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Development of the micro-patterned 3D neuronal-hydrogel model using soft-lithography for study a 3D neural network on a microelectrode array*

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***Abstract*— In vitro patterned neuronal models have been studied as one of the strategies to investigate the relationship between structural connectivity and functional activity of neural network. Despite the importance of three-dimensional (3D) cell models, most of these studies have been performed on two-dimensional models. In this study, we present a technique to construct the micro-pattern to 3D neuronal-hydrogel model using a micromolding in capillaries (MIMIC) technique on microelectrode array (MEA). Our technique was suitable to prevent the deformation of micro-patterned collagen model against the neuronal contracted tension during the network formation. The relationship between the growth directions of glial cells and micro-pattern direction was investigated. Lastly, we confirmed that our 3D model had synchronized activity among neurons in 3D. This model is expected to be used as a tool to study the relationship between structural connectivity and functional activity in the 3D environment.**

I. Introduction

The relationship between structural connectivity and functional activity on neural circuits have been studied to understand brain function. In vitro neural models have been used as one of the strategies of neurophysiological research [1]. It could provide the advantage of reducing difficulty for analysis than in vivo tissue. In particular, engineering techniques for patterning neurons in vitro to control structural connectivity have been developed such as lift-off [2], microcontact printing [3], [4], microfluidic devices [5]. Despite the emerging importance of the cultured neuronal network in 3D environment, most of these research models have been performed on two-dimensional models [6], [7]. The patterned 3D neuronal model can be used as useful tool to research neurophysiology.

In order to construct the 3D environment with neurons, extracellular matrix (ECM)-based hydrogels such as collagen and Matrigel were used as the scaffold which supported cells to 3D space. ECM-based hydrogel provided the microenvironment like in vivo tissue [8], [9]. Also, it had properties such as easily controllable stiffness, pore size and gel shape [10]. These properties could produce the suitable condition to construct the 3D model with micro-pattern. Moreover, the functional activities of 3D neuronal-hydrogel model have been recorded using several techniques such as calcium imaging and microelectrode array (MEA) [11], [12]. Patterning techniques were presented to control the structural connectivity of the 3D neuronal hydrogel model. However, only models with macroscale patterns (millimeters) were reported [13]. Because neural networks had connections in a micro-scale, technology is needed to give micro-patterns to 3D neuronal hydrogel models.

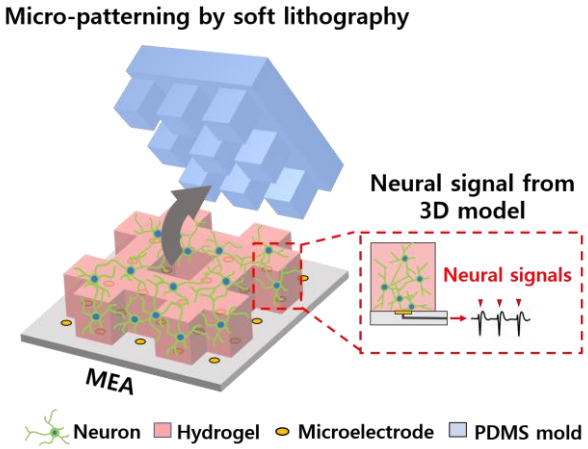

Fig. 1. The scheme of constructing the micro-patterned 3D neuronal-hydrogel model on MEA using soft lithography. Neural signals could be recorded from MEA.

In this study, as Fig.1, we constructed a micro-pattern for a 3D neuronal-hydrogel model. We built a 3D environment using ECM-based hydrogel such as collagen and Matrigel and constructed the micro-patterns on the 3D neuronal-hydrogel model using a micromolding in capillaries (MIMIC) technique on an MEA. To immobilize the micro-patterns of hydrogel on MEA, we used polyDA could provide adhesion to collagen and Matrigel [14]. Then, we investigated the properties of our micro-patterned 3D neuronal-hydrogel model. The relationship between cell growth direction and micro-pattern direction was analyzed by using immunostaining 3D image of cells in cultured 3D model. And

