Aligned Microcontact Printing of Micrometer-Scale Poly-L-Lysine Structures for Controlled Growth of Cultured Neurons on Planar Microelectrode Arrays

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***Abstract*—We describe a method for producing high-resolution chemical patterns on surfaces to control the attachment and growth of cultured neurons. Microcontact printing has been extended to allow the printing of μm-scale protein lines aligned to an underlying pattern of planar microelectrodes. Poly-L-lysine (PL) lines have been printed on the electrode array for electrical studies on cultured neural networks. Rat hippocampal neurons showed a high degree of attachment selectivity to the PL and produced neurites that faithfully grew onto the electrode recording sites.**

***Index Terms*—Hippocampal neuron culture, microcontact printing (μCP), patterned protein, planar microelectrode.**

I. Introduction

FOR STUDIES of neurons in culture, it is desirable to control the attachment and growth of the neurons. Surface modification with chemical and topographical techniques have been performed to influence the attachment and growth of cells in culture [1]–[4]. Chemical modifications have generally been made using photolithographic methods to pattern alkylsilanes with different functional end groups on surfaces including glass, silica, and silicon [3]–[6]. Chemical patterns have been used to investigate surfaces that are conducive to long-term neural cell network cultures [7], [8] and to control nerve cell polarity [9], [10].

Microcontact printing (μCP) is a newer technique that has been used to pattern surfaces with alkylsilanes for control of cell attachment [2], [11]–[13]. μCP has recently been extended to print synthetic polypeptide and protein layers [14]–[16]. This technique has advantages over photolithography in patterning these sensitive biomolecules because it does not require the use of harsh solvents and bases. Patterned protein layers have applications in areas such as enzyme kinetics [17], antibody–antigen complex studies [18], and biosensing of environmental chemicals [19], [20].

Extracellular substrate electrodes have been used for a variety of neuroscience applications: the correlation of intracellular calcium transients with electrical activity in neural networks [21], stimulation/recording from spinal cord slices and cell networks [22]–[24], detailed evaluations of theoretical models of the neuron/electrode interface [25], and biochemical sensing [26], [27]. Extracellular electrodes present specific advantages over their intracellular counterparts in that the latter are invasive, difficult to use for making multiple simultaneous measurements, and can often maintain connection with cells for only several hours. Extracellular electrodes are noninvasive, long-term in duration, and have been successfully used to stimulate and record from neurons; however, signal-to-noise ratios are much worse due to decreased sealing impedance, and it is not always possible to achieve one-to-one electrode-neuron correspondence. Several methods have been presented for extracellular electrodes to enhance culture longevity and cell-electrode coupling such as positioning contacts on cells [28] and topographic modification to guide cells to electrode sites [29]. We present a method for combining chemical patterning and extracellular electrode arrays in order to address issues of long-term cell cultures, extracellular electrode-cell coupling, and multisite extracellular recording from single cells.

II. Description

Several factors are instrumental to extend μCP to a wide-range of applications. The flexible nature of the elastomer stamp allows the stamp to make good contact with the surface, but it can also be a source of limitation in geometrical control. We have recently shown that elastomer collapse is a limiting factor on the allowed printable geometries [14], particularly when printing small isolated features. Although the lateral compliance of the elastomer has been used purposely to alter the pattern dimensions [30], it can limit the reproducibility of printing aligned molecular layers. Extending μCP to produce aligned chemical modifications of adjacent surfaces would offer an ability to greatly increase control of cell attachment and process outgrowth. This is especially important for cultures

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where electrical properties of individual neurons in neuronal circuits are to be measured.

In thin-stamp μCP of fine isolated features, the following stamp dimensions become critical (Fig. 1): the stamp thickness, the stamp corrugation depth, and the gap dimension (the distance between printing features). Problems arise when protruding stamp features become much smaller than the stamp corrugation depth. Removal of the stamp may destroy fine features and high aspect ratio features will lack structural stability. During printing, the applied pressure can cause the features to buckle, causing poor printing and low reproducibility.

We introduce an innovation to address geometrical control of the stamp topography: a multilayer master that provides a further improvement in structural support for isolated, μm-scale features. We have successfully patterned a grid of 1.0-μm-wide poly-L-lysine (PL) lines printed at a 200-μm spacing using thin-stamp μCP. The rigid backing involved in thin-stamp μCP also provides the needed lateral rigidity to the stamp that is essential for aligned printing of multiple layers. Finally, an alignment tool was constructed to align μCP protein features with microelectrode sites on a substrate.

