Master's Thesis
석사 학위논문

Spatial representation neuronal network modeling and in vitro formation on a substrate using a PDMS based physical structure

Minjung Kim(김 민 정 金 旼 貞)

Department of Robotics Engineering

로봇공학전공

DGIST

2015

Spatial representation neuronal network modeling and in vitro formation on a substrate using a PDMS based physical structure

Advisor : Professor Sangjun Moon

Co-advisor : Professor Min-soo Kim

by

Minjung Kim
Department of Robotics Engineering
DGIST

A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Robotics Engineering. The study was conducted in accordance with Code of Research Ethics[1]

01. 09. 2015

Approved by

Professor Sangjun Moon (Signature)
(Advisor)

Professor Min-soo Kim (Signature)
(Co-Advisor)

[1] Declaration of Ethical Conduct in Research**:** I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

Spatial representation neuronal network modeling and in vitro formation on a substrate using a PDMS based physical structure

Minjung Kim

Accepted in partial fulfillment of the requirements for the degree of Master of Science.

12. 01. 2014

Head of Committee 문 상 준 (인)
Prof. Sangjun Moon

Committee Member 김 민 수 (인)
Prof. Min-soo Kim

Committee Member 김 기 태 (인)
Prof. Keetae Kim

MS/RT
201323001

김 민 정. Minjung Kim. Spatial representation neuronal network modeling and in vitro formation on a substrate using a PDMS based physical structure. Department of Robotics Engineering. 2015. 39p. Advisors Prof. Sangjun Moon, Co-Advisors Prof. Min-soo Kim.

ABSTRACT

Spatial representation network in the brain is operated by place cell and grid cell network interactions. For study about these cognitive functional mechanism, computational neuronal network modeling and in vitro neural network analysis platform are designed in terms of network motif. Computational modeling was tried to designed by neuron simulation tool, and for verifying this computational model, real pyramidal neuron was cultured in vitro that was extracted and dissected from rat. In vitro neuronal network pattering on microelectrode array (MEA) is the neuronal network analysis platform for investigating their neuronal activities, especially PDMS physical structure pattern on MEA for single cell level in vitro neuronal network for verifying previous computational neuronal network model. These single cell resolution modeling and analysis platform can be a tool for verifying and investigating about brain cognitive functions in terms of neural circuit approach of neuroscience.

Keywords: Spatial representation network, Neuron modeling, Neuron patterning, In vitro neuronal network, Primary cell culture

Contents

List of Figures

Ⅰ. INTRODUCTION

1.1 Spatial representation system in brain

Spatial cognition is self-localizing and navigating function of mobile animals[1]. This functions are critical for the survival of these animals, and it is a representative high-level cognitive task. Since experimental psychology and neuroscience were emerged, it has been able to analyze the organization and development of spatial cognition experimentally[2]. Behavioral scientists assumed that animals move with an abstract map of space inside their brain[3]. It was demonstrated that activity of specific neurons is role of the abstract map that are relative to behavior[3]. Therefore, this spatial cognition study in physiology is a clue that understand about its mechanism of high-level cognition ability of animals. This spatial cognition concept was appeared and studied for centuries[2]. In the 1940s, Edward C. Tolman who is behavioral psychologist, proposed an internal representation concept of the outside world for spatial cognition in rat's behavior experiment[1]. This internal representation concept was revealed in the form of activity of brain in rat experiment that the microelectrodes was implanted in rat's brain region of hippocampus for recording activities of neurons[1]. Using this experiment method, In the 1960s, O'Keefe J. et al. found that memory about an environment can be stored by activity of individual different neuron. It is called place cell located in hippocampus, this individual different place cell fires when rat passed by specific place (Place field, Figure 1-1a)[4]. And in the 1990s, Hafting T et al.,

found that hexagonal array-shaped activity pattern of neurons when also rat explored by specific place. It is called grid cell located in entorhinal cortex[5], Individual different grid cell generates its own grid pattern activity according to different place. (Grid field, Figure 1-1b) Place cell in hippocampus is directly taken input signal from grid cell in layer III entorhinal cortex, and transmit the signal grid cell in Layer V entorhinal cortex physiologically[6], (Figure 1-1c-d) It is considered that place cell signals are generated by the inputs of integration of several grid cell activity according to complicated transformation methods[7]. But it is unknown how process their input and output in each cell. Research about these a certain relationship with input and output of place cell and grid cell is a key work that describes the spatial cognition ability mechanism in terms of neuroscience.

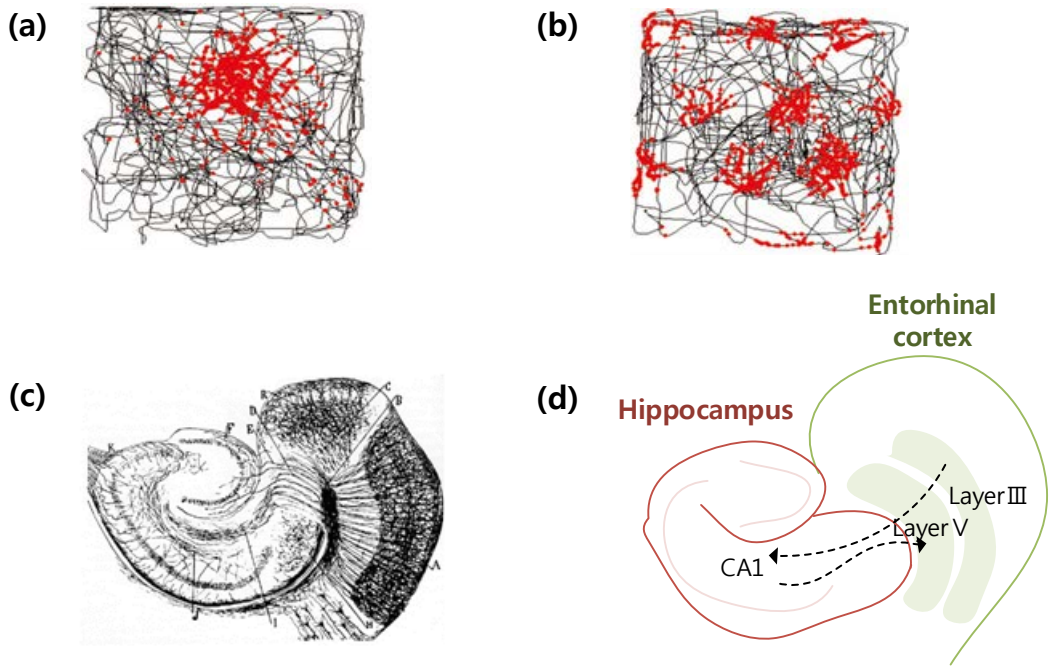

Figure 1-1. Place field and grid field and anatomical place cell and grid cell network

Spike locations(red) on the animal's trajectory(black). (a) is place cell firing pattern, place field and (b) is a grid cell firing pattern[2]. Each place cell has different place field, and also each grid cell has different grid field. These each overlapped fields help spatial representation. (c) Anatomical structure of hippocampus and entorhinal cortex[6] and (d) schematic diagram of that and their anatomical connection. Place cell in CA1 of hippocampus is directly connected with grid cell in LayerIII and LayerV. Grid cell of Entorhinal cortex layerIII generates input signals, and transmit signals to place cell of hippocampus CA1 region, processed signals are transmited grid cell in entorhinal cortex layerV.

1.2 The motif of neuronal network

In terms of system biology, neuronal network can be regarded as complex network, and this consists of building blocks; network motif. Network motif is a small set of recurring regulation patterns[8]. It consists of nodes and edges that represent the network (Figure 1-2a). These network motifs were firstly defined in the transcription network of Escherichia coli (E. coli). The same network motifs have been found in common with organisms of other creatures [9-11]. The network motifs were found in the other several networks, especially in neuronal network, neurons and synaptic connections also make some network motifs. In anatomical connectivity structures of C. elegans neural network, three and four nodes motifs (feed-forward loop and bi-fan) were found[12], and these motifs were also in common with the transcriptional gene regulation networks. This similarity of motif design may relate to a functional similarity in information processing of the two types of networks[8]. These hypothesis was implied by comparing real transcription network and random network; concentration of feed-forward loop motif in real transcription network was about same while concentration of same motif in random network was decreased according to increasing the complexity of network[8]. With this approach, C. Y. Dong et al. did systematic analysis about neuronal network comparing with computational model and real neurons culture[13].

In this paper, these network motif approach was used as basic idea to study about place cell and grid cell network. Spatial representation network may have network motif related to its function. It is assumed that there is three nodes motif by electrophysiological connection: Layer III grid cell, CA1 place cell, Layer V grid cell. (Figure 1-2b) Modeling and in vitro experiment is performed for investigating this simple network as cue about its function.

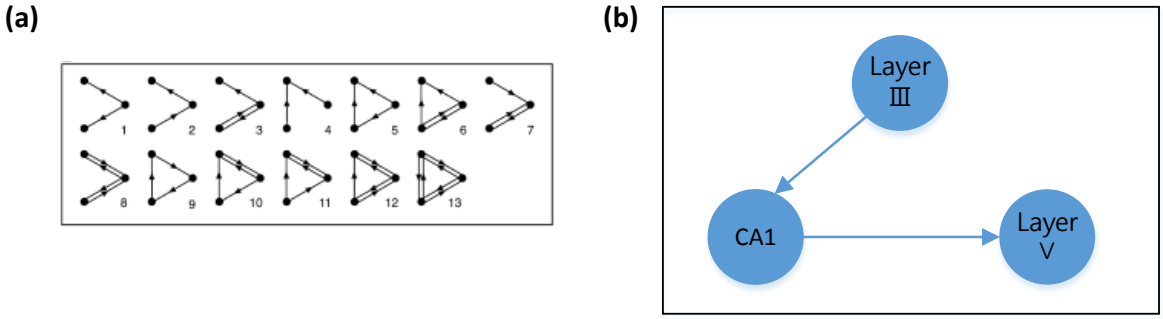

Figure 1-2. Three nodes network motif description and spatial representation neuronal network motif

(a) Three nodes network motif description[8]. Various network motif exgist in same node size. (c) three nodes network motif of spatial representation neuronal network. Layer III grid cell, CA1 place cell, Layer V grid cell is the nodes, their connection is the edges.