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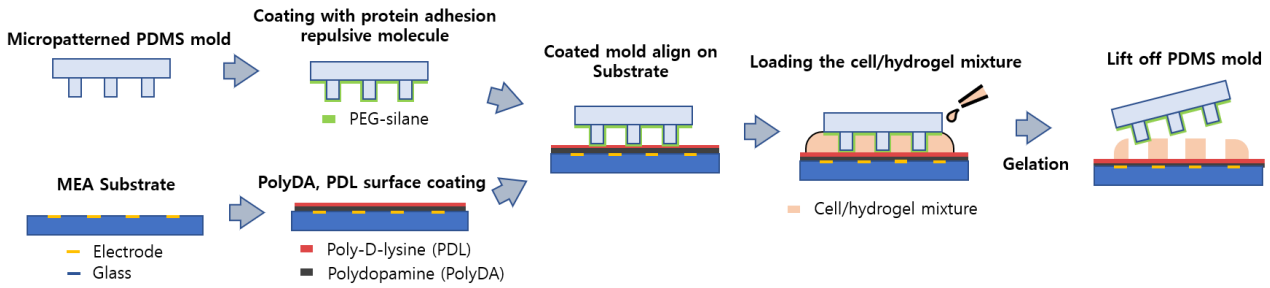

Fig. 2. Procedure to construct the micro-patterned 3D neuronal-hydrogel model on MEA.

we confirmed that neural signals from MEA presented the signals from 3D neural network.

II. Material and Methods

A. Cell Culture within ECM-based hydrogel

Cell/hydrogel mixture was made using the dissociated cortical neurons of E18 Sprague-Dawley rats and collagen type 1 (1.0 mg/mL) or Matrigel (7.5 mg/mL). Final cell concentrations were 3.0×10^7 cells/mL. These models were cultured in incubator (5 % CO_2, 37 ·C, humidified).

B. MEA surface coating

MEA was coated by using polyDA and PDL [15]. In this study, substrate was coated with polyDA (2 mg/mL in Tris-buffer pH 8.5) for 6 hours and then washed substrate using 3rd DI water. PDL (0.1 mg/mL in Tris-buffer pH 8.5) was loaded on substrate which coated with polyDA for 1 hour.

C. Fabrication of PDMS mold with Micro-pattern

The micro-patterned polydimethylsiloxane (PDMS) mold was fabricated by soft-lithography. We made the designed silicon wafer by photo-lithography. The designed pattern was grid pattern which was one of the simplest patterns. Then, PDMS was cured on the designed silicon wafer. Our PDMS mold had square-pillar pattern (300 × 300 μm) and spacing each pattern was 100 μm. Pattern scales were designed to fit the electrode spacing (200 μm) of MEA. Height of pattern was 100 μm. If height was lower than 50 μm, cells were almost composed monolayer. Therefore, a relatively high height is suitable to represent the properties of 3D model. This mold can make the grid patterned model on an MEA by MIMIC.

D. Micromolding In Capillaries (MIMIC) Procedure

Fig. 2 shows the procedure to construct the micro-patterned 3D neuronal-hydrogel model on an MEA. A PDMS mold was coated with PEG-silane (50 % (v/v) in EtOH). PDMS mold was aligned on an MEA using microscope. Then, cell/hydrogel mixture was loaded in gap between mold and MEA. PDMS mold was lift off after gelation in incubator (5 % CO_2, 37 ·C, humidified).

E. Hydrogel adhesion test w/ and w/o cells

Hydrogel droplets (collagen, Matrigel) were placed on substrates such as bare glass, glass coated with PDL, polyDA, PDL + polyDA . Then these were shaken by a shaking incubator (120 RPM during 24 hours).

