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Synaptic Connectivity of a Low Density Patterned Neuronal Network Produced on the Poly-L-Lysine Stamped Microelectrode Array

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Abstract: Rectangular networks of rat hippocampal neurons have been produced on microelectrode arrays (MEAs). The crossing points of networks were located at the recording electrode sites by aligned microcontact printing (μCP) technique. Polydimethysiloxane (PDMS) stamp was fabricated to print fine poly-L-lysine (PLL) patterns of 2μm-width lines for neurites and 20μm-diameter circles for cell bodies. Different densities of neurons were applied on the PLL-stamped MEAs to find how a low density of neurons still has the functional connectivity. From the neural network applied with a density of 200 cells/mm^2, we could observe signal propagation among spontaneous activities. Electrical responses were also evoked by 200μA current pulse stimulation with 50μs pulse width. Immunocytostaining was employed to identify dendrites, synapses, and nuclei in the patterned neurons.

I. Introduction

For more than 20 years, scientists have tried to detect and analyze neuronal electrical activities in vitro. Especially planar microelectrode arrays (MEAs) enabled long-term, simultaneous recording of electrical activities from several neurons [1,2]. But the activities can be obtained only from the cells which are very close to the electrode sites. To locate the neurons near the electrode sites, several methods have been developed by controlling the attachment of neurons by surface modification. Among these technologies, microcontact printing (μCP) has been shown to be able to precisely and easily pattern proteins which can influence the attachment and growth of cells in culture [3,4].

So far, a number of investigators have reconstructed neuronal networks on the planar microelectrode arrays and recorded the electrical activities from the cells and stimulated them [5,6,7]. In most of previous studies, collective characteristics such as firing rate were usually analyzed. On the other hand, synaptic connectivity between individual cells has not been well studied, although the connectivity is one of the most important properties in neuronal network. In order to investigate the connectivity precisely, it is needed to make network with the small number of neurons and to pattern the cells precisely. But an appropriate density of neurons should be cultured, which is lower but still maintains functional synapses because neurons can't survive for long time and the activities are decreased under the condition of low density.

Here, we showed that individual cells and their synaptic connectivity can be analyzed with more resolution in a low density of neurons grown on the planar microelectrode array stamped with poly-L-lysine. We believe this study is fundamental and also important for understanding functional circuits of neuronal networks and developing cell-based biosensors.

II. Methods

A. Microelectrode Array

The MEAs were fabricated by the semiconductor process mentioned below. Titanium (30nm) as adhesion layer and gold (300nm) were deposited on the substrate (Pyrex #7740). Another titanium layer (10nm) was deposited on the gold layer for the adhesion to the upper insulation layer. These metal layers were patterned by wet-etching with HF (1%) and HCl, HNO_3 (3:1) solutions. The triple stack ($SiO_2/Si_3N_4/SiO_2$) was deposited by plasma enhanced chemical vapor deposition with the stress compensated thicknesses of each layer [8]. The insulation areas for the signal detection from neurons and for the contact to the lead-out were etched by reactive ion etching. The upper titanium layer was etched at the same time. Ring-shaped teflon was attached on the MEAs for making culturing chamber. The MEA has 32 recording sites and the impedance of the sites was 112±41kΩ after platinization.

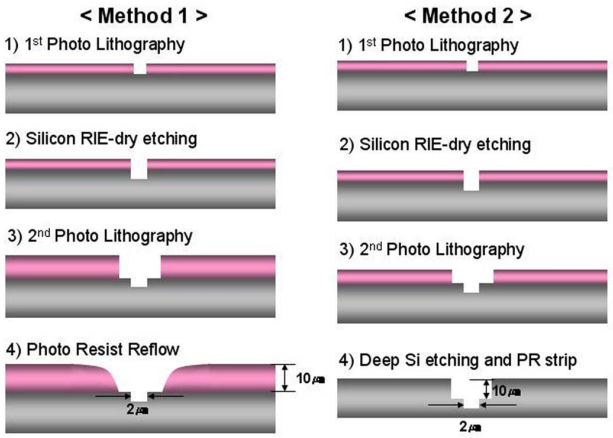

Fig. 1. Stamp masters fabrication methods for molding PDMS stamps

B. *Microcontact stamping*

The polydimethylsiloxane (PDMS) stamp was molded from the stamp master which was designed to match the patterns of MEAs as depicted in Fig. 1. The stamp maters were made by two different methods. Method 1 was using thick photoresist which had been reported previously [5], and method 2 was using two consequential dry etching. The fabricated PDMS stamp has the pattern of 2㎛-wide line and 20㎛ circles at the crossing points. Flourescein isothiocyanate (FITC) labeled poly-L-lysine (PLL) was applied to the PDMS stamp for 1 hour after dissolved at 1mg/1ml in borate buffer solution. After blowing off PLL, the PDMS stamp was mounted at the custom-made alignment tool [5] and made to contact with the surface of the MEA for more than 1 hour.