1.3 Computational modeling of spatial representation network motif

Computational modeling and simulation was used for design and verifying the spatial representation network motif. But, realistic modeling of electrophysiological property of neuronal network is computationally expensive. Therefore simplification is required in modeling process. In NEURON, that is neuron modeling simulation tool, real biophysical neuron can be simplified to conceptual model; Ball and stick model is conceptual model used in NEURON (Figure 1-3a), that defines the complex anatomical structure of neuron as simple cylinders that are reflected some anatomical information (e.g. diameter, length) And topologically determined ball and stick model can be represented as electric circuit model with the electrophysiological properties of neuron membrane. (Figure 1-3a) Spatial representation neuronal network motif can be modeled with this procedure, (Figure 1-3b) especially CA1 pyramidal neurons can be modeled largely three parts, soma, apical dendrite, besal dendrite, spine. It was modeled soma, apical dendrite and spine for modeling hippocampus network[14]; CA1, CA3, interneuron. It was modeled with NEURON, it will be used at spatial representation neuronal network modeling.

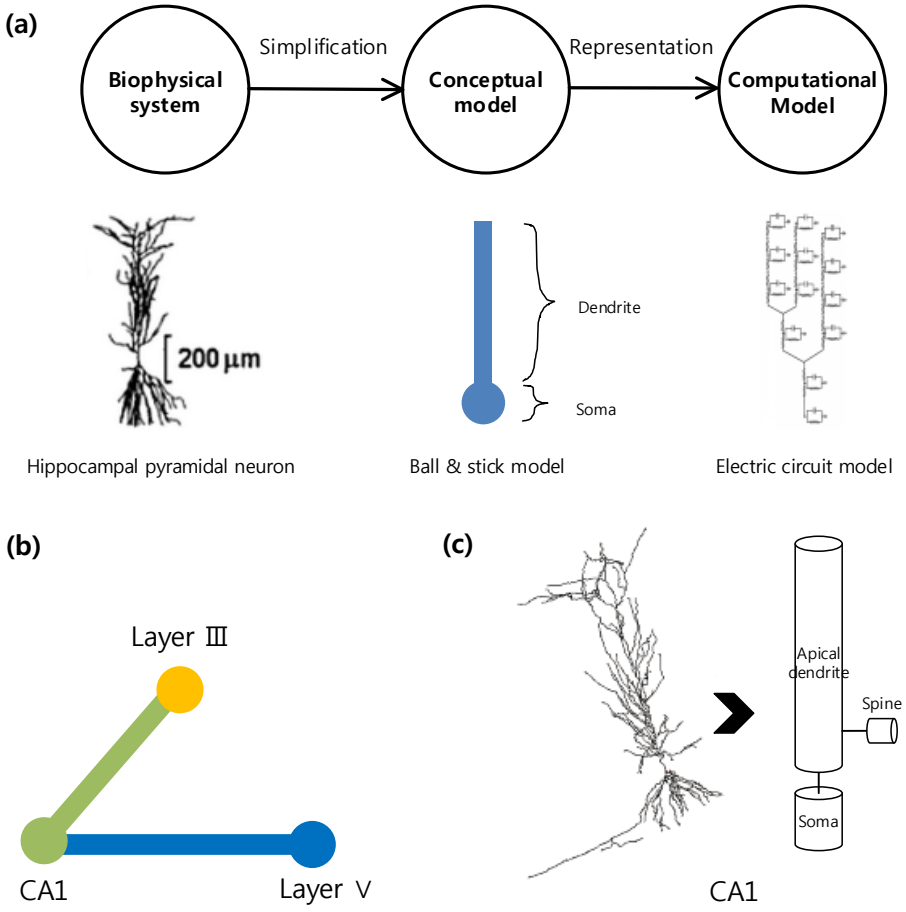

Figure 1-3. Computational neuron modeling process and spatial representation neuronal network motif modeling

(a) Computational modeling procedure. Biophysical neuron[15] is simplified to conceptual model, and representated electric circuit model reflected electrophysiological properties of various chnnels. (b) Spatial representation neuronal network motif. It is designed that each neuron have a dendrite and a soma, and connected (c) CA1 pyramidal neuron modeling[14] for spatial representation neuronal network motif model. It is simply modeled as cylinders, also represented with membrane electric properties. ("n123" pyramidal neuron from NeuroMorpho database. neuromorpho.org)

1.4 In vitro neuronal network formation

1.4.1 In vitro neuronal network

For verifying computational neuronal network motif modeling, real neuronal network activity signals are required. To study about these brain research, neuronal network can be cultured in vitro to overcome the difficulty of physical accessing and monitoring in vivo neurons[16], and test various neuroscience experiment that is difficult to implement in vivo environment[17], such as neurotoxicity research for biosensor or development therapy[18], neural network dynamics[19] and learning mechanism research[20]. Also in vitro cultured neurons have similar physiological properties with in vivo neurons that generate the action potential, secrete neurotransmitter and form the network for signal transmission, though its condition not correspondence with in vivo condition.

1.4.2 Neuron patterning

Especially, for the functional neuronal network study such as spatial representation, in vitro cultured neuronal network should be patterned, because primary neurons disconnect the original connection during dissection procedure, so these neurons are formed random network during culture period.

Neuron patterning complement this randomness by inducing specific connection. Neuron patterning technology utilize cell growth nearby extracellular matrix(ECM), that are extracellular molecules secreted by cells. It helps cell structure and biochemical support to the surrounding cells[21]. Cell adhesion is one of the function of ECM[22], this cell-to-ECM adhesion is regulated by specific cell-surface cellular adhesion molecules (CAM) such as fibronectin and laminin that are cell-surface proteins, it binds cells to ECM structures[23]. For

in vitro culture, cells are exposed non-biological substrate surfaces. So to utilize these adhesion properties of cells for patterning in vitro, artificial chemical and topographical properties of substrate have investigated to mimic the ECM[24]. another cell adhesion mechanisms is utilizing the negative charged glycocalyx that is the extracellular protein of most animal cell membranes that network of proteoglycans, glycolipids and glycoproteins[25]. It is attracted to cationic polyamino acids like poly-L-lysine(PLL). For guiding cellular adhesion and growth, chemical and topographical surface modification strategies are employed[26].

Topographical modification is method to modify the surface roughness and creating micro-sized structures such as wells, pillars, and grooves. Nano-scale roughness on silicon, pillar structure on silicon, parallel grooves on PDMS was tried. (Figure1-4a) These trials discovered that micrometer-sized surface structures induce the cellular responses: the cells adhered and changed their shape depending on the geometry of the features[27]. In contrast, surface chemical modification deals with changing the chemical composition of a surface by chemically binding different protein or cell adhesive molecules to the surface. (Figure1-4b) SAM formation, dip-pen nanolithography (DPN), photo-lithography, soft-lithography, inkjet deposition technologies were used for surface chemical modification patterning. Particularly, one of the soft-lithography, micro contact printing (μCP) was the most widely used method for this. It was introduced by the Whitesides group in 1993, and they produced a pattern of self-assembled monolayers of alkanethiols onto gold substrates using elastomeric stamps[28]. The principle of is that an inked material is transferred from an elastomeric stamp onto the surface of substrate through conformal contact. It has advantages of low cost, reusability, and

high throughput whereas low resolution (>1um) when it compares with other methods. Polydimethylsiloxane (PDMS) is widely used in uCP as stamp, because of low cost, good mechanical properties, easy fabrication process[29]. This material is also biocompatible, so it has been used in many bio applications.

1.4.3 Neuron patterning on microelectrode array (MEA)

Also for analysis of electrophysiological properties, neural interface device is needed that acquires the neural activity signals and also stimulate neurons, vice versa. And neuronal network should be possible to be recorded and stimulated at multiple sites simultaneously for long-term period because of full maturation of neuronal network[30]. (e.g., more than 3 weeks) For these needs, microelectrode array (MEA) has developed. MEA is non-invasive plat neural interface device that can record and stimulate neurons simultaneously at multiple sites[31]. (Figure 1-4c.) These non-invasive interface leads to long term culture. Also this allows investigating learning processes and memory mechanism through development of neuronal network.

But electric neural signals can be only detected by electrode that is nearby these neurons[32], so neuron positioning is needed by neuron patterning technology. But, still it has been difficult to record the same neuron signal consistently over a long period of time. This difficulty is due to neuron mobility, especially in the first few weeks, neurons migrate on a flat substrate over time[32]. Thus in order to reach the full maturation of the network, the preservation of the spatial confinement of neuronal structures is required over time[30]. (i.e., more than 3 weeks in vitro.) (Figure 1-4e.) Also for the mapping with model, identified with

single cell resolution (one neuron-one electrode) neuronal network pattern is required[32, 33].

