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PARALLEL FORCE MEASUREMENT IN CELL ARRAYS

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ABSTRACT

The primary goal of this work is to establish a robust, repeatable method for growing forebrain nerve cells in a parallel manner by stretching them using a microfabricated PDMS beam array and printing arrays of neurons .The highly compliant, transparent, biocompatible PDMS micro beam array may offer a method for more rapid throughput in cell and protein mechanics force measurement experiments with sensitivities necessary for highly compliant structures such as axons. 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 build a neuronal-based, biosensor device capable of acting as a cell-based sensor. We present preliminary results for microbeams attaching to nerve cells. The attachment ratio the life-length and the axon lengths of the chick forebrain cells on microprinted spots will also be compared with an equivalent protein coated area of cells.

INTRODUCTION

Nerve cells are electrically excitable cells in the nervous system that function to process and transmit information (Eric R. Kandel 2000). 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 (Chada, Lamoureux et al. 1997). 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 e.g. (Hasaka, Myers et al. 2004).

An experimental environment for the growth of nerve cells requires an adhesive substrate and a physiological liquid (De Silva, Desai et al. 2004). The most popular protein substrate for forebrain neurons poly-lysine (Heidemann, Reynolds et al. 2003). The most common methods for protein printing are microcontact printing (Sgarbi, Pisignano et al. 2004) and soft lithography (Kane, Takayama et al. 1999). 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 (Sgarbi, Pisignano et al. 2004) to biosensor applications (Lamoureux, Zheng et al. 1992),.

Forebrain nerve cells have cell bodies of approximately 20 μm diameter, axons of a few micrometers in diameter and are dissociatable (Eric R. Kandel 2000). Recently our group proposed a protein array printing method (Sasoglu, Bohl et al. 2007) 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 (Dennerll, Joshi et al. 1988; Dennerll, Lamoureux et al. 1989; Chada, Lamoureux et al. 1997; Lamoureux, Ruthel et al. 2002). By stretching axons in parallel using the previously microfabricated beam array by our group such that each beam will adhere to a single cell. 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 have been demonstrated for cell patterning (Chang, Brewer et al. 2003). Typically, the areas that are not unstamped are treated with an inhibitor prior to cell patterning (Wheeler, Corey et al. 1999). Polydimethylsilixone (PDMS) stamps may also be used for protein patterns onto a PDMS substrate that permits cell attachment and growth without applying any inhibitors (De Silva, Desai et al. 2004; Sasoglu, Bohl et al. 2007).

For the study of neurite extension characteristics, glass microneedles have been used to apply forces *in vitro* to initiate neurites (Bray 1984). Magnetically induced forces have also been used to initiate neurites by applying forces at an accuracy of a few piconewtons per micrometer (Fass and Odde 2003). Other recent work on massive parallel arrays of axons have also shown that escalating rates on growth may be achieved without loss of function (Pfister, Iwata et al. 2004).

METHODS

A. Protein Printing

Poly-DL-lysine was dissolved in sodium tetraborate decahydrate at a concentration of 1mg/mL and kept in 100μL 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 100μL aliquot mixed with 900μL 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).

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 (Sasoglu 2007).

Size of the printing area

The printed area vs. total cell medium area plays an important role in axon growth of cells since the floating dead cells highly affect the attached alive cells. The cells are dissociated at a density of 10^6 cells/ml solution. The total required printed area is calculated as follows:

$$A_{printed\ area} = A_{fully\ coated\ area}$$

From this, a circular area of 1mm is equivalent to 2500 printed spots with 20μm diameters. To test this hypothesis, two areas were printed side by side to obtain the same experimental conditions and environments. The 20μm spots were printed using the nanoarrayer whereas circular area of 1mm diameter was obtained by coating the slide with a micropipette (Figure **1**).