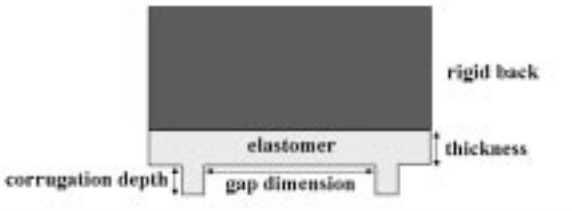

Fig. 1. Important design dimensions for improving μCP techniques.

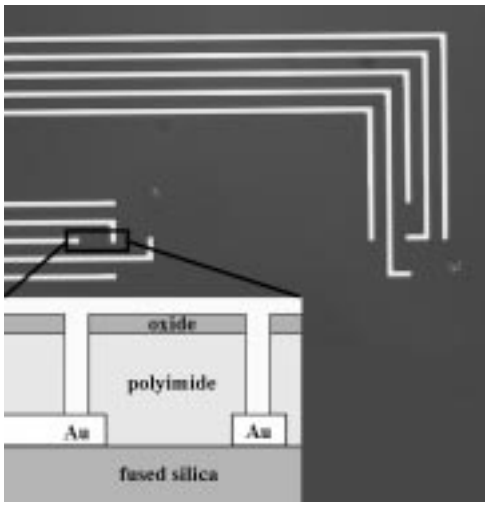

Fig. 2. Top view optical micrograph of two planar microelectrode arrays. Inset diagram shows a cross section of the wafer.

III. Methods

A. Planar Microelectrode Array Fabrication

Planar microelectrodes were fabricated at the Cornell Nanofabrication Facility. Titanium (5 nm) was deposited on fused silica wafers by electron-beam evaporation, followed by thermal evaporation of gold (500 nm). The gold/titanium layer was patterned into 25 arrays of five gold traces, as shown in Fig. 2, using an argon ion mill and a PECVD oxide mask. Wafers were insulated and planarized with a 6.0-μm layer of Probimide 9753 polyimide. The polyimide was dried for 30 min in a 90 °C oven, and finally cured for 15 min in a 175 °C oven. A final PECVD oxide layer (300 nm) was deposited to provide a glass-like surface for cell culturing. Holes on both ends of each electrode trace were etched. Preliminary measurements of electrode impedance using a method described by Jung *et al.* yielded a value of approximately 650 MΩ-μm^2 at 1 kHz for bare gold in a 0.9% NaCl solution [31].

B. Multilayer Resist Profile

We have developed a process yielding a profiled master for μCP. This fabrication process employs two photolithography steps (Fig. 3). The first step creates a 1-μm-wide trench in a silicon wafer, and the second step creates a 1.5- to 3.0-μm-wide trench in 10-μm-thick resist (Shipley 1075). Following this step, the master is placed on a hotplate at 115 °C for 1 min to allow the resist to reflow, resulting in the profile shown in the optical micrograph in Fig. 3. Polydimethylsiloxane (PDMS) elastomer stamps are then produced from this master using Sylgard 184 cured at 60 °C for 3 h with an optical flat as a rigid back support. The elastomer on the support is trimmed to an area of approximately 1 cm^2.

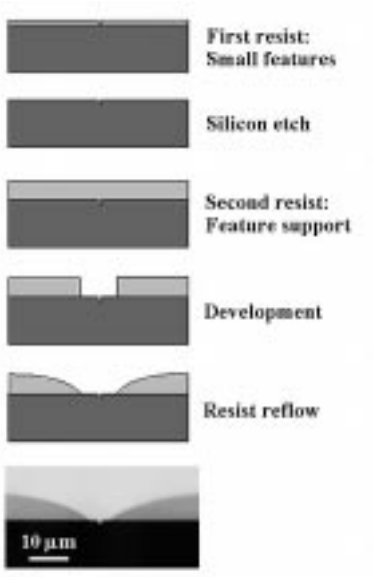

Fig. 3. Photolithographic steps for producing masters with geometrically stable μm-size features. The final image is an optical micrograph of the profile of a μCP master.