For using MEA system module, and customizing neuron patterning, reproducible method is needed that does not transform substrate, and removable from substrate. Therefore, surface chemistry is usually used patterning neural network on this MEA platform. As a top-down approach, surface chemical modification of the microelectrode surface was used via self-assembled monolayers (SAM) of cell attractive or repulsive molecules[34] or patterned deposition of adhesion-promoting proteins as by micro contact printing[35]. (Figure 1-4d) Also physical structure was developed on MEA. As a bottom–up approach, there were poly-imide picket fences[36], parylene neurocages[37]. (Figure 1-4f) They prevented migration of neurons, and increased the soma-electrode contact.

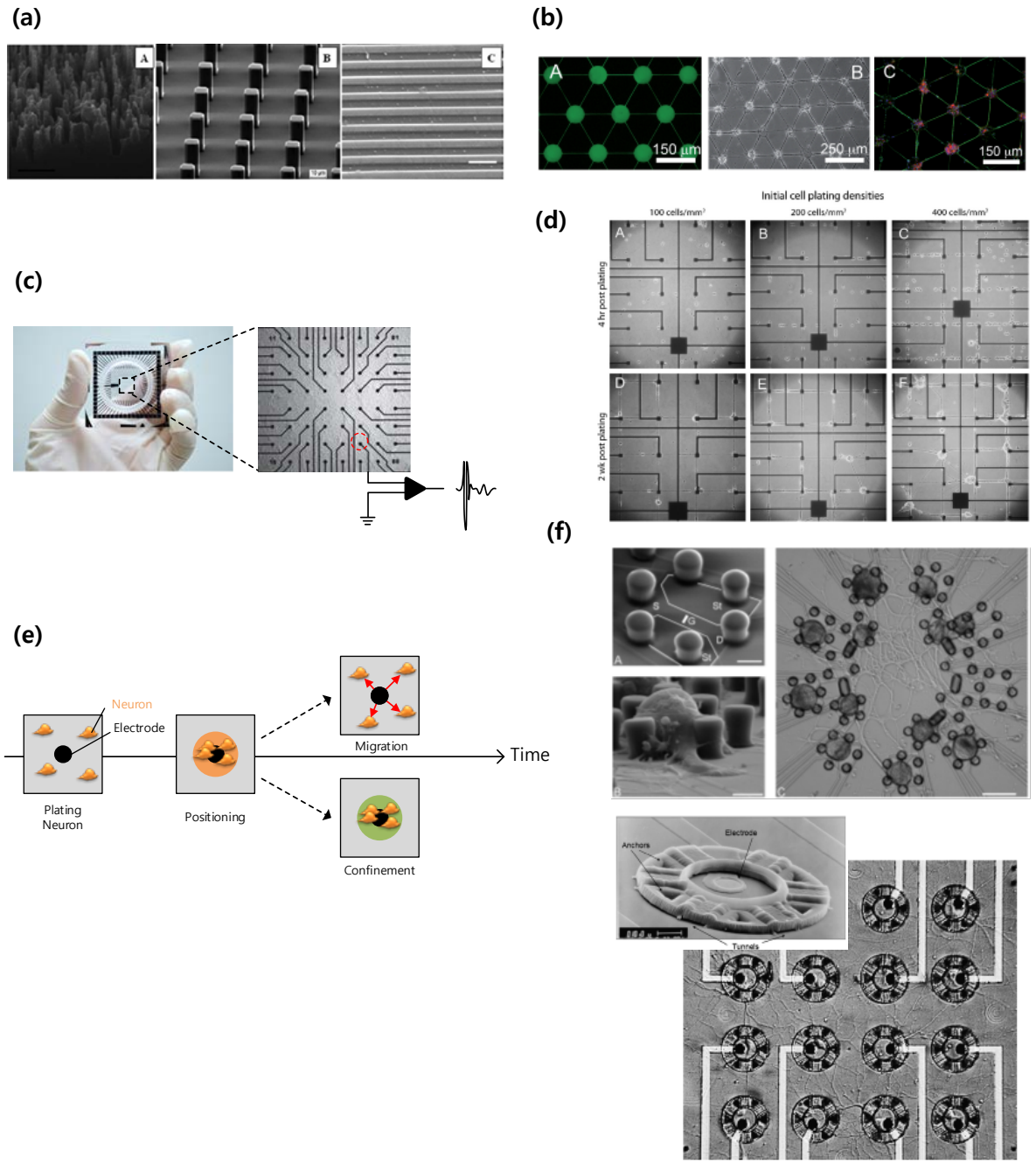

Figure 1-4. Neuron patterning and microelectrode array (MEA)

(a) Surface topographical modification for neuron patterning. Nano-scale roughness on silicon, (scale bar 20nm) pillar structure on silicon, (scale bar 1 um) parallel grooves on PDMS was tried. (Scale bar 5um) (b) Surface chemistry modification for neuron patterning. PL was patterned by microcontact printing on PLL-g-PEG. Primary neurons localized to the adhesion nodes and extended neurites for the formation of internodal connections. Fluorescent immunostaining shows nuclei stained with DAPI (blue), b-III-tubulin (green)

and MAP-2 (red)[38]. (c) In microelectrode array (MEA) chip, there are 8x8 electrodes array and this electrodes are connected to digital system. Neurons are cultured on these electrodes, this chip is integrated in MEA system for acquiring neural activity signal to digital signal. (d) Chemical confinement on MEA. Neural networks formed on patterned poly-l-lysine. (e) Schematic to explain the need of confinement neurons on electrodes. Neurons plated on MEA, are random position in the beginning. Neuron positioning is achieve as time goes by, using surface chemical modification. For a long culture period (e.g., more than 3 weeks), neurons tend to migrate around. (f) Phyisical confinement. Using physical factor, confinement of neurons is achieved on MEA surface. Polyimide picket fence and parylene neurocage confine single neuron on electrode.

1.5 Problem and Hypothesis

For the functional neuronal network study like spatial representation network, neuronal network motif modeling and patterned in vitro neuronal network culture on MEA is needed in terms of network motif. However, chemical patterning method do not perform consistently single cell scale for a long-term period. And physical patterning method, the fabrication of physical traps, required complicated procedures, and processing is time-consuming work[32]. Also additional network connection control is needed.

For the complement each other, uCP that is chemical patterning method and physical structure are integrated. The liquid PDMS is micro-patterned on substrate as ink, using microcontact printing, and cured[39]. (Figure 1-5a.) This method can print a patterned PDMS structure on substrate as top-down approach, and it is easy and good in vitro culture platform to make functional in vitro neuronal network research. The PDMS structure pattern was designed as shown in Figure 1-5b, c.

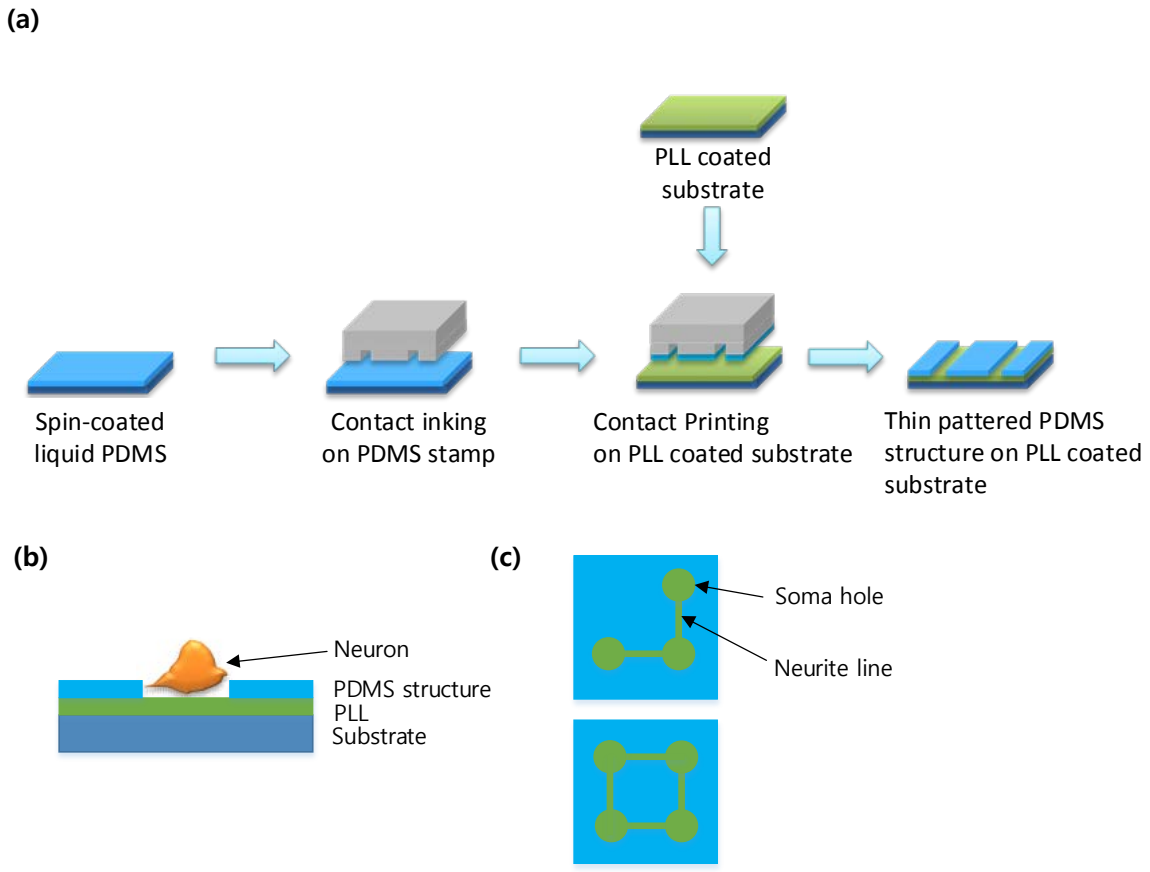

Figure 1-5. A PDMS physical structure fabrication method and design

(a) The liquid PDMS uCP procedure. The liquid PDMS is spin coated and Inked the PDMS stamp, and contact printed on PLL coated substrate. After that, it is cured, micro-sized PDMS structure pattern is fabricated. (b) Cross section of PDMS physical structure design. Substrate is coated with adhesive molecules (e.g. PLL) and PDMS structure is built on that. PDMS structure hight is about ~30um that can operate as structure. (c) Top view image of PDMS physical structure design. Soma of single neuron is confined in soma hole, and neurites of neruon grow along a neurite line that is the space between the soma hole, for connecting each neurons.

