CONSTRUCTION AND ANALYSIS OF AN ARTIFICIAL NEURONAL NETWORK USING A NEURON-COLLECTING, MICRO-PATTERNING METHOD BASED ON A MULTI-ELECTRODE ARRAY SYSTEM

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Abstract: We developed three techniques to make artificial neuronal networks constructed from rat hippocampal neurons. 1) a method of non-invasively collecting primary cultured neurons and their deposition, 2) a technique for microprocessing agarose for the purpose of assembling artificial neuronal networks, 3) a multi-electrode array system for measurement of the multi-point extracellular potential of neurons. The three techniques allow us to assemble and evaluate artificial neuronal networks constructed from particular cells. We can manipulate neuro-transmission pathways and investigate roles played by the innate period or stability information for each individual cell in the framework of physiological mechanism. It is thus possible to construct and demonstrated the actual neuronal networks simulated by the computed neural networks.

1 INTRODUCTION

Neuronal networks in the brain form acceptable patterns for external information such as long term potentiation (LTP) or long term depression (LTD) (Pelletier JG, 2008). Therefore, it is thought that cells in the neuronal networks form acceptable or resistible spatial patterns for external information.

So far, neuronal networks have been analysed both *in vivo* and *in vitro*, but it was very difficult to analyse informational hysteresis because neurons in the brain and a culture dish make a self-formation of synapse that we can't manipulate. If an artificial neuronal network can be constructed with desired neuron types and synapse direction, the informational hysteresis of neurons in neuronal networks can be analysed very easily.

With this aim, we developed three techniques for making artificial neuronal networks, 1) a technique for picking cells from a group of primary cultured neurons in a non-invasive fashion using a digestible thin sheet and depositing the selected cells in a micropattern, 2) a technique for microprocessing agarose for assembling artificial neuronal networks through manipulation of the direction of neurotransmission on a culture dish, 3) a technique for measuring the multi-point extracellular potential of neurons with a multi-electrode array system, these cells can be also stimulated during measuring if necessary.

The three techniques allow us to assemble and evaluate artificial neuronal networks constructed from particular cells. We can manipulate neuro-transmission and investigate the physiological mechanism such as the innate period or stability information for each individual cell.

2 METHOD

2.1 Neuron Preparation and Cultivation

Dispersed cultures of hippocampal cells were

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prepared from E18 of Wistar rats according to the National Institutes of Health guidelines for laboratory animal care and safety. The hippocampal formation was dissected out from anesthetized animals in ice-cold Hanks balanced salt solution. Then hippocampal formation was treated with 0.25% trypsin (Wako) and 0.01% DNase I (Sigma) at 37°C for 30 min. After adding Fatal bovine serum, cells were centrifuged at 1,000 rpm for 5 min. The remaining cells were dispersed in 2 mL Neurobasal (Invitrogen Neurobasal medium) supplemented with 2% B-27 (Invitrogen) and 1% penicillin-streptomysin at 37°C. For primary cultures and recultures, neurons and glias were plated onto a 35-mm culture dish coated with poly-L-lysine (Iwaki) at a cell density of 1.0×10^5 cells / cm^2 at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

2.2 Formation of a Cell Collection Dish for Primary Neurons and Neurons Network

80 µL of 1.5% sodium alginate was put on a 35-mm culture dish and sodium alginate was spin-coated at 3,000 rpm for 10 sec, and dried. Subsequently, the sodium alginate was gelled by applying 1.5% $CaCl_2$, and a calcium alginate thin layer was made on the culture dish. Next, 200mM poly-l-lysine hydro-borate (PLL) and 4% polyethyleneimine（PEI）was coated.

2.3 Detaching and Transferring the Cells

For detaching and transferring cells, a glass capillary whose internal diameter was 0.6 mm was heated, pulled, and fire polished to make the internal diameter around 80 µm using a puller (Narishige) and a micro forge (Narishige). The micro-capillary was fire polished and siliconized by sigmacote (Sigma). To detach the cells, the capillary was filled with a culture medium with 5mM EDTA・2Na (Dojindo Laboratories)

2.4 Re-culture of the Collected Cells

Releasing of the medium containing EDTA from the microcapillary and retrieving cells was controlled by adjusting the air pressure in the microcapillary using a pneumatic manual micro-injector (Eppendorf). The retrieved cells were put onto another culture dish and cultivated. A second cultivation dish for neuron was coated with PLL.

