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TWO-LAYERED MICROWELL-ARRAY DEVICE FOR PREPARATION OF SINGLE-NEURON CULTURE SAMPLES

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Abstract

When a single neuron is cultured in isolation from other neurons, its axon connects with its own dendrites to form a simple, independent network with no synaptic inputs from other neurons. This culture system enables detailed analysis of synaptic function and morphology change in neurites at the single-neuron level, which is useful for elucidating the pathogenesis of neurological diseases and for evaluating the efficacy of therapeutic drugs for them. However, there was previously no device technology capable of simultaneously forming multiple single-neuron samples while allowing co-culture with astrocytes, which is essential for culture of a single neuron isolated from other neurons. In this study, we propose a novel microwell-array device for preparing single-neuron samples. The device consists of an upper layer for cell seeding and a lower layer for cell culture. Each layer has 16 × 16 microwells, and the bottom of each well is made of a 1 μm thick silicon nitride membrane. The membrane of the upper well has one microhole for seeding a single neuron, and the lower membrane has multiple microholes for interaction between a single neuron and astrocytes which are co-cultured back-to-back on both sides of the membrane. When neurons are seeded into the upper well, only one of them passes through the microhole in the upper membrane and falls onto the lower membrane. We evaluated a seeding efficiency of single neurons by changing seeding hole diameter and seeding density. The results showed that the yield of more than 20% was obtained regardless of the seeding density when the seeding hole diameter was 13 μm. We also confirmed that single neurons seeded in this manner and co-cultured with astrocytes developed neurites and formed synapses. These results demonstrated the usefulness of this device for the preparation of single-neuron culture samples.

Keywords: Single neuron, Silicon nitride, Microwell, Microhole, Co-culture

1. Introduction

When a large number of neurons are cultured, they intricately connect with each other, forming an extremely complex and heterogeneous network with various excitatory and inhibitory synapses. This complex network prevents a detailed understanding of the function, behavior, and structure of individual neurons. On the other hand, when a single neuron is cultured, its axon connects with its own dendrites to form synapses, which are generally called “autapses” [1, 2]. This single-neuron culture sample forms a simple network, which enables detailed quantitative analyses of synaptic function and

morphology change in neurites at the single-neuron level by visualization of receptors, synaptic vesicles, primary dendrites, dendrite branches, and spines through immunofluorescent staining [3–5]. Recently, the single-neuron culture sample has been applied to elucidate the pathogenic mechanisms of neurological diseases such as epilepsy and Alzheimer's disease and to evaluate the efficacy of therapeutic drugs for them [6, 7].

Co-culture with astrocytes, which support physiological activity of neurons, is essential to form a single-neuron culture sample [8]. The general co-culture method is to culture a single neuron on an astrocyte microisland which plays a role as a feeder layer to limit a neuronal culture area. A large number of microislands of 200–300 μm in typical diameter are formed by seeding astrocytes on collagen grids stamped by the microcontact printing method, which allows multiple single neuron samples to be obtained simultaneously. However, the microisland culture method has a major issue that it is difficult to maintain single neuron culture for more than about two months due to deterioration of astrocyte microislands [4, 9]. This is extremely detrimental to long-term analysis, especially with human iPS cell-derived neurons, which take time to mature.

On the other hand, various types of microfluidic devices have so far been developed for capturing multiple single cells and arranging them in an array [10, 11]. In particular, microfluidic devices having a microwell array can be widely employed for high-throughput analyses of single cells [12]. However, because the microwell size of these devices is designed to be close to a single cell, it is impossible to culture a single neuron which needs a large culture area for its neurite elongation. Also, some devices have large-area culture wells for cell proliferation and co-culture [13–15]. However, these devices are also dedicated to seeding single cells into the wells, and a single neuron and multiple astrocytes cannot be seeded into the same well. In addition, some devices can capture single neurons in a culture area large enough for neurite elongation [16–18]. However, most of these devices are designed for connection between neurons after single neuron culture, and are not intended to culture a single neuron isolated from other neurons. Even devices capable of culturing an isolated single neuron are likely to be limited to short-term culture because it cannot be co-cultured with astrocytes, which is essential for long-term culture of an isolated single neuron. In other words, there are no devices that allow for both culture of an isolated single neuron and its co-culture with astrocytes.

In this study, we propose a two-layered microwell-array device to solve the limitation of astrocyte lifetime in the microisland culture method and the difficulty of co-culturing an isolated single neuron with astrocytes in a microfluidic device. The microwell array of the upper layer is used to seed a single neuron through a single microhole formed in each well. In the microwell array of the lower layer, a single neuron is co-cultured with astrocytes on both sides of a silicon nitride (SiN) membrane at the bottom of each well. A large number of microholes opened in the membrane provide good cell-to-cell communication between a single neuron and astrocytes. The front side of the membrane in each well has an area large enough to culture a single neuron which elongates numerous neurites. The back sides of all well membranes share a common square-shaped culture surface of 14 mm on a side, which is large enough for mass culture of astrocytes. In this way, it is possible to prepare multiple single neuron culture samples simultaneously without using the astrocyte microisland method. In order to demonstrate the usefulness of the present device, we evaluated the seeding efficiency of single neurons and observed states of co-cultured single neurons and astrocytes.