Ⅱ. MATERIALS AND METHODS

2.1 In vitro experiment

2.1.1 Fabrication of PDMS mold and stamp

PDMS mold is made with considering shrinkage ratio that after peeling off the PDMS from mold, the PDMS shrinks toward center[40]. A PDMS mold and stamp was fabricated using soft-lithography in Figure 2-1a. First, The PDMS mold was fabricated. HMDS (HexaMethylDiSilazane), a pre-treatment of the silicon wafer is coated for 60s at 100℃, for obtain maximum process reliability. AZ GXR 601 photoresist (PR) was spin-coated on a 6-inch silicon wafer at 1000rpm for 3s, 1600rpm for 32s and 1000rpm for 3s to obtain 1.5um thickness. And then it was soft baked at 100°C for 60s and cooled at 23°C for 30s. The soft-baked wafer was exposed to UV light at 310 mJ/cm2 under the chrome photomask using mask aligner (MA8-GEN3, Suss Microtec), and baked at 110°C for 80s and cooled at 23°C for 30s. It was developed in AZ300 developer, rinsed with de-ionized (DI) water and blow-dried with nitrogen gas. Then it was hard baked at 120°C for 90s and cooling at 23°C for 30s finally.

After mold fabrication, PDMS stamp was fabricated. PDMS mold and a few drop of silanizing reagent, Trimethoxy (3, 3, 3-trifluoropropyl) silane (Cat. 91877, Sigma) in a glass bottle were placed in vacuum desiccator (AS.1-068-01, As One) for 24 hours, for silanization of the surface of the mold. Then PDMS pre-polymer base solution and curing agent

(Sylgard-184, Dow Corning) are mixed at a 10:1 weight ratio and poured over the silanized PDMS mold with 5 mm thickness. In the degassing process, the dissolved gas is removed from the mixed PDMS solution, then the mixed PDMS solution is cured in an oven (65˚C for 4 hours, JSOF-150, JSR). When the PDMS solution is solidified, desired part of the cured PDMS that is defined as the PDMS stamp was cut out using a surgical knife. Then the PDMS stamp bonded with a slide glass by a plasma system (CUTE-B, FemtoScience) that generates oxygen plasma (20s, 50W).

2.1.2 Micro alignment system for micro contact printing (μCP)

Figure 2-1b-c shows the schematics of micro alignment system and the basic μCP process using micro alignment system. Micro alignment system is divided into an actuator part which composes of um resolution manual XY stage, motorized Z stage, motorized rotation stage, and a controller part which control the actuator part programmed by LabVIEW (National Instruments, Ver. 2010) software, by providing vision feedback from camera. The actuator part and camera are integrated with an inverted microscope (CKX41, Olympus). PDMS stamp is attached on the vacuum holder of the actuator part tightly, using vacuum chuck and user adjusts the position of stamp by checking images from microscope in XYZ direction and even rotates the stamp. A few um unit movement along Z rotation stages controlled by micro-stepping motors eliminate the vibration and errors according to manual stamping. And micro alignment system provides fixed stamping pressure and maintains uniform contact surface between stamp and substrates for a certain period of time. In the controller part, image processing module make it possible to multiple stamping. This module

can get only the pattern images of stamp and overlap with camera image so that pattern align is possible against other pattern.

2.1.3 Noise Reduction of MEA

Optimizing MEA cleaning condition was verified by statistical analysis. Five tergazyme solution loading time conditions were compared for finding optimization cleaning condition. (2h, 5h, 10h, 24h 48h) Random noise is represented to probability density function, and standard deviation is indicator of noise magnitude. 9 electrode, is specimen under one condition. Each electrode represent noise to probability density function, and after cleaning, each electrode represent noise to probability density function, one more. Than subtraction of standard deviation before and after, are averaged and its standard error is calculated.

2.1.4 MEA recording system and signal processing with beta cell

Beta cell extracellular signals can be recorded by the MEA2100-System (Multi Channel Systems, Germany) for test MEA recording system. (Figure 2-1d-e) Beta cell is in the pancreas and the cell that contribute the islets of Langerhans. This cell can generate extracellular signal rapidly without maturation whereas nerve cells need the maturation for generating extracellular signal. Beta cell sense high blood glucose concentration, and glucose molecules move into the cell, down its concentration gradient. In the cell, glucose is decomposed and that metabolism produces ATP. The ATP-sensitive potassium ion channels close when ATP ratio rises. As a result, the membrane potential becomes more positive, this change opens of the voltage-gated calcium channels that calcium ions enter into the cell to

reduce their concentration gradient. Beta cell line, INS-1 cell was used in this experiment. This INS-1 cell cultured in RPMI1640 medium, experiment was processed when cell passage was 20. RPMI1640 medium was used as recording medium. In the RPMI1640 medium, glucose 11mM is contained that beta cell can response. The MEA chip was inserted into the headstage (2× Gain, 2^{14} resolution) and data were recorded by MC Rack software at a sampling rate of 10 kHz. A low pass digital filter (cut off frequency is 100 Hz) was also applied to obtain the local field potential (LFP). The system also includes a heating system connected to a temperature controller (TC02, MCS GmbH) that keeps the MEA chamber at 37 ◦C. Beta cell was seeded as concentrating random seeding.

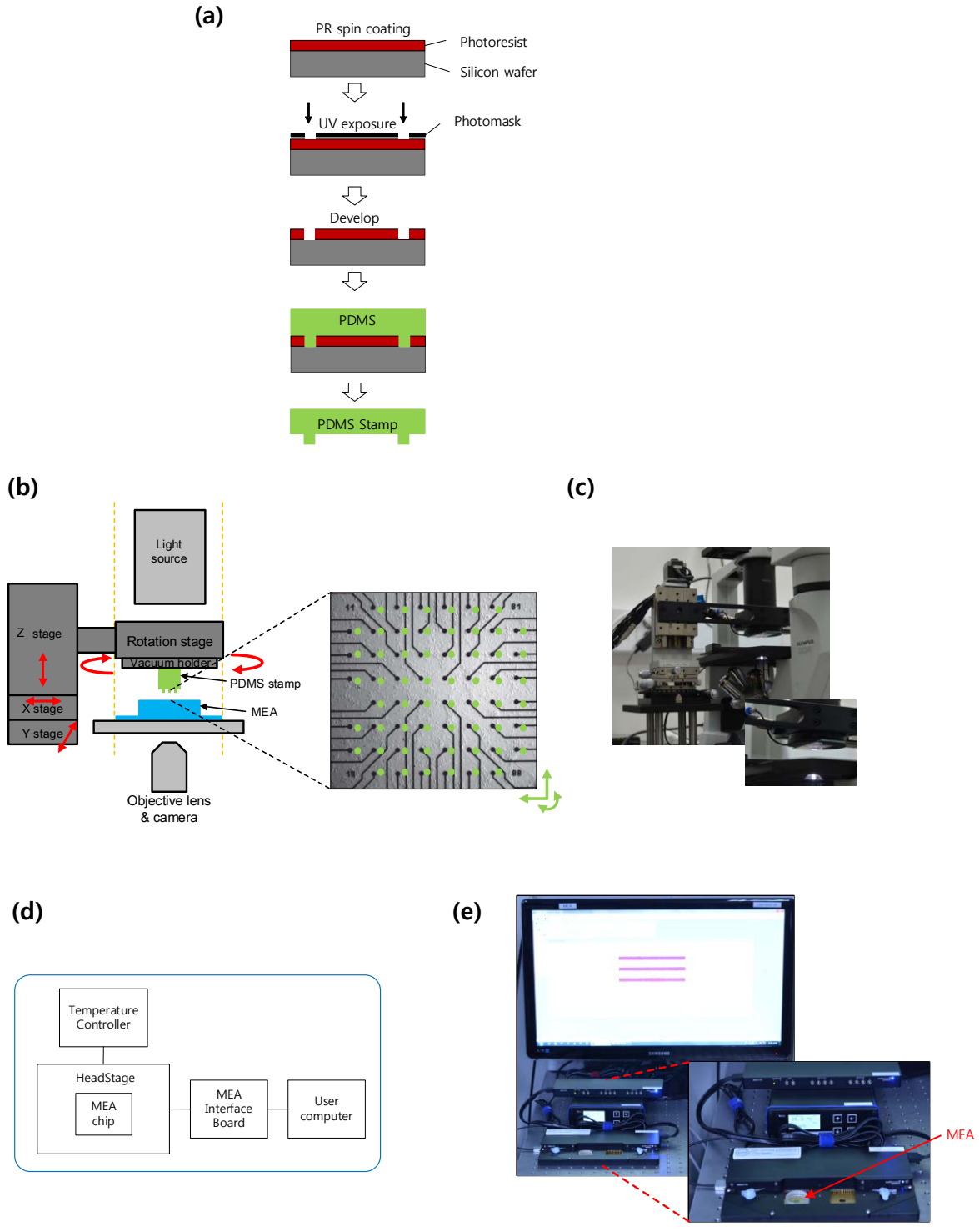

Figure 2-1. Schematics of fabrication of a PDMS stamp, Micro alignment system and MEA system