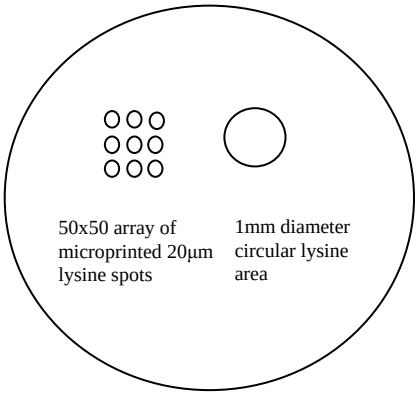

Figure 1. Equivalent lysine printed areas using the eNabler and a micropipette

B. Cell Printing

Embryonic day-8 chick forebrain neurons were harvested, dissociated and plated on indexed German glass coverslips (Bellco Glass, Vineland, NJ) at a concentration of 10^6cell/ml (Dennerll, Lamoureux et al. 1989). Coverslip surfaces were pre-treated to promote attachment. Indexed coverslips were used for tracking sets of individual neurons over time via phase contrast microscopy and post hoc immunocytochemistry (Lamoureuxet al. 2002). Cultures were maintained in supplemented M199 medium (Invitrogen, Carlsbad, CA) and incubated (5% CO_2; 37°C) for 4-6 days before experimentation. Culture medium was changed every other day. HEPES buffered, CO_2-independent, supplemented Ham's F12 medium (Invitrogen) was used as experimental medium during live imaging experiments

C. Microbeam height consistency determination

In order to achieve full contact with the cells during stretching, the microbeams have to be same length. The out-of-plane misalignment of the microbeams may reduce the probability of attachment of some of the microbeams to the cells. For this purpose, the length of the microbeams were determined using the Zygo 6000 optical profilometer. The lengths of the microbeams are 118.4 ± 0.2 μm. The maximum and minimum beam heights were 118.9 and 118.0 μm (n = 64) (Figure 2).

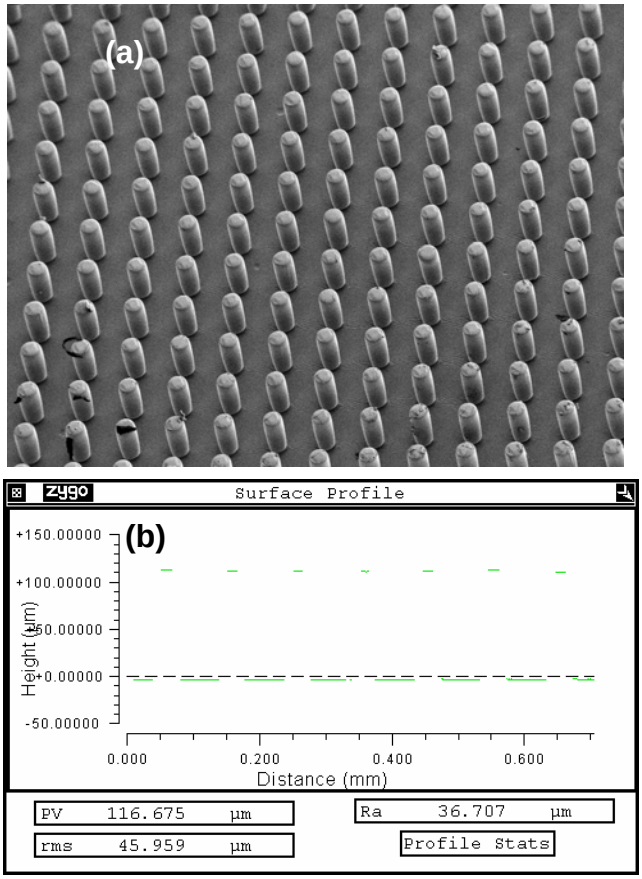

Figure 2. a) An SEM image of the microfabricated PDMS microbeam array. b) A sample surface profile of the microbeams using optical profilometer.

D. Stretching Cells using a PDMS Microbeam Array

The microbeams are prepared by photolithography followed by replica molding (Sasoglu, Bohl et al. 2007). The cells were dissociated and the cells printed onto the spots (Sasoglu 2007). The PDMS microbeams were manipulated on the attached cells using an Eppendorf NK-2 micromanipulator with has 40-nm precision. A rod was attached to the micromanipulator and glass slide fixed to the rod with microbeams glued to one the edges of the glass slide. Microbeams were first aligned above the nerve cells and lowered slowly toward the cells until the attachment between the cells and the microbeams occurred (Figure 3).

