

Towards a Method for Printing a Network of Chick Forebrain Neurons for Biosensor Applications

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Abstract— The primary goal of this work is to establish a robust, repeatable method for printing arrays of neurons. This work has two endpoints. One is to use a neural array as an experimental testbed for investigating neuronal cell growth hypotheses. The other endpoint is to enable the next generation of cell-based sensors. Herein we compare microcontact printing results previously published by our group with a new method of dip-pen printing. We present preliminary results for neurons growing on these microprinted arrays, assessing contact frequencies and growth characteristics.

I. INTRODUCTION

Nerve cells are electrically excitable cells in the nervous system that function to process and transmit information [1]. While it is well known that neurons rely on the electrical properties of their ion channels and membranes to transmit information, they also rely on their sophisticated cytoskeletons to maintain growth and structural dynamics [2]. Neurons are typically composed of a soma (cell body), a dendritic tree, and an axon. The majority of vertebrate neurons receives an input of information to the cell body from the dendritic tree, and then output this information via the axon. Most vertebrate neurons receive input on the cell body and dendritic tree, and transmit output via the axon. We would like to exploit this property by printing neurons in a predetermined configuration, controlling contact points and investigating communication rates through a neuronal network with a quantifiable degree of connectivity.

There is great heterogeneity throughout the nervous system and the animal kingdom of neuron size, shape and function. Another overarching goal of this research is to understand the mechanical processes that drive neuron morphology, development, and maintenance. This includes understanding how the cytoskeleton of the neuron interacts with the membrane to produce directed cell growth, and how pharmaceutically induced cytoskeletal alteration affects this growth.

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An experimental environment for the growth of nerve cells requires an adhesive substrate and a physiological liquid [3]. The most popular protein substrate for forebrain neurons poly-lysine [4]. The most common methods for protein printing are microcontact printing [5] and soft lithography [6]. These methods have been applied to numerous neuron types for a variety of purposes ranging from gaining a better understanding of the supramolecular organization of proteins [5] to biosensor applications.

Forebrain nerve cells have cell bodies of approximately 20 μm diameter, axons of a few micrometers in diameter and are dissociatable [1]. Recently our group proposed a protein array printing method [7] for obtaining mechanical data from a large group of cells, with the intent of alleviating many of the painstaking one-cell-at-a-time methods previously pioneered by other groups calibrate and execute [2, 11-13]. By stretching axons in parallel using the previously microfabricated beam array by our group such that each beam will adhere to a single cell [7] we hope to achieve the goals of both investigating the roles that specific cytoskeletal and molecular motor proteins play in neuronal growth mechanics and directing neuronal growth for cell-based sensor technologies.

Microstamping using PDMS stamps onto glass substrates has been demonstrated for cell patterning [8]. Typically, the areas that are not unstamped are treated with an inhibitor prior to cell patterning [9]. Polydimethylsiloxane (PDMS) stamps may also be used for protein patterns onto a PDMS substrate that permits cell attachment and growth without applying any inhibitors [3, 7].

For the study of neurite extension characteristics, glass microneedles have been used to apply forces *in vitro* to initiate neurites [10]. Magnetically induced forces have also been used to initiate neurites by applying forces at an accuracy of a few piconewtons per micrometer [11]. Other recent work on massive parallel arrays of axons have also shown that escalating rates on growth may be achieved without loss of function [12]

II. METHODS

A. Protein Printing

Poly-DL-lysine was dissolved in sodium tetraborate decahydrate at a concentration of 1mg/mL and kept in 100 μL aliquots at -4°C . The aliquots were kept at room

temperature 20 minutes prior to the application. It was then diluted in borate buffer at a ratio of 1:9 (each 100 μ L aliquot mixed with 900 μ L borate buffer). The solution was then used to pattern on glass cover slides (Bellco Glass, Vineland, NJ) specifically designed for nerve cells or hard-to-attach cells. A desired pattern was obtained with the Nano eNabler (BioForce, Ames, IA). It is a multi-faceted platform which is capable of printing femtoliter volumes of solutions to the defined locations. It uses microfluidic channels which constantly deliver the liquid to be transferred on the surface and are called surface patterning tool (SPT) (Fig 1).