(a) schematic diagram of the fabrication of PDMS mold: positive photoresist was spin-coated on the HMDS treated 4-inch silicon wafer for 50um thickness and then PR spin-coated wafer

was soft-baked. And then the patterned photofilm mask was put on the wafer and the wafer exposed to UV light. Partially exposed wafer was baked in a convention oven again and developed in PR developer for removing the UV-exposed PR from the wafer. Finally the wafer was rinsed with de-ionized (DI) water and blow-dried with nitrogen gas. (b) Schematic diagram of stamping procedure: First cured PDMS stamp was ready for stamping and it was cleaned with acetone, IPA and DI water for 5 min respectively in ultra-sonication bath and dried by nitrogen stream. Then oxygen plasma treatment turned hydrophobic surface of PDMS stamp into hydrophilic one and then protein solution was loaded on the patterned surface of stamps for 5 min. Filtered nitrogen gas removed the extra protein solution and dried the stamp surface. Finally protein-loaded stamp attached upside-down developed 1st and 2nd aligned patterning results using the accurate adjustment provided by micro alignment system. (c) MEA system. Headstage and interface board and temperature controller and software. The MEA chip was inserted into the headstage and signal acquisition, amplification and digitalization are processed. Interface board process signal processing and MEA software MC Rack display the data and control the each hardware. A temperature controller is connected the headstage.

2.2 Cell culture

2.2.1 Primary pyramidal neuron culture

For formation of spatial representation network, pyramidal neuron in hippocampus and stellate neuron in entorhinal cortex are required. For getting the most similar responses with a practical living things, primary cells are needed, that are straight obtained cells dissected from tissue of living thing. In the spatial representation network, pyramidal neuron in hippocampus and stellate neuron in entorhinal cortex are role of place cell and grid cell. Among these cells, primary pyramidal neuron culture is well known and well used in many neuroscience research. There is various reason. For useful model of in vitro culture system, there are necessary conditions: (i) its properties and behaviors close to nature (in vivo), (ii) homogeneous cell group culture is required to minimize the impact according to different cell types in biochemical and molecular biology research, (iii) cells are easy to handle. To get the almost homogeneous cell group, hippocampus is also used, because they consist of high purity of one cell type; it consists of about 90% pyramidal cell and 10% interneuron and glial cell. Also hippocampus save memory so that is suitable for neural plasticity and learning mechanism research as important brain function, and there are extensive database about hippocampal culture for 20 years, also pyramidal cell is relatively distinguished their morphology well then other cell types.

Primary pyramidal neuron culture is the first step of spatial representation network formation. Surgical procedure is explained as follow protocol paper[41]. First, the surgical instruments are sterilized with 70% ethanol, and 4~5 the early postnatal Sprague-Dawley rat pup are euthanized by decapitation. Place the head on a dish that contains dissection medium

(Hank's buffered saline solution (HBSS, Invitrogen, cat. no. 14175095), 11 mg ml^{-1} sodium pyruvate (Invitrogen, cat. no. 11360070), 20% glucose, 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich, cat. no. H-4034)), brain is taken out by incising the head in that dish (Figure 2-2a.), Extracted brain is moved on operating table grapping cerebellum part using forceps then hippocampus is extracted from the brain. (Figure 2-2b.) 8-10 hippocampi are put in new dissection medium. After hippocampi wash gently with new dissection medium, incubated at 37℃ with trypsin solution for 20 min. Hippocampi also incubated with DNase(Sigma-Aldrich, cat. no. DN25) solution at room temperature for 5 min. After gentle washing with plating medium (MEM Eagle's with Earle's BSS (Invitrogen, cat. no. 21010046), heat-inactivated fetal bovine serum (FBS), 20% gloucose, 11 mg ml^{-1} sodium pyruvate, 200mM glutamine, Penicillin/streptomycin, hippocampi are triturated by pipet. This cell suspension filtered through cell strainer (70um filter) and after cell counting, cells are plated in maintenance medium (Neurobasal medium (Invitrogen, cat. no. 21103049), B27 (Invitrogen, cat. no. 17504044), 200mM glutamine, Penicillin/streptomycin). (65 cells per mm2). 4 hours after plating, whole maintenance medium is gently exchanged. After that, half maintenance medium exchanged twice a week.

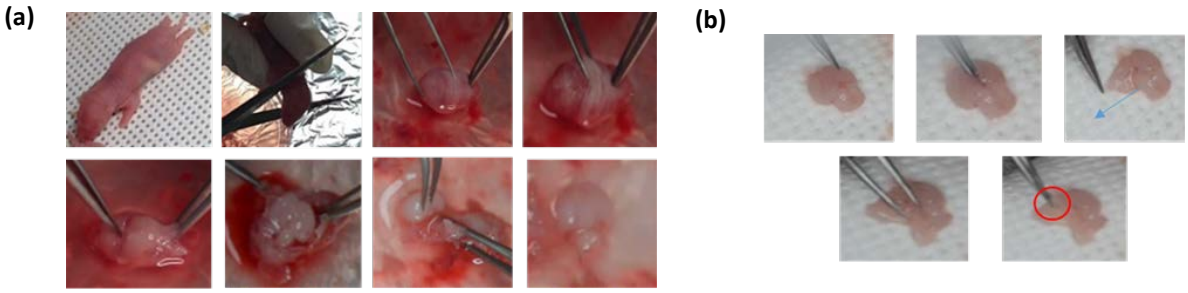

Figure 2-2. Hippocampus extraction from P0 rat pup

(a) Extraction of brain from p0 rat pup. Head is devided from body, immersed in dissection medium and skin and bone are gently removed out. (b) Extraction of hippocampus from the brain. Brain is moved on the operating table, and cerebral hemisphere is splited and hippocampus is extract out carefully. This all procedure must be performed in 2-3 min preventing death of many neuronal cells.

Ⅲ. RESULTS AND DISCUSSION

3.1 In vitro experiment

3.1.1 PDMS mold and stamp fabrication

Before PDMS stamp fabrication, chrome photomask patterns are designed with AutoCAD software. (Figure 3-1-1b) Pattern design is fitted with MEA layout (8x8 micro electrodes, electrode diameter is 30um, an interval length between electrodes is 200um) (Figure 3-1-1a) Fabricated mold pattern size is confirmed, node size is about 30um, edge width is about 2um. (Figure 3-1-1c) The fabricated PDMS stamp (fabricated on 1.07% magnified PDMS mold) were aligned on MEA, using micro alignment system. The nodes were located on the electrodes, and using image processing, MEA electrode pattern was captured and overlapped on microscope image. (stamp image) (Figure 3-1-1d) We can assume that the PDMS stamp was fitted well with MEA layout. With this result, it is known that the optimizing condition is 1.07% magnified PDMS mold in this protocol.

