FORMING OF 3D NEURONAL PATHWAY BY NEURONAL BLOCK ASSEMBLY

M. Kato-Negishi¹, H. Onoe¹,², Y. Morimoto¹,² and S. Takeuchi¹,²

¹Institute of Industrial Science, the University of Tokyo, Japan
²Takuchi Biohybrid Innovation Project, Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology (JST), Japan

ABSTRACT
This paper describes a method of three-dimensional (3D) neuronal pathway formation in vitro by bottom-up neuronal tissue block assembly (Fig.1). We first formed the neuronal tissue blocks by molding uniform-sized neurospheroids into the PDMS mold chambers. We then assembled a millimeter-sized cortex-hippocampal tissue by connecting cortical and hippocampal tissue blocks. We found that cortical and hippocampal neurons extended their axons to each other, and that 3D neuronal pathway was successfully formed in the 3D environment. We believe that our neuronal block assembly enables the fabrication of in vivo-like neuronal pathway, and that this system is useful tool for neuronal tissue engineering, pharmacological assay for the screening of drugs and toxins to neuronal pathway.

KEYWORDS: 3D tissue engineering, neuron, cortex, hippocampus, neuronal pathway, Ca²⁺ imaging

INTRODUCTION
In vitro reconstruction of 3D neuronal pathway is needed to know the mechanism of neuronal pathway formation in vivo. The co-culture of dissociated cortical and hippocampal neurons on the 2D condition has been reported [1], but this method is difficult to make an in vivo-like neuronal pathway because of low cell density, low synaptic density and non-assembled culture system. On the other hands, the co-culture system of cortical and hippocampal tissue dissected from brain have some risk for neuronal pathway fabrication because of neuronal damage by dissection. Here, we describe a method to fabricate a 3D neuronal pathway by bottom-up neuronal tissue block assembly. Figure 1 shows a concept of our study that consists of the following 4 steps: (1) Preparation of the dissociated cortical and hippocampal cells from dissected brain regions, (2) Formation of uniform-sized neurospheroids using a PDMS microchamber array, (3) Formation of tissue block using the millimeter-sized PDMS mold chamber and (4) Assembly of the different types of tissue block. We also demonstrate that assembled neuronal tissue blocks connect strongly to each other and make a neuronal network.

**Figure 1.** Concept illustration of our “bottom-up” neuronal tissue fabrication methods. Neuronal pathway is formed by neuronal tissue assembly.
FABRICATION OF NEURONAL BLOCKS

To assemble neuronal tissue blocks, we first prepared the uniform-sized neurospheroids on a PDMS microchamber array using the previously reported method [2]. PDMS microchamber array is generated using conventional photolithography and soft lithography techniques (Fig. 2). Briefly, SU-8 negative photoresist were span onto a silicon wafer and soft baked. The resist was exposed to UV light through a patterned photomask using a mask aligner and developed using SU8-developer solution. This process creates an SU8 master that could be used to mold PDMS. The PDMS microchamber was formed by pouring PDMS (10:1, prepolymer: curing agent) onto the master and curing for 2 h at75°C. Removal of the PDMS from the wafers yielded PDMS microchamber array for cell culture.

The cerebral cortices and hippocampus were dissected from Wistar rats (embryonic day 16-19) and cultured using previously described methods [3-5]. Briefly, the cerebral cortices and hippocampus were dissociated with papain and triturated through a pipette. The cortical and hippocampal cell suspensions were seeded onto the PDMS microchamber array (diameter: 100 or 150 μm) and non-dropped cells were removed after 30 min by changing the medium (Fig. 1). Cultured cells were maintained at 37°C under a humidified atmosphere of 5% CO₂ and 95% air. After 48-72 h of culture, we molded uniform-sized neurospheroids into a PDMS mold chamber and cultured (Fig. 3b). Neurospheroids connected to each other at 24 h culture. After 72 h of culture, millimeter-sized neuronal tissue blocks could be formed and released from chamber (Fig. 3c).

We next investigated whether neuronal tissue block had neuronal activity and formed neuronal network. In the 2D culture system, there are many reports that cortical neurons form a neuronal network because of showing synchronized [Ca²⁺]i oscillations [6-7]. Using the Ca²⁺ imaging system, we observed synchronized [Ca²⁺]i oscillations in the cortical tissue blocks. The frequency was approximately 0.1 Hz. These [Ca²⁺]i responses conformed to the responses of cortical neurons in primary dissociated 2D culture. Therefore, neurons of the tissue block had functional synaptic connections each other and formed a large-scale neuronal network (Fig. 4).

NEURONAL BLOCK ASSEMBLY

We found that different types of neuronal tissue blocks could be easily assembled and could fabricate large-scale neuronal tissue (Fig. 5). We first prepared different types of neuronal tissue blocks, cortical tissue block and hippocampal tissue block. Then, we assembled the cortical tissue block with hippocampal tissue block into a PDMS mold chamber for forming millimeter-sized tissue. After 72 h of culture, we observed that neuronal tissue blocks adhered tightly to each other and many neurons extended a large number of axons in the other neuronal blocks. Finally we examined whether...
cortical neurons and hippocampal neurons in the cortex-hippocampal tissue connected to each other with synapses as in vivo neuronal pathway. After 3-5 days of culture, the cortex-hippocampal tissue showed synchronized [Ca^{2+}] oscillations (Fig. 6c), indicating that cortex tissue block and hippocampal tissue block were connected to each other and formed 3D cortex-hippocampal neuronal pathway.

CONCLUSION
This study describes a strategy for formation of 3D neuronal pathway in vitro by neuronal tissue block assembly. We developed an efficient technique, bottom-up neuronal tissue fabrication methods, to form the millimeter-sized neuronal tissue blocks using the uniform-sized neurospheroids. We also demonstrated that cortical-hippocampal neuronal tissue blocks had functional synaptic connections each other and made a large-scale neuronal network. Therefore, our neuronal block assembly approach would be an extremely effective method for fabrication of functional neuronal tissue and for formation of 3D neuronal pathway.

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CONTACT
Prof. Shoji Takeuchi, tel: +81-3-5452-6650; takeuchi@iis.u-tokyo.ac.jp