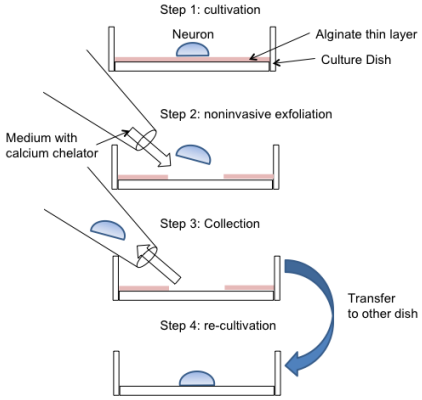

Figure 1: The procedure of collecting a cultured single neuron.

2.5 Agarose Microprocessing System for the Single Cultivation and Regulating the Direction of Neurite

Microstructures (microchamber and microchannel) to fix cell positions, guide neurites and form the network patterns were created using a photothermal etching method. A 1480-nm infrared laser beam was focused on the agar thin layer through the objective lens of the microscope on the culture dish, causing the agar at the focal point to melt (Fig.2).

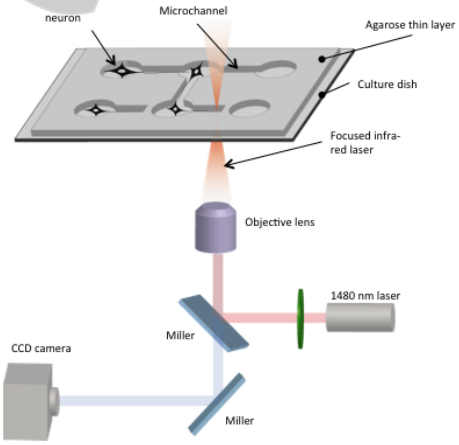

Figure 2: Device configuration of an agarose microprocessing system.

2.6 An agarose Microprocessing and Multi-electrode Array System for the Measurement of an Action Potential of an Artificial Neuronal Network

A multi-electrode array (MEA) chip was formed on a glass slide consisting of either a 8×8 or 16×4

electrode array. This system enables measurement of extracellular action potentials of single neurons and a sampling rate of 100 kHz per channel can be used. The MEA chip was made of indium tin oxide (ITO) whose transparency facilitate subsequent microfabrication of microchamber and microchannel, so that this system allow us to fabricate agarose microchambers and microchannels on the MEAs chip. To measure action potential of artificial neuronal network of neurons, agarose microstructures was made on the MEAs chip.

3 RESULTS

3.1 Preparation of a Collection Dish for Primary Neurons

Sodium alginate was put on a culture dish, and coated by spin-coater, and then dried in air. Subsequently, sodium alginate was gelled by applying $CaCl_2$ solution. A thin calcium alginate sheet was dried and washed. Subsequently, PLL and PEI were coated.

Primary neurons adhered onto the PLL-micro-contact-printed alginate dish and extended the neurites and axons very well.

3.2 Collection of Cultured Primary Hippocampal Neurons and Re-cultivation

Primary hippocampal neurons were initially cultured on the detaching-culture dish for neurons. After a few minutes, neurons started to adhere on the detaching-culture dish. After 1 day, hippocampal neurons extended their neurites. After confirming that neurites of cells were extended, the medium containing EDTA was loaded by a micro-capillary. Then calcium alginate around the target cell was immediately solated, and target cells were released from the layer. The released neurons were collected with a pipette very easily. They were cultured on another dish coated with PLL. All steps from cell collecting to re-culturing required less than 2 min. The collected neuron retained their shapes and did not shrink, and re-cultivated neurons are extended their neurites immediately (Fig3).

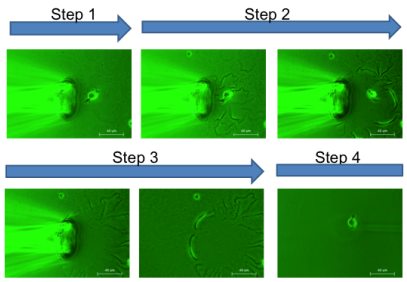

Figure 3: Micrographs of each step for collecting cultured neuron.