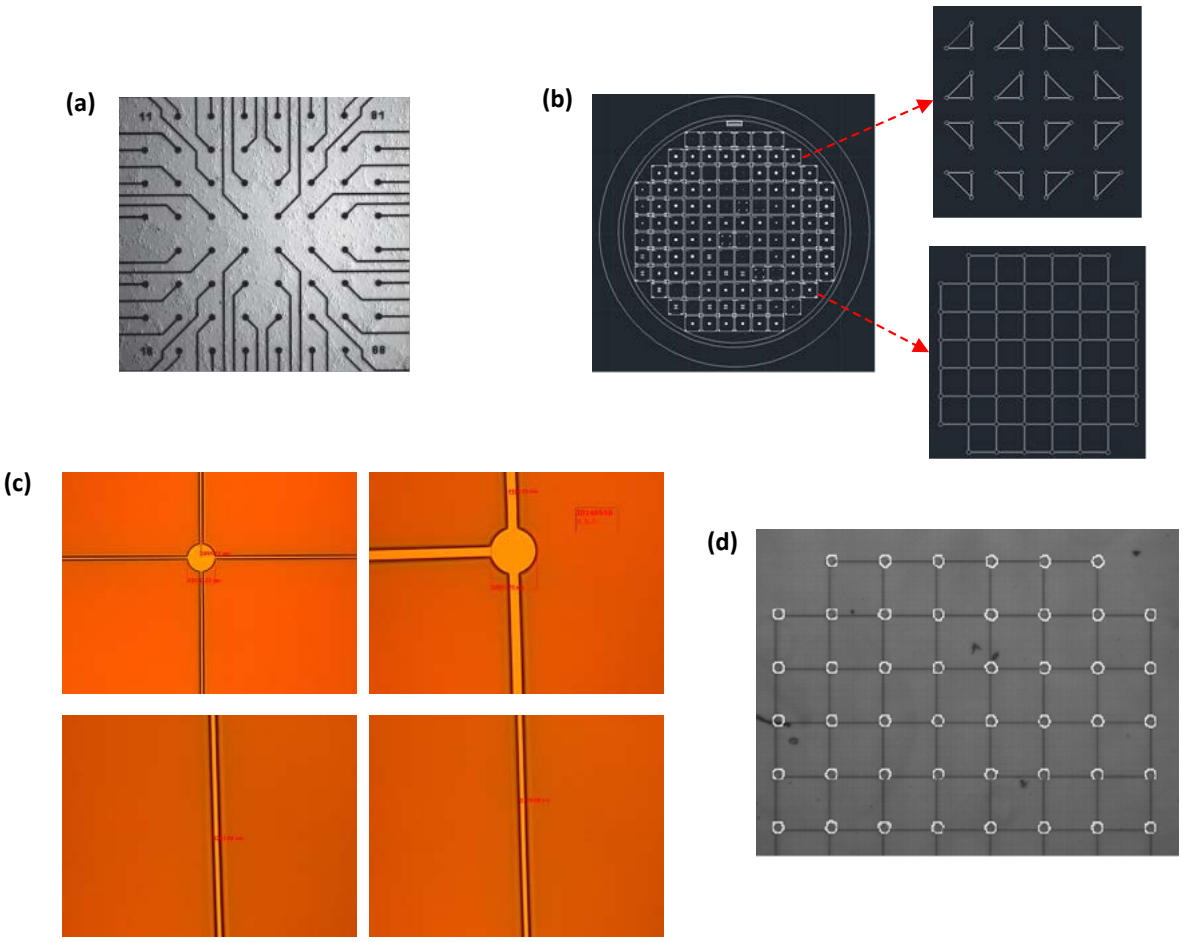

Figure 3-1-1. Photomask design and fabricated mold and stamp

(a) Standard MEA layout. (8x8 micro-sized electrodes) (b) Photomask AutoCAD design for three nodes network motif pattern. (c) After photolithography, Fabricated mold images. Measured diameter closes to bout 30um, and line width is 2um. (d) Overlapped microscope image of fabricated PDMS stamp (gray grid) on 1.07% magnified PDMS mold, and MEA electrode pattern (white) by image processing of micro alignment system.

3.1.2 MEA cleaning condition optimization

MEA noise reduction data are shown in Figure 3-1-2. In this result, tergazyme solution best cleaning time is about 24h. (Figure 3-1-2a) At 2h, noise reduction rms(root mean square) magnitude is about 0.3407 and standard erreor is 0.0374, at 5h, noise reduction rms magnitude is about 0.0507 and standard erreor is 0.0571, at 10h, noise reduction rms magnitude is about 0.5443 and standard erreor is 0.03616, at 24h, noise reduction rms magnitude is about 1.0908 and standard erreor is 0.039, at 48h, noise reduction rms magnitude is about 0.912 and standard erreor is 0.0798. (Figure 3-1-2c) As result of this, At 24h, MEA noise is lowest. After 24h cleaning, MEA noise is remarkably reduced. (Figure 3-1-2b)

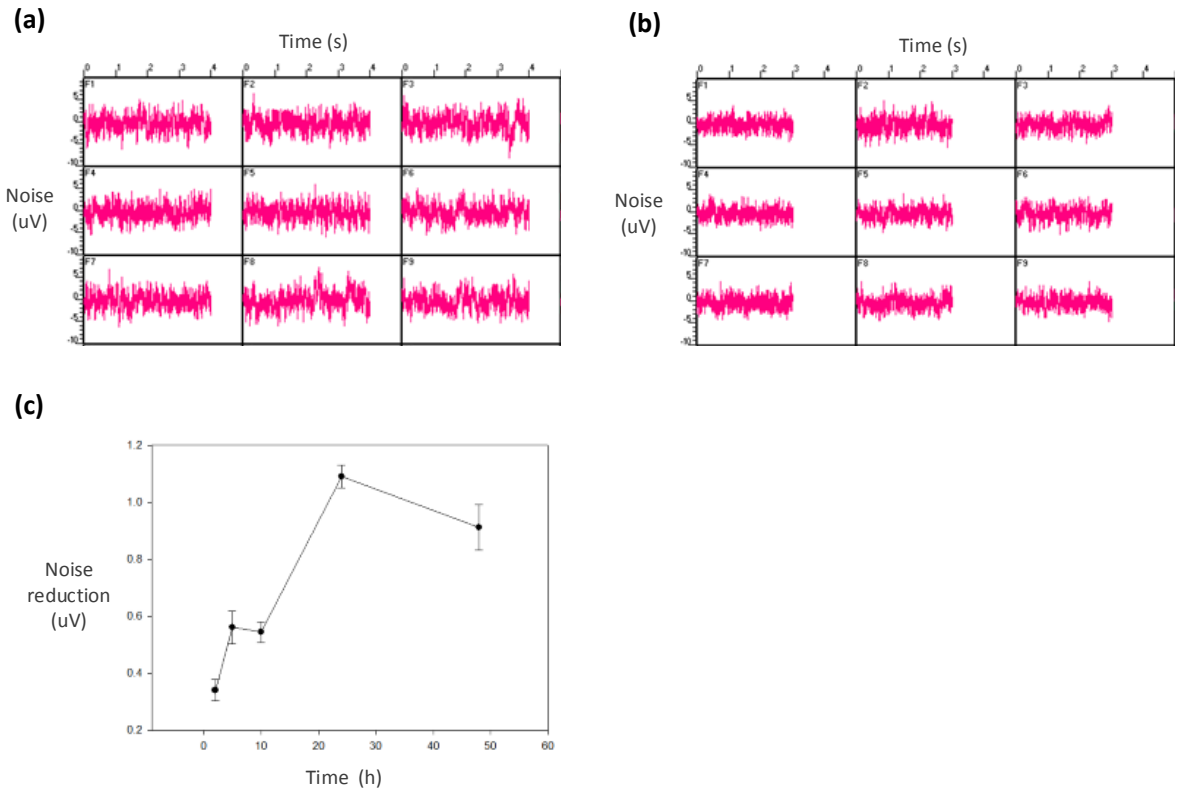

Figure 3-1-2. Optimization of MEA noise reduction condition with tergazyme solution

(a) Base noise recording of 9 electrodes before the cleaning treatment. (b) Base noise recording of 9 electrodes after the cleaning treatment. (c) Noise reduction degree according to loading time changes after MEA tergazyme solution cleaning. (2h, 5h, 10h, 24h, 48h) At 24h, noise reduction magnitude is about 1.0908 and standard erreor is 0.039.

3.1.3 Recording the local field potential of beta cell with MEA system

MEA system set up is tested by beta cell recording. Beta cell was concentrating random seeded on 6 well MEA (Figure 3-1-3a) because non-patterning surface MEA cannot positioning the cell bodies. Figure 3-1-3c is beta cells local field potential recording data at electrode A8 for about 60min. Figure 3-1-3d also is same data at electrode A4 for about 60min. A4 electrode is more far from the cells than A8 electrode. So the magnitude of signal is weak. And this show base noise line is about -10~10uV and extracellular local field potential magnitude is about -15 ~ -20uV. It is similar value the reference[42]. (Figure 3-1-3b) But noise is still remain as problem for recognizing reasonable data.

