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Microfabrication, surface modification, and laser guidance techniques to create a neuron biochip*

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Abstract

In this report we illustrate our application of soft lithography-based microfabrication, surface modification, and our unique laser cell-patterning system toward the creation of neuron biochips. We deposited individual forebrain neurons from Day 7 embryonic chicks into two rows of eight in a silicon microstructure aligned over a microelectrode array (MEA). The polydimethylsiloxane (PDMS) membrane with microstructures to confine cells and guide network connectivity was aligned to the electrodes of a MEA. Both the MEA and the PDMS membrane were treated with O₂ plasma, Poly-L-Lysine, and Laminin to aid in cell attachment and survival. The primary advantage of our process is that it is quicker and simpler than previous cell-placement methods and may make highly defined neuronal network biochips more practical.

One of the current emphases in neuroscience research is to study neuronal network dynamics and how network structure contributes to memory consolidation and information processing. This focus aims to fill the gap between what is known at the cellular and molecular levels of neuroscience and what is known at the higher anatomical level, where cognitive functions are mapped to brain regions. Biochips composed of neuronal cells cultured on top of microelectrode arrays (MEA) have become a popular and powerful research tool for studying the electrical activity of neuronal networks.

So far, the most successful method for creating neuronal biochips has used a large number of neurons cultured randomly over the MEA as a monolayer. Experiments using this method have shown how overall network-activity dynamics such as mean firing rate, bursting rate, and spike synchronization are affected by different stimulation or training protocols^[1-3]. Some groups have studied the changes in network activity in the presence of different chemicals or biomolecules^[4-6]. While in vitro random monolayer cultures allow for better stimulation and recording to the electrical activity of neurons, each neuron that synapses with many other neurons for the network connectivity is analyzed and correlated with functions of the network.

Neuronal networks may be simplified by forcing the geometry of networks into a defined pattern and reducing the number of connections between neurons. Microcontact printing and stenciling techniques can be used to achieve a partially-defined network^[7], while more

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highly-defined networks involve aligning a single neuron to each electrode^[8]. At this level of control, recording and correlating the activity of each neuron with its pre- and post-synaptic counterparts should be straightforward, allowing for more accurate models of how neuronal network dynamics arise from and depend on the network connectivity. For example one may study how specific “memories” are encoded in the network by correlating each neuron’s activity with the activity of every neuron it synapses with.

While several groups have successfully created defined neuronal networks of varying degrees^[7-13], most of these publications are still focused on technical design or aimed at characterizing the effects of patterning on overall network activity. We suspect that the delay in moving the research concentration towards signal processing analysis of neuronal networks is due to the limitations of current cell-deposition techniques (for a review see Ref. [14]), which may be too difficult and time consuming for practical use in daily research.

We have addressed this problem with our own process of combining microfabrication, surface modification, and our unique laser-cell-guidance techniques to create a neuronal biochip. Soft lithography techniques were used to create a microstructure over a commercially available microelectrode array. Surface-modification techniques, including plasma treatment, bioactive molecules, and biological-factors coating, were used to aid in cell attachment to the microelectrode array, which is vital to survival and development.

A laser guidance technique was employed, which used a weekly-focused laser beam to capture cells and propel them toward the spot on the substrate. This technique is similar to optical trapping or laser tweezers, but offers a much increased spatial range especially in the z dimension (the vertical axis parallel to the axis of the laser beam). With the increased range, we could guide cells injected into the cell-deposition chamber to many electrodes that spread across the substrate to create a neuronal biochip.

The MEA used in this experiment was specially ordered from Multi Channel Systems (MCS), a commercial producer of microelectrode arrays. The MEAs are nearly identical to the stock MCS 200/30/ ito/ir MEAs, with 60 circular electrodes measuring 30 μm in diameter arranged in a square configuration with a spacing of 200 μm . The electrodes and leads are made of Indium tin oxide (ITO), which is an optically transparent conductor. To our specification, the electrodes were not padded with a rough gold coating, which is usually applied to decrease the impedance and to reduce noise. Gold or platinum electrode pads absorb too much of the laser’s energy and boil the media above the electrode. The bubbles and burns thus formed obstruct imaging at the electrode during the patterning process and during later analysis and damaged the substrate modifications and most likely the cell.