3.3 Neuron Cultivation on the Agarose-micropatterned Chamber

Primary neurons dissected from hippocampal formation were initially cultured in an agarose microchamber. Neurons transferred by a micro-pipette adhered onto the microchamber. After 1 day, neurite of neurons extended along the microchamber. Neuron didn't adhere onto the bottom of the small chamber whose diameter was 20μm. Furthermore, neurons didn't extend neurites along the microchannel whose width was less than 7μm.

3.4 Measurement of the Extracellular Action Potential of Micropatterned Single Neurons

A microchamber was made on top of each electrode and these microchambers were interconnected via micro-channels. The Primary hippocampal neurons were initially cultured on the detaching culture dish for neuron. After a few minutes, neurons started to adhere onto the detaching culture dish. After 1 day, hippocampal neurons extended their neurites. After 7days, the extracellular action potential was recorded (Fig.4).

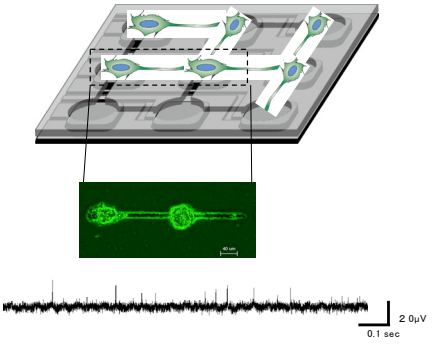

Figure 4: An extracellular action potential recording from single neuron on the micropatterned structure.

4 DISCUSSION

Alginic acid is a viscous gum derived from algae and is composed of β-D-mannuronate and α-L-guluronate. Calcium alginate, which is a salt of alginic acid, is harmless to cells and used as a scaffold in tissue transplantation (Heise, 2005). However, cells cannot adhere to intact calcium alginate. In this study, alginate sheet that have both properties that transform sol/gel state and adhesiveness of cells could be made. Using this sheet, a specific cell can be collected without exfoliating surrounding cells.

Recently, Okano et al. have developed techniques, which allow us to detach cells from culture dishes without using digestive reagents9. Temperature dependent polymer, poly (N-isopropylacrylamide) (PIPAAm) changes the hydrophilic/ hydrophobic property in a temperature-dependent manner (Masuda, 2008). PIPAAm is hydrophobic at 37°C and hydrophilic at 20°C, so that cells on the PIPAAm coated culture dish can be detached from the culture dish without perturbing the extracellular matrix and intercellular connection such as tight junctions. Such a method gives us cell sheets that retain intercellular connections. Using this technique, the stick cardiac tissue stacked mono-layered cardiac cell sheet can be made.

However, individual cells that have specific property cannot be collected with this method because temperature cannot be controlled on the scale of micrometer. In fact, dispersed cultured cells have heterogeneous properties while if averaging the physiologically property, dispersed culture cells are apparently homogeneous. So that, to align the physiological properties homogeneously, it must be necessary to develop a method to collect each single cell from culture dishes non-invasively.

On the other hand, our method is suitable for collecting single cells or small clusters of cells. Therefore, for example, if there are several types of differentiated or undifferentiated cells derived from ES or iPS cells in the culture dish, our method can allow us to collect only targeted cells.

Moreover, primary neurons were cultured on the agarose-micropatterned chamber. The cultured neurons extended neurite along the microchannel. Furthermore, the extracellular action potential of single neuron can be measured by an agarose-micropatterned multielectrode array.

The results of three techniques; the noninvasive collection method of neuron, agarose microproceesing method and multielectrode array, allow us to make artificial neuronal networks using neurons regulating direction of neurotransmission, and to measure the activity of artificial neuronal networks. The next stage of the study is to construct basic components working in the actual brain.

5 CONCLUSIONS

We developed three techniques 1) a non-invasive neuron collection method, 2) an agarose micro-processing technique, 3) a multielectrode array system. These techniques allow us to construct and demonstrated the actual neuronal networks simulated by the computed neural networks.

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