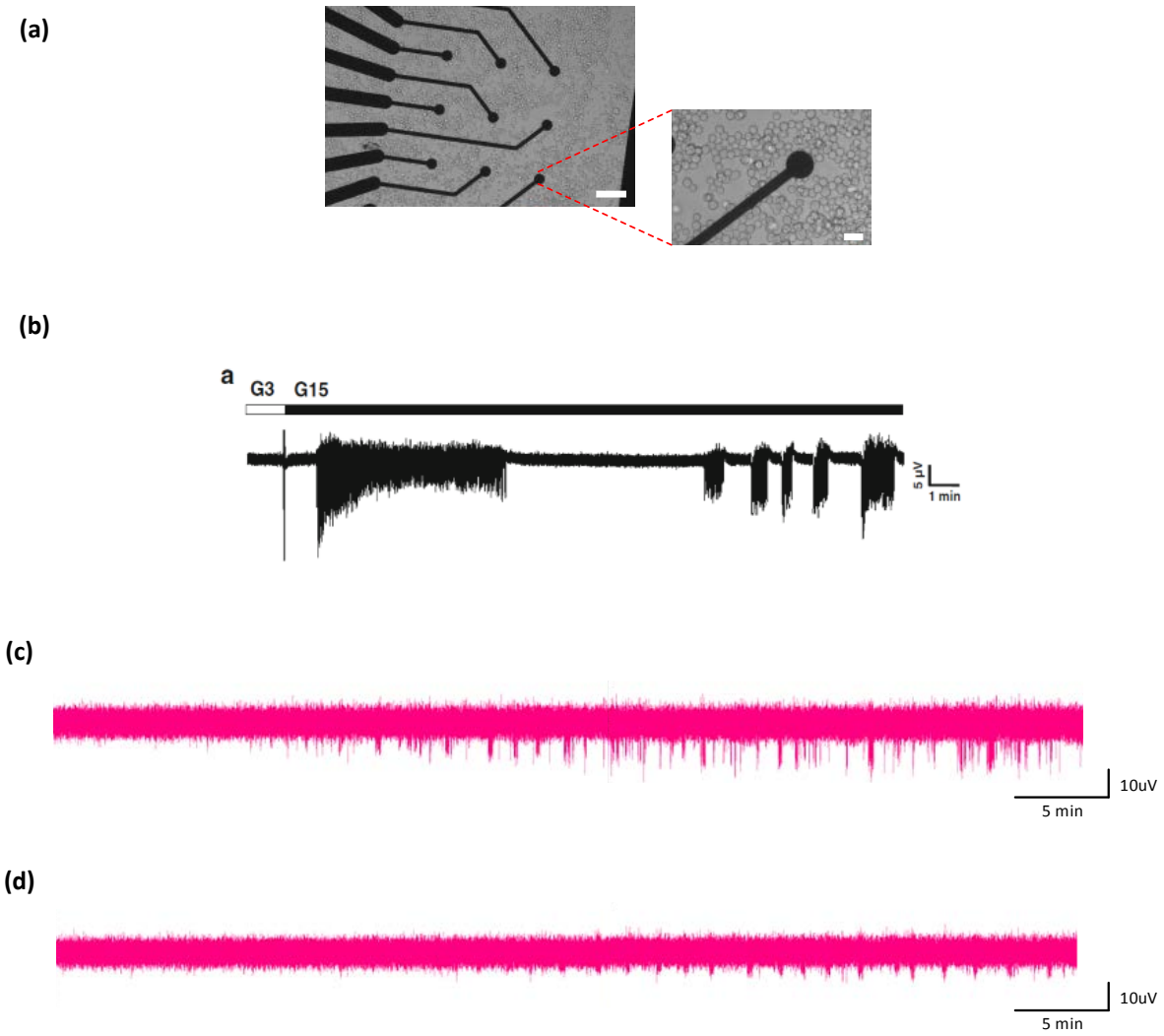

Figure 3-1-3. Beta cell recording on MEA

(a) Concentrating random seeding Beta cell on 6 well MEA. Scale bar is 100um, 20um. (b) Beta cell reference signal with MEA. (c) Beta cells local field potential recording data at electrode A8. Extracellular signal is monitored among the noise. (d) Beta cells local field potential recording data at electrode A4.

3.2 Primary pyramidal neuron culture

Figure 3-2 is cultured pyramidal neuron pictures. This primary neurons can live for up to 1 month. But in this paper, primary pyramidal neurons survived for 14 days on coverslip in 6 well dish. It may because of plating more cells than reasonable count or fail of medium change. Medium color was changed very fast, It drove sudden death of neurons. Figure 3-2a is a single pyramidal neuron picture during 0-14 days, and Figure 3-2b is multiple pyramidal neurons and connections during same days. Pyramidal neurons grew well for 14 days, single neuron morphology is remarkably changed. All microscope image was taken pictures at day 0 (4 hours after plating the cells), days 1, days 2, days 5, days 10, days 14.

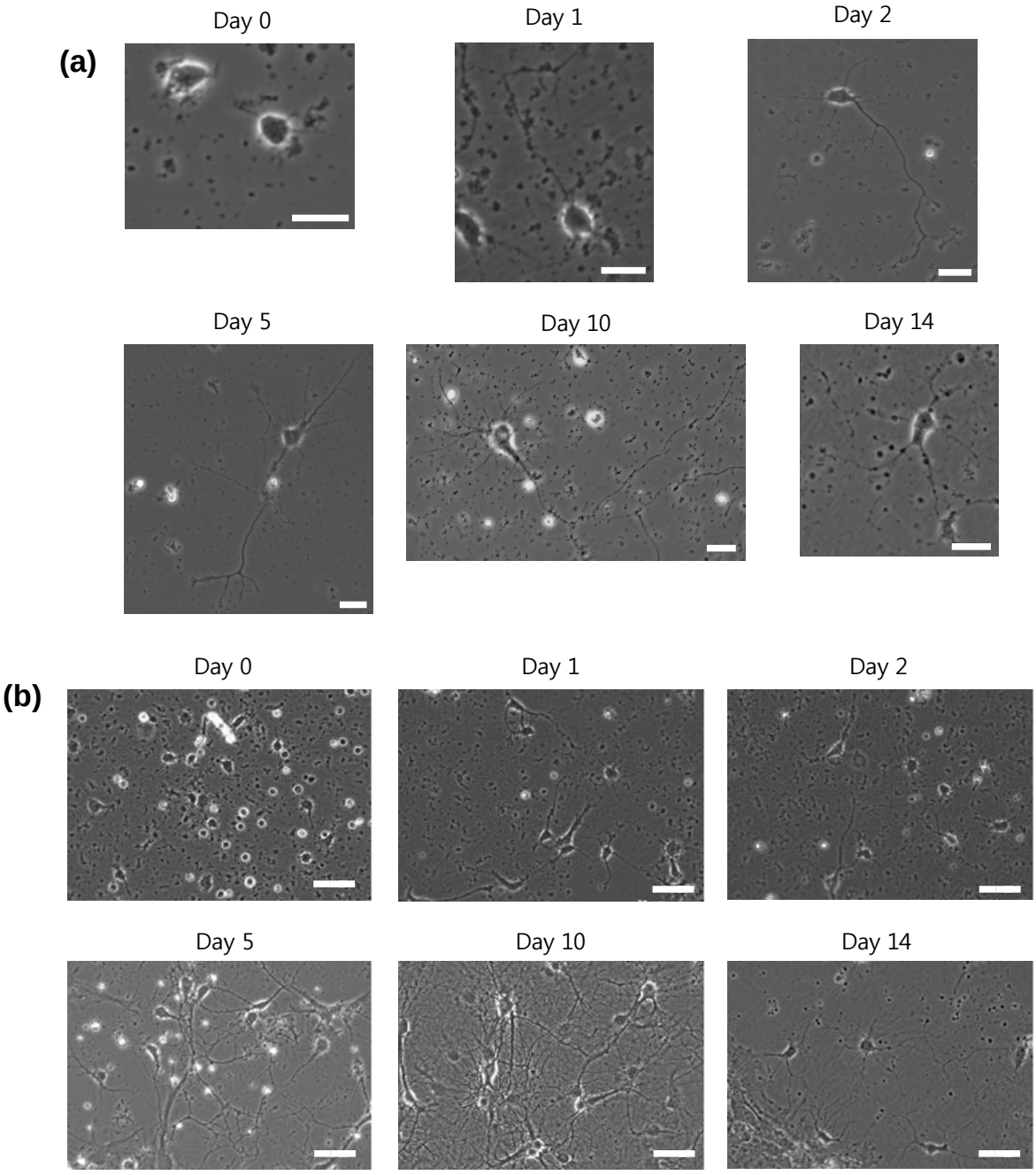

Figure 3-2. P0 primary pyramidal neurons culture

(a) A single pyramidal neuron picture at day 0 (4 hour after plating the cells), days 1, days 2, days 5, days 10, days 14. All of scale bars are 20um. (b) The multiple pyramidal neurons and connections at day 0, days 1, days 2, days 5, days 10, days 14. All of scale bars are 50um.

Ⅳ. CONCLUSION

4.1 Conclusion

The main purpose of this paper is making analysis platform about spatial representation neuronal network by modeling and in vitro neuronal network formation on a substrate using a PDMS based physical structure with motif idea. Spatial representation neuronal network could be modeled as network motif based on anatomical connection cue. First, for the modeling of the network motif, NEURON program was introduced with simple network models, and in the future, this network motif will be modeled with hippocampus and entorhinal cortex properties. Second, for the in vitro experiment, micro contact printing aligner and MEA system was set up, PDMS mold and stamp was fabricated for patterning PDMS physical structure. Using a PDMS physical structure, the network motif will be fabricated later. Third, for the in vitro neuronal network model, primary culture was implemented. Pyramidal neuron was cultured on coverslip for 14days. After verifying these all step, primary neurons can be patterned on MEA not substrate, and this step will contribute to study about mechanism of spatial representation neuronal network.

References

[1] E. Marozzi and K. J. Jeffery, "Place, space and memory cells," *Curr Biol,* vol. 22, pp. R939-42, Nov 20 2012.

[2] E. I. Moser, E. Kropff, and M. B. Moser, "Place cells, grid cells, and the brain's spatial representation system," *Annu Rev Neurosci,* vol. 31, pp. 69-89, 2008.

[3] A. Abbott, "Neuroscience: Brains of Norway," *Nature,* vol. 514, pp. 154-157, 2014.

[4] J. O'Keefe and J. Dostrovsky, "The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat," *Brain Research,* vol. 34, pp. 171-175, 11/12/ 1971.

[5] T. Hafting, M. Fyhn, S. Molden, M. B. Moser, and E. I. Moser, "Microstructure of a spatial map in the entorhinal cortex," *Nature,* vol. 436, pp. 801-6, Aug 11 2005.

[6] T. W. Blackstad, "ON THE TERMINATION OF SOME AFFERENTS TO THE HIPPOCAMPUS AND FASCIA DENTATA," *Cells Tissues Organs,* vol. 35, pp. 202-214, 1958.

[7] B. Poucet and F. Sargolini, "A Trace of Your Place," *Science,* vol. 340, pp. 35-36, April 5, 2013 2013.

[8] R. Milo, S. Shen-Orr, S. Itzkovitz, N. Kashtan, D. Chklovskii, and U. Alon, "Network motifs: simple building blocks of complex networks," *Science,* vol. 298, pp. 824-7, Oct 25 2002.

[9] T. I. Lee, N. J. Rinaldi, F. Robert, D. T. Odom, Z. Bar-Joseph, G. K. Gerber, *et al.*, "Transcriptional Regulatory Networks in Saccharomyces cerevisiae," *Science,* vol. 298, pp. 799-804, October 25, 2002 2002.

[10] S. Mangan, A. Zaslaver, and U. Alon, "The Coherent Feedforward Loop Serves as a Sign-sensitive Delay Element in Transcription Networks," *Journal of Molecular Biology,* vol. 334, pp. 197-204, 11/21/ 2003.