C. High-Resolution Aligned Patterned Protein Layers

FITC-labeled PL was dissolved at 1 mg/ml in phosphate-buffered saline. Stamps were treated in an radio-frequency plasma cleaner for 30 s to produce a hydrophilic surface, and then were coated with the protein solution for ten minutes. Stamps were subsequently blown dry with a filtered nitrogen gun and placed over the objective of the inverted microscope.

The critical components of our μCP aligner are indicated in Fig. 4. The vacuum chuck holds the substrate to be pat-

terned, and the stamp, which has been coated with protein, is fastened underneath to the stage of an inverted Olympus IX70 optical microscope. The microscope objective is held below the transparent stamp, allowing both surfaces of the substrate and the stamp to be visualized on a video screen. Then, the distance between the two surfaces is measured at several locations by focusing on the two surfaces and noting the distance in the travel of the objective piece. Wedge error between the two surfaces is removed using the three θ_x–θ_y micrometers [Fig. 4(b)] to alter the tilt of the substrate. The x and y translational stages are used to position the substrate, and the θ_z rotary stage with a micrometer-replacement actuator is used to remove rotational error. A digital crosshair allows the x, y, and θ_z positioning of stamp features to gold traces while alternating the focus between the two surfaces. Fig. 4(c) shows a cross section of the alignment tool arm as the z stage brings the wafer to the surface of the stamp. Both the z and θ_z stages are controlled by motorized picomotor drivers. A kinematic mount (with an opening in the center to allow for the neck of the chuck to pass through) was fixed to the θ_z stage. Before contact, the three tilt micrometers rest in the kinematic mount, and when the stamp surface is reached, the chuck loses contact with the kinematic mount [Fig. 4(d)] as the θ_z stage continues moving in the $-z$ direction. The chuck then remains on the stamp under the force of its own weight (400 g). After contact, the stamp was allowed to remain for 15 min, and then the substrate was lifted off and rinsed in PBS and deionized water.

D. Neurons Grown on Patterned Electrode Array

Cultured rat hippocampal neurons were isolated as previously described [32] and grown on the PL printed electrode array using the following methodology. Briefly, hippocampi were dissected from the brains of embryonic 18-day-old rats, incubated in trypsin, and then dissociated by vigorously pipetting up and down with a Pasteur pipette. Cells were plated at a low density of 3000–4000 cells/cm^2 onto the electrode array surface in a 100 mm dish containing minimal essential medium [MEM] supplemented with 10% horse serum. Once the neurons became attached (2–4 h), the electrode was transferred (neurons facing up) into a new 100-mm dish containing four 18-mm coverslips on which a confluent layer of astroglia were growing. The neurons were maintained in glial conditioned serum-free medium [MEM containing N2 supplements [33], pyruvate (0.1 mM) and ovalbumin (1 mg/ml)]. Neurons were fixed one day later in 4% formaldehyde/4% sucrose in phosphate-buffered saline (pH 7.4, warmed to 37 °C, 15 min). Images were acquired using a Leica DM-R microscope, interline charge-coupled device camera, and Metamorph software.

IV. Results

The multilayer μCP master has enabled the production of thin elastomeric stamps with the following attributes: a 1-μm feature supported on a mechanically stable pedestal with a corrugation depth of ∼11 μm. A scanning electron micrograph of an elastomer stamp is shown in Fig. 5. The profile increases the geometrical control of the stamp, reduces the lateral compliance of the fine features, and facilitates an easier release of