C. *Cell Culturing*

Hippocampal neurons were obtained as previously described [3]. Hippocampi were dissected from the brains of embryonic 18-day-old rats, incubated in trypsin, and dissociated mechanically with a Pasteur pipette. Neurons were plated at the densities of 100, 200, and 400 cells/cm^2 on the PLL-patterned MEAs in minimal essential medium (MEM) supplemented with 10% horse serum and 0.1% pyruvic acid. After 4 hours, the medium was replaced with serum-free neurobasal media (Gibco) supplemented with B27 (Gibco) and 0.5mM L-glutamine. Cultures were maintained at 37℃, 5% CO_2. Half of the media were replaced with fresh media twice a week.

D. *Electrophysiology*

Before electrophysiological experiments, the media was replaced with recording media (HEPES). MEAs were mounted into the custom-made connector, which was placed on a resistively-heated stage. An Ag/AgCl wire was placed in the culture chamber as the reference electrode for extracellular recording and stimulation. The signals from the recording sites were amplified with the gain of 10,000 and filtered (0.3~5 kHz, 40 dB/decade) by A-M systems differential AC amplifier model 1700. The signals were sampled at the frequency of 20 kHz and digitized by data acquisition device (NI 6024E, National Instruments). Recording was performed under the background noise less than 10 ㎶rms.

Using isolated stimulator (ISO-Flex, AMPI, Israel), electrical current with the pulse width of 50 ㎲ was applied through a electrode site for stimulation. The amplitude of the pulses were increased from 50 ㎂ to 200 ㎂ until the evoked potential appeared.

E. *Immunocytochemistry*

After electrophysiological experiments, the neurons were fixed with 4% paraformaldehyde at 37℃ for 15 minutes, made permeable with 1% triton in HEPES Hank's buffer for 15 minutes at room temperature (RT), and incubated in 6% bovine serum albumin for 30 minutes at RT to block nonspecific binding. Cells were exposed to a mixture of rabbit polyclonal anti-MAP2 (1:500) (Sigma) and mouse monoclonal anti-synaptophysin (1:200) (Sigma) for 1 hour at 37℃. In order to identify these primary antibodies, Texas Red goat anti-rabbit (1:200) (Molecular Probes) and Alexa 488 goat anti-mouse (1:200) (Molecular Probes) were used as secondary antibodies. MAP-2 is a maker for the identification of the neuronal cell bodies and dendrites, and synaptophysin

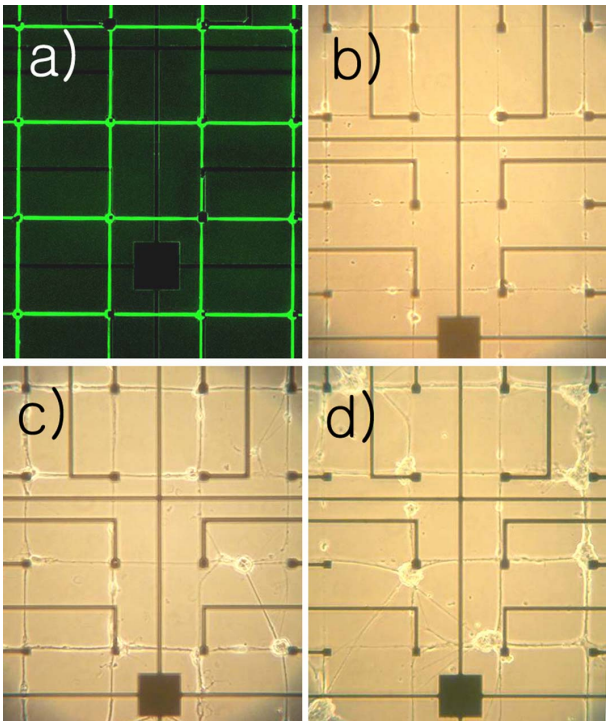

Fig. 2. (a) Fluoresent micrograph showing FITC-labeled poly-L-lysine patterns aligned stamped on the MEA (b) Phase-contrast micrograph of cultured neurons on the stamped MEA at the plating density of 100 cells/mm^2 (c) 200 cells/mm^2 (d) 400 cells/mm^2