A microfabricated polydimethylsiloxane (PDMS) membrane was employed in our chip with the aim of confining neurons to the electrode once they have been laser deposited there, to guide the axon outgrowth of neurons to form the desired network. The PDMS membrane was created using modern soft lithography techniques. Photolithographic masks were designed 4.2% larger than the dimensions matching the microelectrode array’s features to compensate for shrinkage of the PDMS and laser photoplotted. A two-layer 3D mold was created on a 50 mm silicon wafer with SU8-2005 and -2025 photoresist from Microchem and a Karl Suss MJB3 microaligner. Two types of features were created on the mold, 70 μm tall circular pillars, which were connected by tapered rails 7 μm tall. The mold was then vacuum-coated with 1H, 1H, 2H, 2H-Perfluorooctyltrichlorosilane 97% (Alfa Aesar #L16606) overnight. The PDMS was spun at 2500 rpm for 30 s to achieve a membrane thickness of about 60 μm , allowing the tallest features to break through the PDMS surface. A thicker annular was created by applying more PDMS around the outside of the feature area and spinning at 400 rpm for 30 s. The mold was baked at 100 °C for 10 min and

allowed to cool. The PDMS membranes were then carefully removed from the mold. Membranes were hung on a line to keep them from folding onto themselves, sticking to each other, or sticking to the sonication chamber. They were then sonicated in 100% ethanol for 15 min, 100% acetone for 1h, and then allowed to dry. They were then baked in a vacuum oven at 120 °C for 4 h. Finally the membranes were soaked in phosphate buffered saline (PBS) overnight.

The microelectrode array and the PDMS membrane were treated with O₂ plasma for 10 min. They were then coated with poly-l-lysine (PLL) for 30 min, and rinsed with DI water 3 times. The MEA and membrane were shaken dry, leaving some water on the surfaces to act as lubricant during the alignment process, which was performed by hand through a 20 × microscope objective. The illumination helped to dry the MEA and membrane, allowing for the alignment to lightly set in a few minutes. To finish the bonding, the substrate was then placed on a 50 °C hotplate for 1 h. The substrate was then plasma coated once more for sterilization after alignment in open air. It was then recoated with PLL for 30 min, after which the remaining PLL was removed. A 800-μm thick PDMS donut was then placed on top and secured with the remaining elements of the cell patterning chamber described previously^[15]. Next the chamber was filled with sterilized DI water and placed in a vacuum desiccator to de-gas the membrane, removing air trapped beneath it, in the channels, and in the clear-through holes. The chamber was then rinsed by circulating sterilized DI water through it. Then the DI water was exchanged for media 199 with 10 mg/ml natural mouse Laminin. The chamber was incubated for 4 h at 37°C and 5% CO₂.

Chick forebrain neurons were harvested from Day 7 embryonic white leghorn chicks. The two lobes were incubated in 1.5 ml 0.1% trypsin in PBS for 5 min. The trypsin was removed and culture media was added. 50 ml of culture media was prepared with 21.5 ml Neurobasal media without phenol red, 21.5 ml media 199, 5 ml fetal bovine serum, 1 ml B27 neural supplement, 0.25 ml Glutamax, and 0.05 ml of nerve growth factor at concentration of 10 μg/ml.

We have previously reported on the design of the laser deposition chamber^[15], which is made of stainless steel and PDMS. This chamber was mounted to a motorized, computer controlled, three-axis stage (AeroTech FiberAlign 90). A World Precision Instruments Micro-pump II was used to feed cells into the deposition chamber. A cell suspension of $3 \times 10^4 - 5 \times 10^4$ cells/ml was loaded into a 50 μl syringe, which was then loaded into the Micropump II. Volumes of 20-100 nl were injected through a hollow optical fiber into the deposition chamber. The chamber was kept at 37°C with heating system composed of a PID controller, a thin wire RTD sensor, and two flexible Kapton heating elements from Minco.

Once a cell was injected into the chamber, the chamber was moved such that the cell was maneuvered inline with a stationary, weakly focused laser beam. The optical forces of this beam trapped the cell radially and propelled it axially toward the substrate. The chamber was then moved relative to the laser beam with the guided neuron until it was deposited at a target electrode. A continuous single-transverse mode Ti:Sapphire laser from spectra physics, tuned to a wavelength 800 nm, was used. The beam was collimated with a two-lens telescopic system ($f = 20$ and $f = 50$), passed through a dichroic mirror, and then passed through a 20 × L plan objective purchased from Mitutoyo. This objective was used for imaging as well as to focus the laser beam. The waist of the beam was steered to coincide with the focal plane by adjusting the position of the first lens ($f = 20$). A laser intensity of 100 mW (measured in the deposition chamber) was used during most of the patterning process. When the laser guided neuron and laser focus were brought close to the electrode, the control software automatically dropped the laser intensity to 15mW to avoid overheating of the electrode.