The suitability of hydrogels was determined by calculating the overlap area ratio to the initial area. Areas were measured

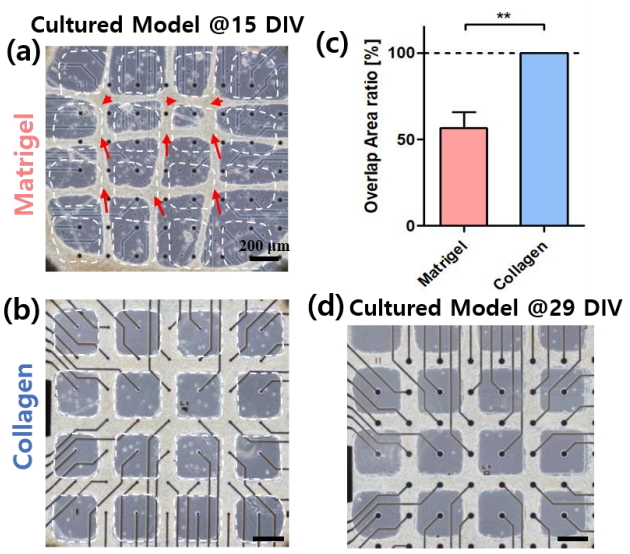

Fig. 3. Test for find the suitable hydrogel type. Image of micro-patterned model using (a) Matrigel, (b) collagen at 15 DIV. Scale bar: 200 μm. White dot line was initial area boundary. (c) Overlap area ratio within initial pattern area. n= 8, 6 chips, mean±sd, Mann Whitney test, **p=0.0016. (d) Image of the cultured model using collagen at 29 DIV.

by phase contrast image of the micro-patterned 3D neuronal-hydrogel models.

$$\text{Overlap area ratio} = 100\ \frac{A_{initial} \cap A_{developed}}{A_{initial}}\ [\%]$$

where, A_{inital}: cell/hydrogel area of micro-patterned 3D model at initial stage, $A_{developed}$: cell/hydrogel area of micro-patterned 3D model at 15 DIV. overlap

F. Immunostaining and Image analysis

We used primary antibody such as anti-beta-3-tubulin, anti-tau1, anti-GFAP, Hoechst 33342. We obtained image using a Zeiss LSM880 confocal laser scanning microscopy with x40 water immersion objective lens. 3D rendering was processed by Imaris software (Bitplane). Orientation analysis was measured using ImageJ software (NIH).

G. Neural signal recording

We used a MEA which had 60 electrodes (diameter: 30, 50 μm, spacing: 200 μm) and an amplifier (gain: 1000, bandwidth: 200~5000 Hz). The threshold level to detect the spike is set at "mean – 6 × standard deviation" of noise signal. For calcium imaging, we used Oregon Green BAPTA-1 (Molecular Probes) calcium indicator. Neural signals from

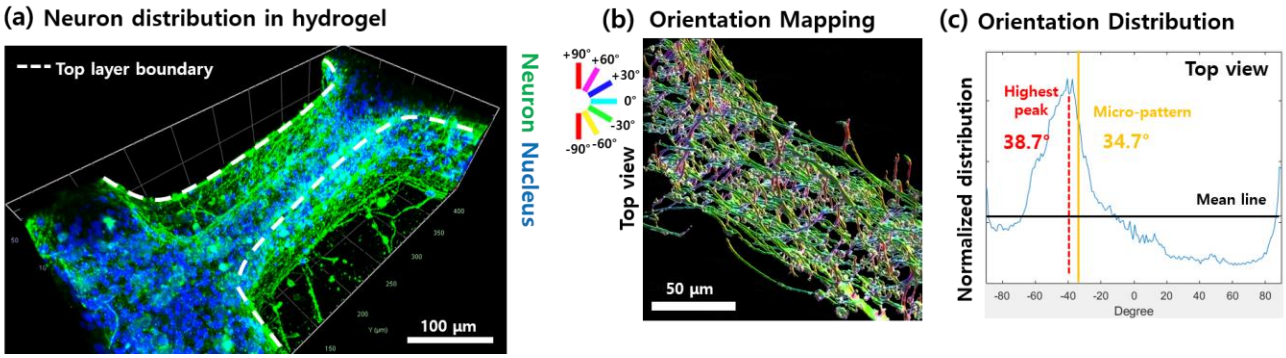

Fig. 4. (a) Immunostaining image of micro-patterned 3D neuronal-hydrogel model. (b) Orientation mapping colored image of glial cells about top view. (c) Distribution histogram of glial cells orientation.

calcium imaging were extracted by using NeuroCa software [16].