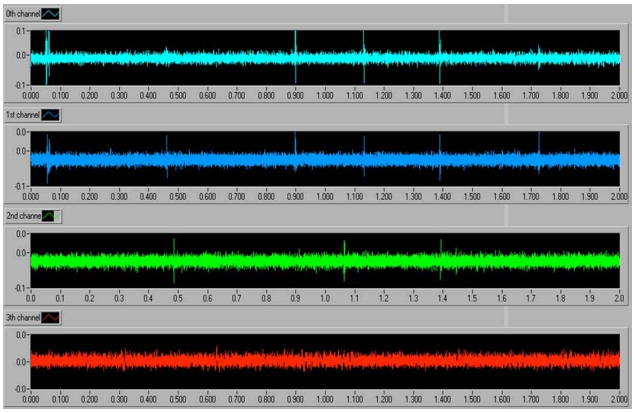

Fig. 3. Spontaneous activities recorded from patterned neuronal network at DIV 21 for 2 seconds

is one of the presynaptic proteins which show the locations of the synapses. After the immunostaining, cells were applied with DAPI in Milli-Q water (18.2MΩ) at RT for 30 minutes for staining nuclei. The sample was mounted under cover glass using mounting media (1:1 HEPES Hanks/glycerol containing n-propyl gallate). Stained sample was observed with an Olympus BX41WI epifluorescence upright microscope and the images were captured by an optronics magnafire CCD camera (Olympus, Melville, NY).

III. RESULTS

In the neuronal network plated with cell density of 100

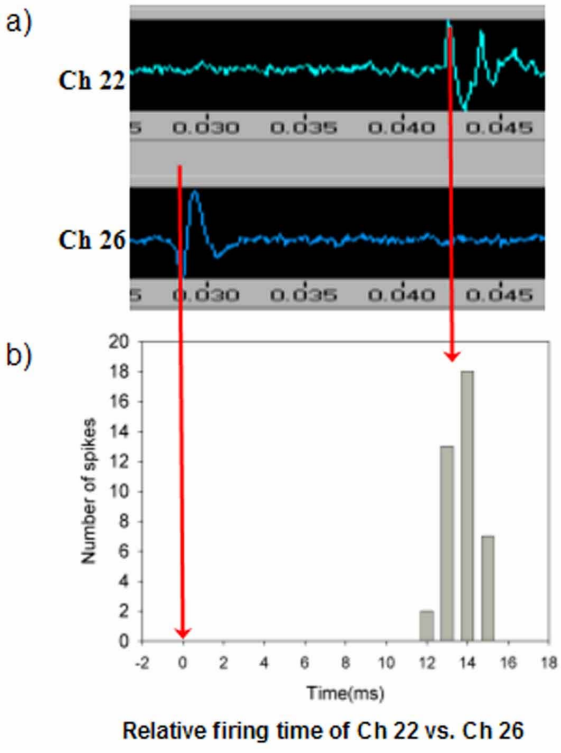

Fig. 4. Activities propagation recorded at two electrode sites

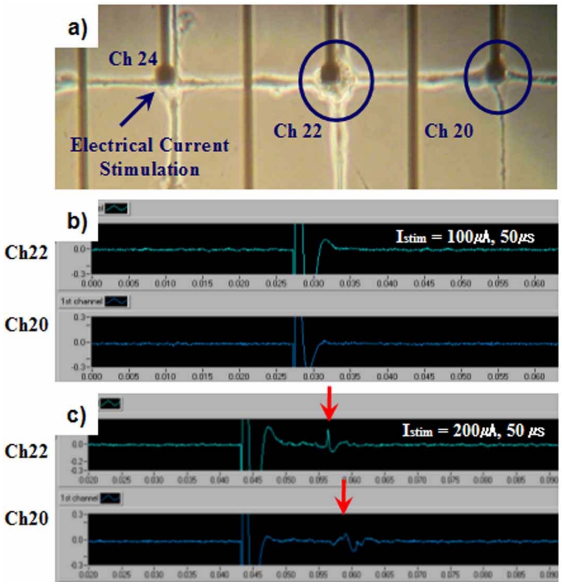

Fig. 5. (a) the location of stimulation and recording (the distance between sites: 200㎛) (b) evoked signals by stimulation of 100㎂, 50㎲ current pulse and (c) 200㎂, 50㎲ current pulse

cells/mm^2, cells were attached very well such that almost all the cell bodies were located on the cross point. But no spontaneous signal was observed. When the cells were plated at 400 cells/mm^2, excessive number of neurons aggregated together that recorded signals couldn't be discriminated for matching spikes to specific cells. From the network plated with 200 cells/mm^2, not only spontaneous activities but signal propagations were also observed although more than one neuron are attached near one site sometimes. Fig. 3 shows the spontaneous activities from the patterned neurons plated at 200 cells/mm^2. Between two recording sites with the distance