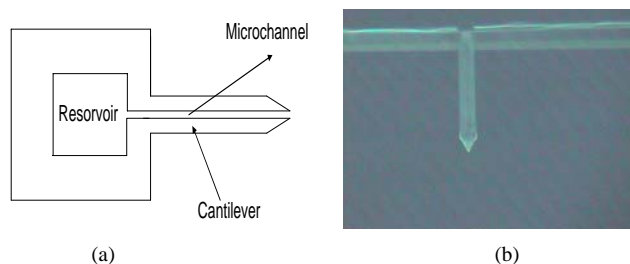


Fig. 1. The surface patterning tool (SPT) for the Bioforce Nano eNabler. a) The lysine is poured onto the reservoir and flows down the microchannel to the tip of the cantilever which contacts with the glass surface. b) SPT cantilever filled with poly-lysine glycerol solution. Cantilever length is approximately 200 μ m.

Poly-lysine was placed into the reservoir where it flows from the reservoir down the channel. When contact occurs with the surface a small volume of liquid is transferred to the surface. The process is repeated automatically until the desired pattern is obtained. The size of the pattern may be controlled by changing the humidity level or by changing the dwelling time. We found a humidity level of 30% and a dwell time of 0.5 sec to be optimal for our process. Glycerol was added at 1:1 ratio to prevent the polylysine from evaporating during the printing process.

B. Cell Printing

Eight-day old chick embryos were used for the chick forebrain neuron culture. Forebrain hemispheres were dissected and dissociated, plated at 10^4 cells/coverslip according to a standard procedure [4]. 2ml of CMF-PBS was added to a 15 ml tube and the samples were placed into the tube and the solution was agitated by pipetting. 3 ml of CMF-PBS was added and the tube was vortexed gently for 15 seconds. Next, the tube was centrifuged for 4 minutes. In the meantime, frozen trypsin-EDTA was incubated in a 37 $^{\circ}$ C in water bath for 10 minutes. The supernatant was aspirated and 0.1mM trypsin in PBS was added. It was then incubated for 7 minutes in a 37 $^{\circ}$ C in water bath, shaking every 2 minutes. Just after the incubation, the tube was spun down for 1 minute. The supernatant was aspirated and the cells were agitated with culture medium before plating. It was again centrifuged for 4 minutes. Cultures were maintained in supplemented M199 medium (Invitrogen, Carlsbad, CA) and incubated (5% CO₂; 37 $^{\circ}$ C) for 2-3 days before experimentation. Culture medium was changed every

other day. Cultures were fixed with glutaraldehyde in PBS (5 μ L/mL) for 15 minutes followed by storage in PBS.

III. RESULTS

SPT-S-C30 tips (BioForce Nanosciences, Inc) were used for protein printing (Fig. 2). It is capable of printing spot sizes between 2-30 μ m. In general, increasing the humidity level and dwell time result in larger spot sizes (Fig. 3). The dependency of printing size, dwell time, and waiting time, at constant humidity was determined. This was done by changing the dwelling time and waiting time during printing using the NanoArrayer. While printing the microspots, waiting time had no affect on the spots size while dwell time and humidity were constant. However; spot size was highly dependent on dwell time at constant humidity and waiting time. Average spot sizes at corresponding dwelling times were determined by averaging 5 sample spots diameters at 19% humidity and 1.0 sec waiting time.

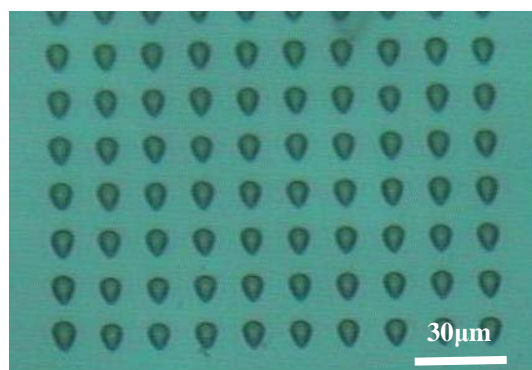


Fig. 2. The printed arrays. The pitch is 15 μ m. The humidity level is 15% and the dwell time is 0.5secs. This resulted in a spot size of approximately 8 μ m.

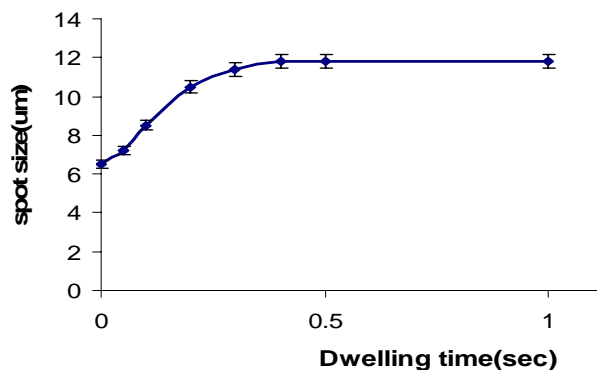


Fig. 3. Increasing the dwell time results in an increase in spot size up to an asymptotic level of 10mm at a humidity level of 15%.