[11] L. A. Saddic, B. Huvermann, S. Bezhani, Y. Su, C. M. Winter, C. S. Kwon, *et al.*, "The LEAFY target LMI1 is a meristem identity regulator and acts together with LEAFY to regulate expression of CAULIFLOWER," *Development,* vol. 133, pp. 1673-1682, May 1, 2006 2006.

[12] J. G. White, E. Southgate, J. N. Thomson, and S. Brenner, *The Structure of the Nervous System of the Nematode Caenorhabditis elegans* vol. 314, 1986.

[13] C. Y. Dong, J. Lim, Y. Nam, and K. H. Cho, "Systematic analysis of synchronized oscillatory neuronal networks reveals an enrichment for coupled direct and indirect feedback motifs," *Bioinformatics,* vol. 25, pp. 1680-5, Jul 1 2009.

[14] H. J. Jang, "Development and assessment of excitatory-inhibitory neuromorphic network model for hippocampal spike timing-dependent plasticity," 국내석사학위논문, 고려대학교 대학원, 서울, 2013.

[15] G. Tesauro, D. S. Touretzky, and T. K. Leen, *Advances in neural information processing systems 7* vol. 7: MIT press, 1995.

[16] F. Morin, N. Nishimura, L. Griscom, B. Lepioufle, H. Fujita, Y. Takamura, *et al.*, "Constraining the connectivity of neuronal networks cultured on microelectrode arrays with microfluidic techniques: a step towards neuron-based functional chips," *Biosens Bioelectron,* vol. 21, pp. 1093-100, Jan 15 2006.

[17] Y. Nam, "Neuron-on-a-chip: Microelectrode array system and neuronal patterning," *Journal of biomedical engineering research : the official journal of the Korean Society of Medical & Biological Engineering,* vol. 30, pp. 103-112, 2009.

[18] A. F. M. Johnstone, G. W. Gross, D. G. Weiss, O. H. U. Schroeder, A. Gramowski, and T. J. Shafer, "Microelectrode arrays: A physiologically based neurotoxicity testing platform for the 21st century," *NeuroToxicology,* vol. 31, pp. 331-350, 8// 2010.

[19] D. A. Wagenaar, J. Pine, and S. M. Potter, "An extremely rich repertoire of bursting patterns during the development of cortical cultures," *BMC Neurosci,* vol. 7, p. 11, 2006.

[20] Y. Jimbo, T. Tateno, and H. P. Robinson, "Simultaneous induction of pathway-specific potentiation and depression in networks of cortical neurons," *Biophysical Journal,* vol. 76, pp. 670-678, 1999.

[21] G. Michel, T. Tonon, D. Scornet, J. M. Cock, and B. Kloareg, "The cell wall polysaccharide metabolism of the brown alga Ectocarpus siliculosus. Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes," *New Phytol,* vol. 188, pp. 82-97, Oct 2010.

[22] M. Abedin and N. King, "Diverse evolutionary paths to cell adhesion," *Trends Cell Biol,* vol. 20, pp. 734-42, Dec 2010.

[23] B. M. Gumbiner, "Cell Adhesion: The Molecular Basis of Tissue Architecture and Morphogenesis," *Cell,* vol. 84, pp. 345-357, 2/9/ 1996.

[24] G. Chan and D. J. Mooney, "New materials for tissue engineering: towards greater control over the biological response," *Trends Biotechnol,* vol. 26, pp. 382-92, Jul 2008.

[25] A. Blau, "Cell adhesion promotion strategies for signal transduction enhancement in microelectrode array in vitro electrophysiology: An introductory overview and critical discussion," *Current Opinion in Colloid & Interface Science,* vol. 18, pp. 481-492, 2013.

[26] S. Khan and G. Newaz, "A comprehensive review of surface modification for neural cell adhesion

and patterning," *J Biomed Mater Res A*, vol. 93, pp. 1209-24, Jun 1 2010.

[27] M. C. Lensen, V. A. Schulte, J. Salber, M. Diez, F. Menges, and M. Möller, "Cellular responses to novel, micropatterned biomaterials," *Pure and Applied Chemistry*, vol. 80, 2008.

[28] A. Kumar and G. M. Whitesides, "Features of gold having micrometer to centimeter dimensions can be formed through a combination of stamping with an elastomeric stamp and an alkanethiol ‘‘ink’’ followed by chemical etching," *Applied Physics Letters*, vol. 63, pp. 2002-2004, 1993.

[29] W. Choi, "A soft-imprint technique for submicron-scale patterns using a PDMS mold," *Microelectronic Engineering*, vol. 73-74, pp. 178-183, 2004.

[30] E. Marconi, T. Nieus, A. Maccione, P. Valente, A. Simi, M. Messa, *et al.*, "Emergent functional properties of neuronal networks with controlled topology," *PLoS One*, vol. 7, p. e34648, 2012.

[31] R. Kim, S. Joo, H. Jung, N. Hong, and Y. Nam, "Recent trends in microelectrode array technology for in vitro neural interface platform," *Biomedical Engineering Letters*, vol. 4, pp. 129-141, 2014.

[32] C. Xie, L. Hanson, W. Xie, Z. Lin, B. Cui, and Y. Cui, "Noninvasive neuron pinning with nanopillar arrays," *Nano Lett*, vol. 10, pp. 4020-4, Oct 13 2010.

[33] M. Maher, J. Pine, J. Wright, and Y.-C. Tai, "The neurochip: a new multielectrode device for stimulating and recording from cultured neurons," *Journal of Neuroscience Methods*, vol. 87, pp. 45-56, 2/1/ 1999.

[34] G. E. Slaughter, E. Bieberich, G. E. Wnek, K. J. Wynne, and A. Guiseppi-Elie, "Improving Neuron-to-Electrode Surface Attachment via Alkanethiol Self-Assembly: An Alternating Current Impedance Study," *Langmuir*, vol. 20, pp. 7189-7200, 2004/08/01 2004.

[35] S. B. Jun, M. R. Hynd, N. Dowell-Mesfin, K. L. Smith, J. N. Turner, W. Shain, *et al.*, "Low-density neuronal networks cultured using patterned poly-l-lysine on microelectrode arrays," *J Neurosci Methods*, vol. 160, pp. 317-26, Mar 15 2007.

[36] G. Zeck and P. Fromherz, "Noninvasive neuroelectronic interfacing with synaptically connected snail neurons immobilized on a semiconductor chip," *Proc Natl Acad Sci U S A*, vol. 98, pp. 10457-62, Aug 28 2001.

[37] J. Erickson, A. Tooker, Y. C. Tai, and J. Pine, "Caged neuron MEA: a system for long-term investigation of cultured neural network connectivity," *J Neurosci Methods*, vol. 175, pp. 1-16, Oct 30 2008.

[38] H. Hardelauf, S. Waide, J. Sisnaiske, P. Jacob, V. Hausherr, N. Schobel, *et al.*, "Micropatterning neuronal networks," *Analyst*, vol. 139, pp. 3256-64, Jul 7 2014.

[39] J.-P. Frimat, J. Sisnaiske, S. Subbiah, H. Menne, P. Godoy, P. Lampen, *et al.*, "The network formation

assay: a spatially standardized neurite outgrowth analytical display for neurotoxicity screening," *Lab on a Chip,* vol. 10, pp. 701-709, 2010.

[40] S. W. Lee and S. S. Lee, "Shrinkage ratio of PDMS and its alignment method for the wafer level process," *Microsystem Technologies,* vol. 14, pp. 205-208, 2008.

[41] G. M. Beaudoin, 3rd, S. H. Lee, D. Singh, Y. Yuan, Y. G. Ng, L. F. Reichardt, *et al.*, "Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex," *Nat Protoc,* vol. 7, pp. 1741-54, Sep 2012.

[42] T. Pfeiffer, U. Kraushaar, M. Dufer, S. Schonecker, D. Haspel, E. Gunther, *et al.*, "Rapid functional evaluation of beta-cells by extracellular recording of membrane potential oscillations with microelectrode arrays," *Pflugers Arch,* vol. 462, pp. 835-40, Dec 2011.

요 약 문

공간 표상 신경망 네트워크의 모델링과 PDMS 물리적 구조물을 이용한 기판 위 체외 신경망 네트워크 형성

뇌 안에 있는 공간 표상 신경망 네트워크는 장소세포와 격자세포간 네트워크의 상호작용으로 장소를 인지하고 나의 위치를 확인하는 기능을 한다. 이러한 뇌의 인지 기능적인 기작을 연구하기 위해서, 네트워크 모티프의 관점에서 신경 네트워크에 대한 계산적 모델과 그것과 비교하기 위한 실제 신경망 네트워크 분석 플랫폼을 만들고자 하였다. 뉴런 시뮬레이션 툴을 이용하여 이 계산적 모델을 설계하려고 하였고, 계산적 모델을 증명하기 위해 체외 신경망을 만들어야 하는데, 이를 위해 실제 쥐의 뇌에서 분리, 박리시킨 피라미달 신경세포를 배양하였다. 신경 인터페이스 칩인 미세전극 어레이 위에 체외 신경망을 형성하기 위해서, 미세전극 어레이 위에서 간단하게 만들 수 있고 제거할 수 있는 PDMS 물리적 구조 패턴을 디자인하였다. 네트워크 모티프 관점의 체외 신경망 모델과 분석 플랫폼은 뇌 과학의 신경회로 연구 관점에서 뇌의 인지적 기능을 조사하기 위한 증명, 분석하는 툴이 될 수 있을 것이다.

핵심어: 공간 표상 신경망 네트워크, 신경세포 모델링, 개별 세포 단위의 신경세포 패터닝, 체외 신경망 네트워크, 초대 pyramidal 신경세포 배양