The correlation between neurons of bottom and top layers was analyzed by signals from MEA and calcium imaging. Pearson-correlation coefficient was calculated by 3 types of pair (intra-pair: pairs with same layer, inter-pair: pairs with other layer, negative-control: pairs with another chip). Each pair of signals was used as raw data with same bin size (66.7 msec). These signals recorded for 300 second.

III. Experimental Result

A. Hydrogel adhesion test on neural interface

We investigated whether the neural interface can induce adhesion between polyDA and hydrogel (collagen, Matrigel) even though coated with PDL on polyDA film. Interfaces which were PDL coated on polyDA and coated only polyDA showed sufficient adhesion with collagen and Matrigel droplets during 24 hours shaking. In case of bare glass surface or that coated only with PDL, all collagen and Matrigel droplets detached within 1 hour. These results indicated that even though collagen and Matrigel doesn't attach to PDL, polyDA under PDL could bring the adhesion between polyDA and both type of hydrogel such as collagen and Matrigel.

B. Suitable type of hydrogel to maintain the micro-pattern.

Test was investigated to find the suitable ECM-based hydrogel about collagen and Matrigel (Fig. 3). The micro-patterns of model which used Matrigel were distorted during the cell growth over 15 days. Area of micro-patterns was maintained only 56.6 ± 9.1 % of initial patterned area (Fig. 3(a), (c)). Whereas, model which used collagen maintained 100 % of initial patterned area (Fig. 3(b), (c)). Collagen was more suitable type of ECM-based hydrogel to fix the structural connectivity of our model. Also, collagen micro-pattern model maintained their patterned area for 29 days of cell cultivation (Fig. 3(d)).

C. Cells type and structural properties

Our micro-patterned 3D neuronal-collagen model presented the positive on neuron marker which was beta-3-tubuline, glial cell marker which was GFAP and axon marker which was tau-1, nucleus marker which was Hoechst. We confirmed that neurites grew within all heights of 3D model at 8 days in vitro (DIV) (Fig. 4(a)). Also, to investigate the relationship between

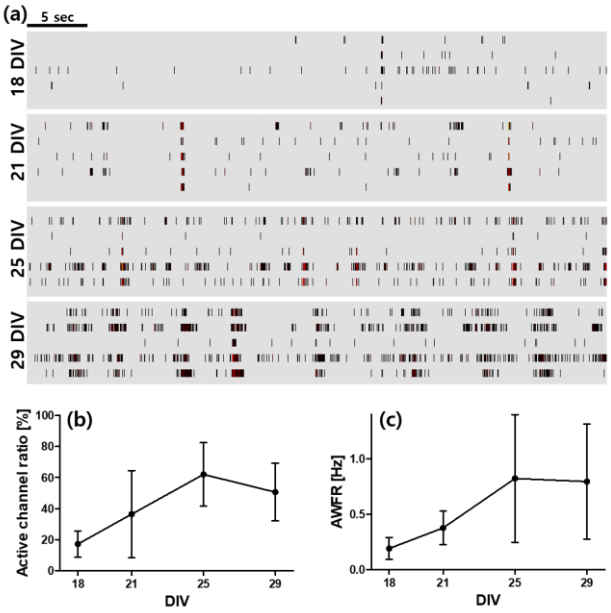

Fig. 5. (a) Raster plots according to DIV. (b) Active channel ratio (Firing rate>0.05Hz), (c) Array wide firing rate (AWFR). n = 4 chips, mean±sd.

cell growing orientation and micro-pattern direction of 3D model, growth orientations of glial cell were measured (Fig. 4(b)). Growth orientations of glial cells were biased to 38.7 ° orientation angle. This angle was similar with orientation of micro-pattern, 34.7 ° (Fig. 4(c)). It indicated that the micro-pattern effected on growth orientation of glial cells.

