protocol for single-cell reverse transcription and PCR could not be established which eliminated contamination (GAPDH specific products were present in negative controls) and could quantify APOE expression.

Conclusions and Future Work:

Microcontact printed patterns allows for guidance of neuronal cells and for analysis of the mechanisms behind neuronal cell polarity. Single-cell PCR can then be used to measure the genetic changes that occur because of pattern geometry or chemistry. In the immediate future, single-cell PCR optimization, additional controls (such as with a solid pattern) and additional data to determine statistical significance are needed to confirm the effects of these genes and show statistical significance. Long term, gene silencing experiments are planned to determine which genes have the greatest impact on neuronal polarity. This analysis adds to the understanding of the mechanisms of early neuronal cell growth and will allow for better control of neuron growth on bioelectronic devices.

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References:

[1] Fricke, Rita, Peter Zentis, Lionel Rajappa, Boris Hofmann, Marko Banzet, Andreas Offenhäusser, and Simone Meffert. "Axon Guidance of Rat Cortical Neurons by Microcontact Printed Gradients." Biomaterials (2010): 2070-2076. Web. 26 July 2011.

[2] Holtzman, D. M. "Low Density Lipoprotein Receptor-Related Protein Mediates Apolipoprotein E-Dependent Neurite Outgrowth in a Central Nervous System-Derived Neuronal Cell Line." Proceedings of the National Academy of Sciences, 92.21 (1995): 9480-484. Web. 9 Aug. 2011.