The control software was written in National Instruments LabView 8.0. The user then could navigate a cell through the 3D space of the cell deposition chamber with wireless game pad controller. Point coordinates were marked and automatically recalled using the program, and in this manner cells could be guided to specific targets on the substrate. The program could display an image of the patterning area with an overlay of navigation cues to direct the user towards marked targets. Other parameters including laser intensity, laser intensity reduction cues, micro injection volumes and speeds, navigation speeds, and interface control schemes, could be set by the users to fit their application and ability.

A pattern of two rows of eight cells was created within rows of electrodes connected by the channels in the PDMS membrane. The entire patterning process took close to 16 min. The end of the microinjection fiber was positioned about 800 μm above the substrate and 1000 μm away from the electrodes in the x - y plane. During normal laser guidance of cells, the final laser beam power is set to 100 mW. This power has been shown to cause no cell damage and provides excellent radial and axial forces for manipulation, allowing cell guidance speeds near 100 $\mu\text{m}/\text{s}$. However, the laser power had to be reduced while passing through the electrodes to avoid overheating. A final power of 15 mW was used for the final stage of patterning. The control application was set to automatically reduce the power to 15 mW when the laser beam focus and captured cell came within 60 μm of the target electrode in the x - y plane and 200 μm above the surface. During the final 200 μm travel, the cell guidance velocity dropped to between 5 and 10 $\mu\text{m}/\text{s}$. This led to a total deposition time of about 1 min for each cell. With an additional minute attributed to the target selection, microinjection, and cell selection, the average total period of a cell deposition was 2 min. The entire patterning process took about 16 min, after which the chamber was transferred to a Zeiss Axiovert 200 microscope equipped with an onstage incubator set to keep 37°C and 5% CO_2 . Images were taken using 10 $\times$ phase and 40 $\times$ long-distance objectives.

The two rows of patterned cells were photographed at 45 min, 4.5, 16, and 24 h after deposition. Within this time there was very little cell migration or neurite extension. Fig.1 shows the excellent conformity of the PDMS membrane to the MEA, with the single neurons visible in the two rows of electrodes. The cells in row A are shown at 40 $\times$, 4.5 h after patterning, in Fig.2. Axon extension can be seen in the 64 $\times$ close-up micrograph taken of cells at 16 hours after patterning (Fig.3).

The micrograph in Fig.1 indicates that we have successfully developed a process for depositing neurons into a microstructure which can confine cells and may be used to guide their connectivity.

The primary advantages of our process over conventional single-cell placement methods are the speed and ease of the process, which make it more practical. The laser guidance system provides high-precision placement (less than 1 μm) with a wide 3D range of motion (greater than 2 mm in every dimension). This ability, combined with the easy and intuitive control provided by the software control application, enables quick and direct patterning of individual cells to the desired position in the biochip substrate.

An additional advantage of our process is that it can be used to pattern multiple cell types in one session with a specific design of the microinjection system^[15] and the wide spatial range of the laser guidance system. This ability may be used to create more complex neuronal circuits that more accurately mimic in vivo circuits composed of multiple cell types, as occurring in the hippocampus and the reflex arc.

The process that we have developed for creating a neuronal biochip, presented here, has several advantages over previous methods. Most importantly, our results illustrate the unique ability of our process to deposit single neuron with the highest level of precision,

quickly and easily. This aspect of our process may lead to its adoption in other biochip fabrication settings and promote more highly defined neuronal network research.

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Fig.1.

The two rows of cells, 45 min after being patterned into the microwells formed by the PMDS membrane and onto the electrodes. Triangular tunnels span the 200-μm inter-electrode distance.

Fig.2.

After 4.5 h, 6 of the 8 cells in row A appear to be healthy. Electrodes are 30 μm in diameter.

Fig.3.

16 h after patterning, a neuron (a) shows significant axon extension (b) into the wide portion of the guidance channel (c). The 20- μ m diameter, clear through hole (d) keeps the neuron in place over the ITO electrode (e).