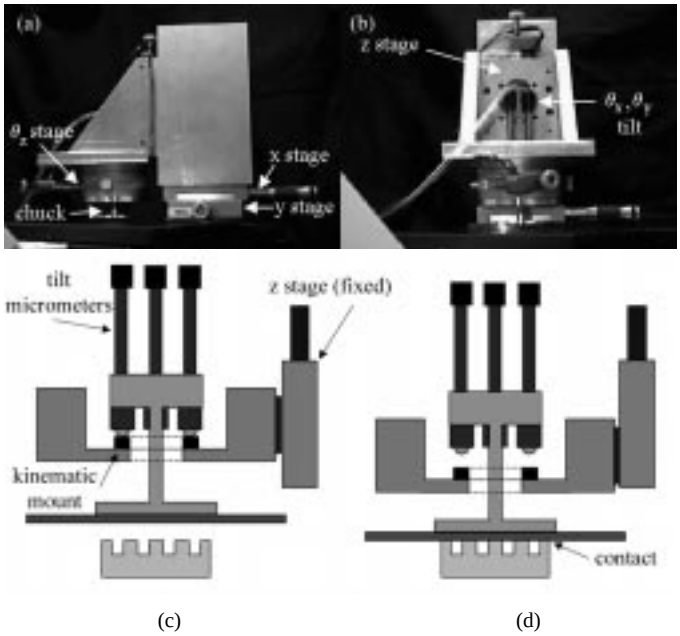

Fig. 4. Device for μCP aligned protein patterns. (a) Indicated are the translation stages for x and y, the rotational stage for θ_z, and the vacuum chuck used to hold the wafer. (b) The stepper motor controlled z-stage and the wafer tilt adjustment micrometers. (c) Drawing of the θ_z and z stages to illustrate the aligned stamping procedure. The chuck holding the wafer is attached to three tilt micrometers that rest on a kinematic mount. (d) As the z stage moves the wafer down, it contacts the stamp and allows the chuck to rest under its own free weight on the stamp.

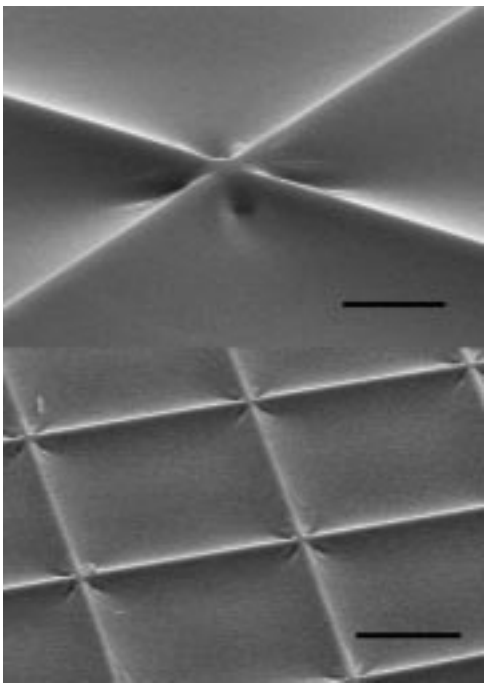

Fig. 5. SEM micrographs of an elastomer stamp cast from the multilayer resist master. Scale bars in the top and bottom image are 20 and 100 μm, respectively.

the stamp from the master. We can consistently produce 1-μm to 2-μm-wide lines spaced by 200 μm in a continuous grid pattern. The μCP aligner produced high-precision overlay alignment of a protein layer to an electrode substrate. Fig. 6(a) is an optical micrograph through a transparent stamp in contact with the electrode surface. A PL grid pattern was printed on the oxide

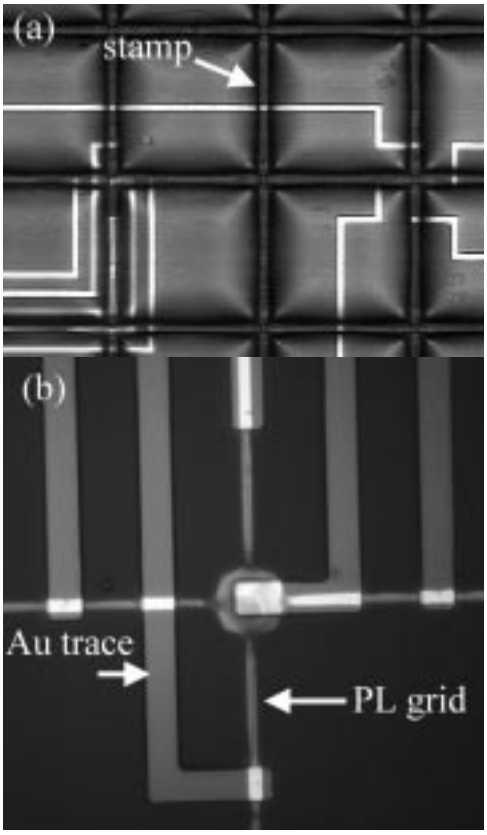

Fig. 6. Aligned μCP. (a) Optical micrograph during the stamping process. The stamp features can be seen making contact with the oxide surface of the wafer. (b) Fluorescent micrograph showing a μCP PL grid printed on the oxide surface of a microfabricated electrode array. Holes through the oxide and insulating layer are at the tips of each trace.