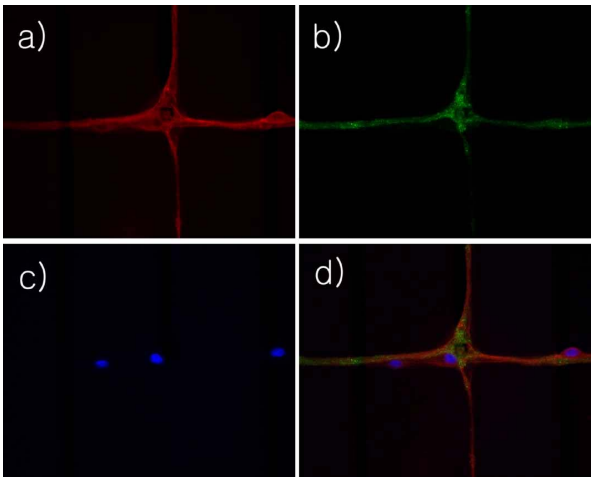

Fig. 6. Fluorescence micrographs illustrating (a) dendrites and cell bodies by MAP-2 staining, (b) presynaptic proteins by synaptophysin staining, (c) nuclei by DAPI staining and (d) merged image

of 400 ㎛, signal propagations were observed as shown in Fig. 4(a). 76% of activities from Ch 26 were followed by the signals from Ch 22, and the time interval was very consistent as shown in Fig. 4(b).

When electrical current stimuli were applied through one electrode site, activities of cells were evoked near the stimulation point as shown in Fig. 5. The responses appeared only at the current amplitude above 200㎂ when the pulse width was fixed at 50㎲.

The dendrites, synapses and nuclei in the patterned network were identified by immunocytostaining. As depicted in Fig. 6, presynaptic proteins were located along neurites and near cell bodies. It shows that synapses have been developed between neurons successfully in this patterned neuronal network.

IV. Summary/Discussion

Neurons were successfully cultured in low density for long term survival. From the neuronal networks cultured in low density, propagation of signal along the network was recorded for the first time. The evoked potentials were also observed following electrical current stimulations. The formation of synapses and the location of cell bodies were identified by immunocytostaining.

Acknowledgment

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References

[1] G. W. Gross, “Simultaneous single unit recording in vitro with a photoetched laser deinsulated gold multimicroelectrode surface,”

[2] Y. Jimbo and A. Kawana, “Electrical stimulation and recording from cultured neurons using a planar electrode array,” *Bioelectrochem. Bioenerg.*, vol 29, pp. 193-204, 1992

[3] C. James, A. Spence, N. Dowell-Mesfin, R. Hussain, K. Smith, H. Craighead, M. Isaacson, W. Shain, and J. Turner, “Extracellular recording from patterned neuronal networks using planar microelectrode arrays,” *IEEE Trans. Biomed. Eng.*, vol. 51, pp. 1640-8, 2004

[4] John C. Chang, Gregory J. Brewer, Bruce C. Wheeler, “Modulation of neural network activity by patterning,” *Biosensors & Bioelectronics*, vol. 16, pp. 527-533, 2001

[5] C. James, R. Davis, M. Meiyer, A. Turner, S. Turner, G. Wither, L. Kam, G. Banker, H. Craighead, M. Isaacson, J. Turner, and W. Shain, “Aligned microcontact printing of micrometer-scale poly-L-lysine structures for controlled growth of cultured neurons on planar microelectrode arrays,” *IEEE Trans Biomed. Eng.*, vol. 47, pp.17-21, 2000

[6] Y. Nam, D. Khatami, B. C. Wheeler, and G. J. Brewer, “Electrical stimulation of patterned neuronal networks in vitro,” *Proceedings of the 25th Annual International Conference of the IEEE EMBS*, Cancun, Mexico, 2003

[7] Alexandra Gramowski, Komstantin Juugelt, Dieter G. Weiss, and Guenter W. Gross, “Substance identification by quantitative characterization of oscillatory activity in murine spinal cord networks on microelectrode arrays,” *European J. of Neurosci.*, vol. 19, pp. 2815-2825, 2005

[8] T. H. Yoon, E. J. Hwang, D. Y. Shin, S. I. Park, S. J. Oh, S. C. Jung, H. C. Shin, and S. J. Kim, “A micromachined silicon depth probe for multi-channel neural recording,” *IEEE Trans Biomed. Eng.*, vol. 47, pp. 1082-1087, 2000