SPT-S-C30 tips (BioForce Nanosciences, Inc) were used for protein printing (Figure. 4). It is capable of printing spot sizes between 2-30 µm. In general, increasing the humidity level and dwell time result in larger spot sizes. The dependency of printing size, dwell time, and waiting time, at constant humidity was determined.

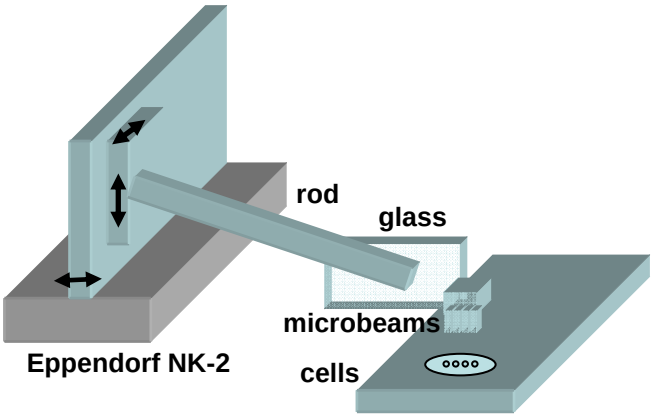

Figure 3. The diagram showing the experimental setup to stretch the nerve cells. The microbeams were lowered onto to the cells and stretched after attachment occurred.

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 (Sasoglu 2007).

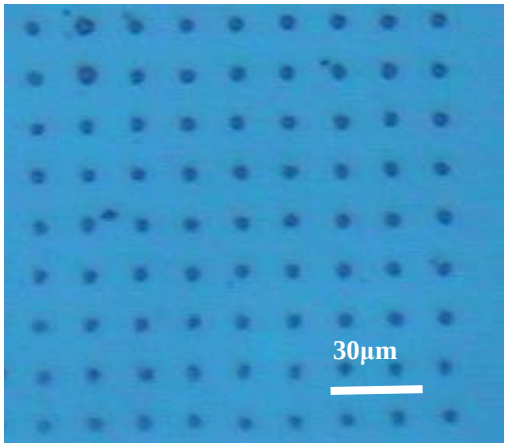

Figure. 4. The printed arrays. The pitch is 25µm. The humidity level is 15% and the dwell time is 1 sec. This resulted in a spot size of approximately 5 µm.

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

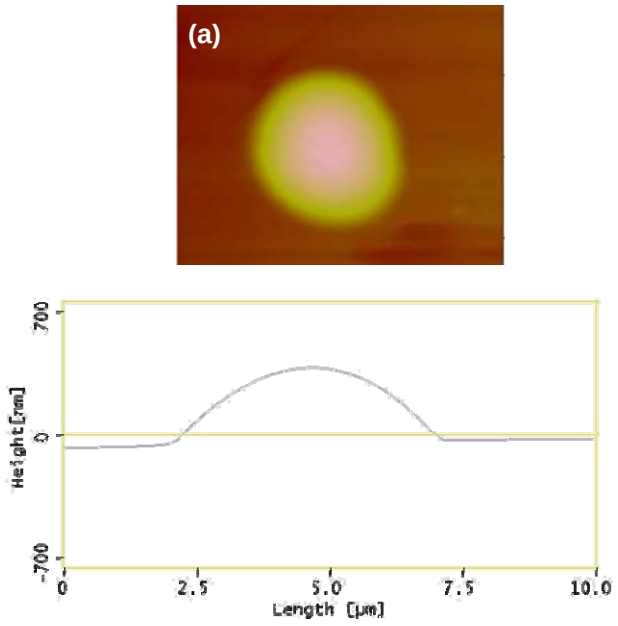

Figure 5. a) A 10-µm AFM image of a single microprinted lysine dot. b) The AFM cross-section of the single printed dot.

The previously fabricated microbeam array was lowered using a micromanipulator on the dissociated nerve cells to stretch them. The cells were observed to be pulled by the beams if a certain close distance, usually a few microns, can be maintained. This helps the cells to be manipulated even though the cells are slightly misaligned with the desired printed locations.