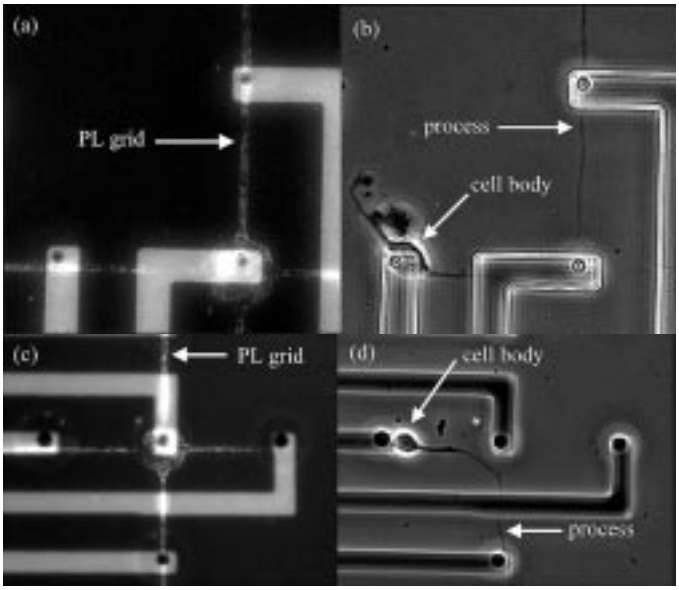

Fig. 7. (a) Fluorescent micrograph showing the PL grid on the oxide surface of the wafer. (b) Corresponding phase-contrast micrograph shows a neuronal cell body and process outgrowth that is guided along the μCP PL grid. Note that the cell body is directly covering an electrode site and that the process makes a 90° turn to follow the protein pattern to a nearby electrode site. (c) Another fluorescent micrograph showing the PL grid on the wafer surface. (d) A phase-contrast image showing a cell body next to an electrode site with a process being guided to another electrode site.

surface of an electrode array to guide the attachment and growth of neurons. Fig. 6(b) shows the electrode array with the printed PL grid pattern printed on the surface in alignment with the underlying Au electrode traces. Positioning to within ±1 μm has been achieved using this apparatus.

The cultured neurons adhered preferentially to the stamped PL pattern. These neurons appeared to have a healthy morphology and produced processes that also followed the stamped pattern. Fig. 7 shows fluorescent micrographs [Fig. 7(a) and Fig. 7(c)] and their corresponding phase-contrast micrographs Fig. 7(b) and Fig. 7(d)] where cells have attached and grown near the electrode sites. Within one day of culturing, a number of the growing processes had extended along the PL patterns and crossed over electrode sites. These results demonstrate that μCP PL provides a favorable substrate for healthy neurons to adhere and survive, and that this μCP pattern is capable of directing process outgrowth to defined points on an electrode array. Preliminary results indicate that cell networks cultured on μCP PL patterns can last at a minimum of four weeks. Further analysis of the viability of longer-term cultures on such patterns will be explored in future research.

V. Conclusion

An effective combination of extracellular electrode arrays and chemical patterning has been accomplished. High-precision alignment μCP of protein layers has been demonstrated to be an extremely useful method for spatially controlling the adherence and subsequent growth of cells on substrates. A design for improving structural stability and fine-feature printing precision has been shown to alleviate several complications with using μCP for μm-scale chemical modification of surfaces. A tool for aligned μCP was constructed and successfully used to pattern protein layers capable of yielding neural networks with μm-scale alignment accuracy on a substrate. Aligned, patterned protein successfully guided neuronal cell bodies and processes into close proximity (<3 μm) with electrode recording sites, offering a significant advantage over uncontrolled neuronal cultures on planar microelectrode arrays. This approach can serve as an effective method for patterning multiple biologically active proteins with a high degree of spatial accuracy. These techniques also present a new method for developing controlled neuronal circuits for long-term electrophysiological measurements that may include multisite recordings from individual cells.

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