The printed spots have a high contact angle presumably because of its hydrophobicity and low wetting properties (Fig. 4). Therefore this makes printing stripes comprised of a series of spots difficult. This also has a disadvantage for cell attachment. It is easier for a cell to attach on a protein patch that has a shallower contact angle.

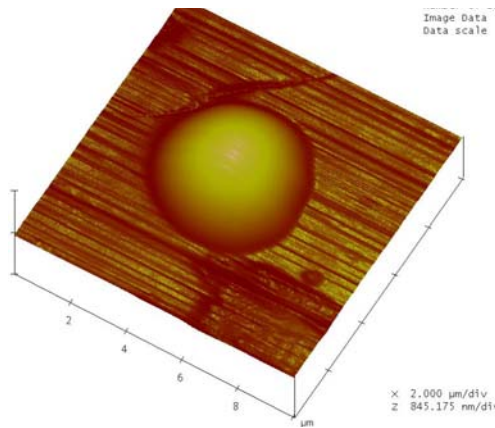


Fig. 4. A 10 μm x 10 μm AFM image of printed polylysine on untreated glass coverslip.

In order to achieve a flatter surface, the glass coverslips were treated under UV light for approximately 30 minutes. The UV changes the hydrophobicity and wetting properties of the glass. Printed stripes were obtained using the Nano eNabler (Fig. 5).

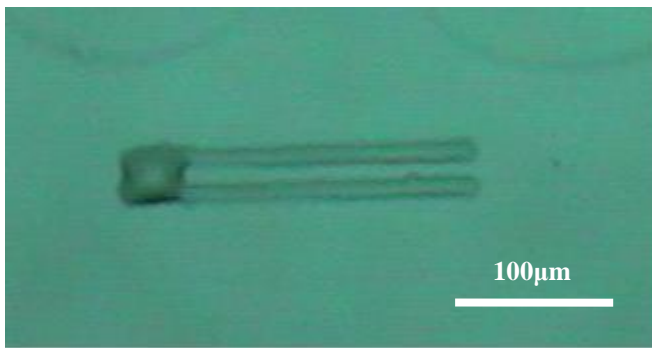


Fig. 5. 200 μm long printed stripes on UV treated glass.

The forebrain nerve cells were grown on polylysine coated cover slides. The cells were fixed using the method explained. The cells were observed with brightfield microscopy and AFM using fluid contact mode (Fig. 6).

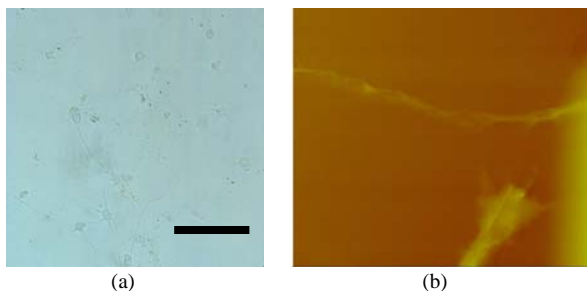


Fig. 6 a) The dissociated forebrain nerve cells on the glass surface under brightfield light microscopy indicate growth patterns typical of lysine-coated slides. Scale bar equals 100 μm . b) 20 μm AFM images of the fixed nerve cells reveal growth cones. The middle portion of the image shows an axon which extends horizontally and bottom portion shows the growth cone of another nerve cell.

IV. DISCUSSION AND FUTURE WORK

A full evaluation of the association rates of neurons with printed lysine is still under evaluation. However our current

results indicate that the size of the printed proteins can be controlled by changing the dwell time in the ink-pen printing method. Additionally, the UV-treated glass allows for flatter, printed spots, which is necessary for proper nerve cell attachment and growth. The desired cell pattern will be obtained by printing lysine in the desired pattern. Nerve cells will adhere to the lysine spots, but they will not adhere directly to the glass slide. Once the cells are patterned, we will be using our previously microfabricated beam array to manipulate the cells. The beam array will allow us to determine the mechanical properties of the patterned cells independently but simultaneously. Future work will include using the arrayed neurons to perform force-transduction experiments in pharmacologically challenged neurons. Pharmaceuticals used will include taxol, vinblastine, and vincristine. Each of these drugs affects the behavior and chemical interactions of the cytoskeletal proteins inside neurons, and we will be determining if these drugs affect the mechanical properties of the cell as well. Furthermore, we will be investigating the potential of the patterned neurons for cell-based sensing applications.

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