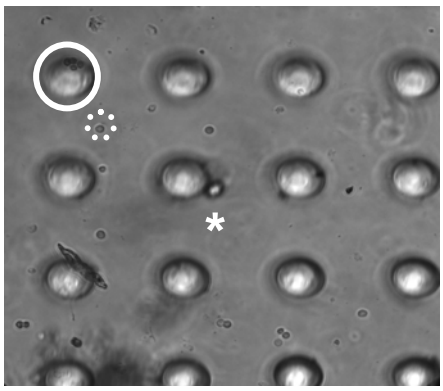

Figure6.Optical micrograph showing the microbeams (solid white circle) lowered onto the immature nerve cells (dashed white circle) and the attachment between a cell and a microbeam (asterisk).

E. Force Measurement

As the microbeam array is actuated in the direction of cell stretching, and with the microscope focused in the plane of the beam tips, images are acquired digitally with a SPOT-RT camera (Diagnostic Instruments, Sterling Heights, MI). The initial images is used as the position of zero force for each cell. As the array is pulled, images are captured at ten to thirty second intervals. The deflection and thus force of the microbeams are then calculated using a Matlab code as follows: The images are converted within Matlab to binary images using thresholding algorithm to obtain the outer borders of the beams (Figure 7). The outer borders are then fitted to ellipsoids using the least squares method.

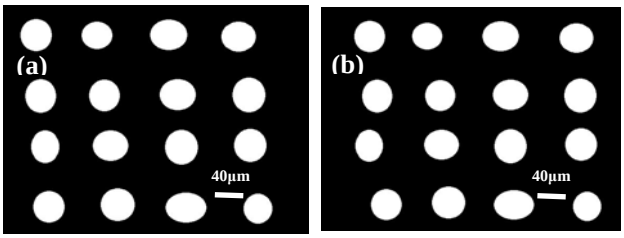

Figure 7. a) The initial (a) and final (b) configurations of the location of the microbeams after converting it to binary form using Matlab.

From this, displacement of each beam is calculated by subtracting the center of the individual circular spots at the initial configuration and the displaced configurations (Figure 8). Having the deformed and undeformed bottom view of the beams under the microscope, the code calculates the displacement of the center of the beams from the images. The code then determines the force applied to the cells using the Euler beam equations with the empirically measured geometry of each individual beam and the average modulus obtained by measuring a representative number of microbeams from the array to obtain the force applied to each cell.

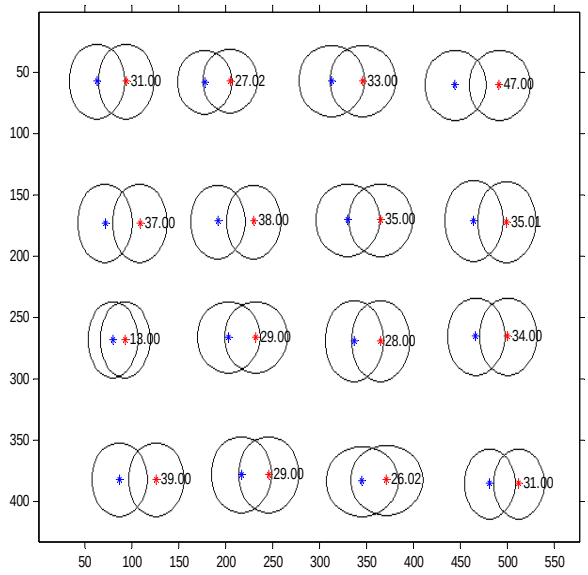

Figure 8. The initial and final configuration of the beams and the displacements. The blue dots show the center of the initial position of the beams where the red spots show the final position the beams.

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. 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. The unattached cells are washed away in order to eliminate the interaction between the alive and dead cells. Once the cells are patterned, we will be using our previously microfabricated beam array to manipulate the cells. Our preliminary results show that once the required distance, which is usually a few microns, is provided the cells attach to the microbeams which makes it possible to manipulate the cells. The beam array will allow us to determine the mechanical properties of the patterned cells independently but simultaneously. Preliminary results show that as soon as the certain distance is maintained, cells are pulled to the microbeams which allow them to be pulled with the array simultaneously. Future work will include using the arrayed neurons to perform force-transduction experiments in pharmacologically challenged neurons. The cells will be stretched at constant directions to direct the growth. 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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