D. Recording the neural signals of 3D model

We recorded electrophysiological signals during 18~29 DIV using MEA. Raster plots from recorded signals at each DIV were plotted on Fig. 5(a). Ratio of active channels (firing rate>0.05 Hz) showed a linear increasing until 25 DIV (62.1 ± 20.0 %) (Fig. 5(b)). Also, Array wide firing rate (AWFR) increased until 25 DIV (0.82 ± 0.57 Hz) (Fig. 5(c)). If electrodes were not located below the patterned 3D model, the neural signals were not measured. In order to investigate the functional connections between neurons of different heights, the Pearson correlation coefficients between neural signals from MEA and calcium imaging were calculated (Fig. 6(a), (b)). Coefficients of inter pairs (0.51 ± 0.07) had significantly higher values that those of negative control (0.01 ± 0.03) (Fig.

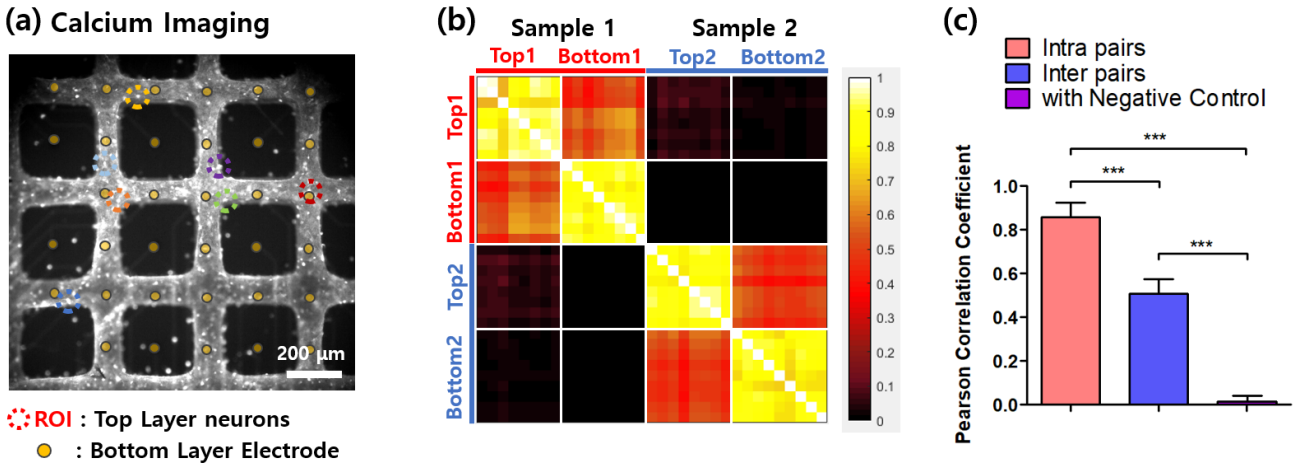

Fig. 6. Neural signals from calcium imaging. (a) Calcium imaging window with region of interest (ROI) and microelectrodes. (b) Heat map of Pearson correlation coefficient. Sample 1, 2 were different chips. Each sample had groups as signals from top layer and bottom layer. (c) Pearson correlation coefficient about each type of pairs. n= 120, 136, 272 pairs in 2 chips, mean±sd, One-way ANOVA, post-hoc: Bonferroni, ***p<0.0001.

6(c)). It indicated that neurons at top and bottom layers had established some functional interconnectivity.

IV. Conclusion

In this study, we developed the technique that worked very well on MEA to construct the micro-patterned 3D neuronal-hydrogel model. The condition that was using collagen hydrogel and neural interface which coated with polyDA and PDL was more suitable to maintain the structure of the micro-pattern than using Matrigel. Our 3D patterning method maintained the micro-pattern for 4 weeks and it was sufficient to record neural signals from cultured neurons. Our method can be used to construct the micropatterned 3D model on an MEA to analyze the relationship between structural connectivity and functional activity on 3D environment.

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