2. Materials and methods

2.1 Device design and fabrication process

The two-layered microwell-array device consists of an upper layer for seeding single neurons and a lower layer for co-culturing single neurons and multiple astrocytes. Each layer has 16 × 16 microwells, and the upper and lower wells correspond one-to-one (figure 1(a)). The well bottoms were made of a 1 μm thick SiN membrane. The membrane of each well of the upper layer has a microhole through which a single neuron is seeded to the lower layer. Because the diameter of neurons in suspension is 8–10 μm, the minimum diameter of the seeding hole was set to 12 μm to prevent two neurons from passing through at the same time. Also, seeding holes with larger diameters of 24 μm, 36 μm, and 48 μm were designed for comparison. As described below, the actual diameters of the microholes were approximately 1 μm larger than the designed values due to the fabrication process (figure 1(d)). The membrane of each well of the lower layer has multiple microholes with a diameter of 3 μm and a pitch of 20 μm for interaction between a single neuron and astrocytes which are cultured on both sides of the membrane (figure 1(e)). Also, in order to align the two layers in the manner described below, concave and convex portions were formed on the back side of the upper layer and the front side of the lower layer, respectively (figure 1(b)). A square frame made of polydimethylsiloxane (PDMS) (Shin-Etsu Chemical Co., Ltd., KE-106) was mounted on the top surface of the upper layer to hold a cell suspension. A polycarbonate square frame fabricated by a milling machine was attached around the lower layer to improve its handling (figure 1(a)).

The procedure of cell seeding and co-culturing is as follows (figure 1(b)). First, astrocytes are cultured on the back side of the lower membrane. Next, the upper layer is overlaid on the lower layer, and the convex portions of the lower layer are inserted into the concave portions of the upper layer. This allows easy horizontal alignment of the two layers, and the gap

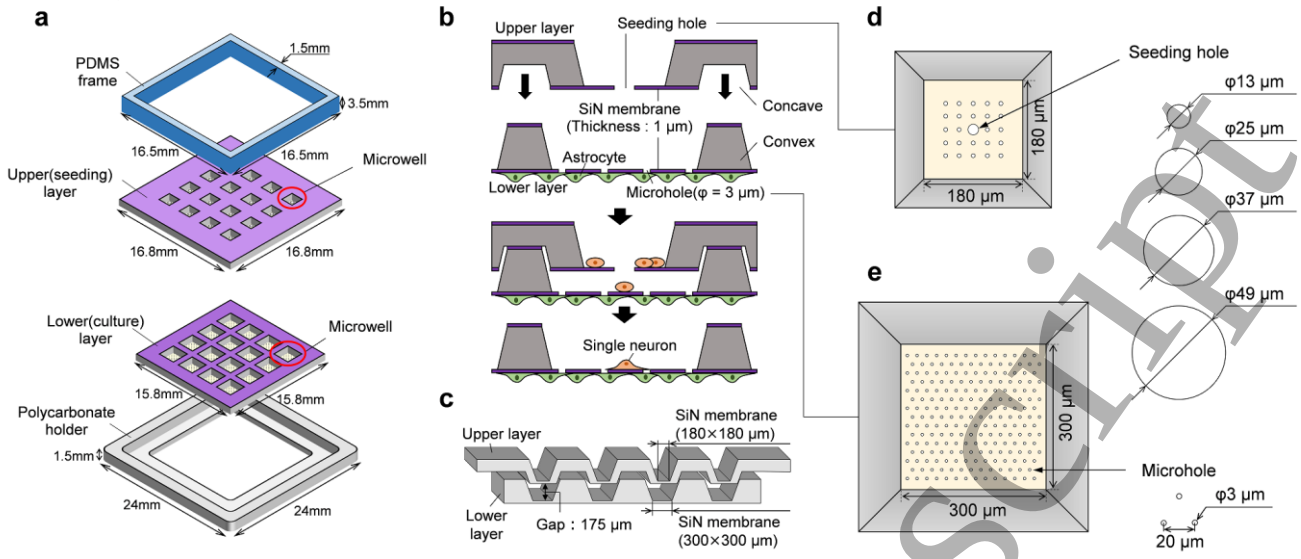

Figure 1. Schematic and design details of a two-layered microwell-array device. (a) Structure of upper and lower layers. Each layer has 16 × 16 microwells. For simplicity, 4 × 4 microwells are shown in the figure. The upper layer has a PDMS frame to hold a cell suspension. The lower layer was placed in a polycarbonate holder to improve handling. (b) Procedure of cell seeding and co-culturing. The bottoms of the microwells were made of a 1 μm thick SiN membrane. Only one microhole for seeding a single neuron was formed in the center of the membrane of the upper layer. The lower membrane had multiple microholes for cell-to-cell interaction. By inserting the convex portions on the front side of the lower layer into the concave portions on the back side of the upper layer, the device was assembled. (c) Partial bird's-eye view of the upper and lower layers. Overlaying the upper and lower layers gives a gap of 175 μm between the membranes. (d) Design of the membrane of the upper layer. Four sizes of seeding microholes (diameter: 13, 25, 37, and 49 μm) were prepared. (e) Design of the membrane of the lower layer. Multiple microholes (diameter: 3μm) were arrayed with a pitch of 20 μm.

between the two membranes is set to approximately 175 μm (figure 1(c)). The gap can be changed mainly depending on the depth of the concave portions. Then, neurons are seeded into the wells of the upper layer. A single neuron passes through a hole in each well, and drops onto the bottom membrane of the corresponding well of the lower layer. After single neurons are confirmed to have adhered to the lower membrane, the upper layer is separated from the lower layer. At this time, unnecessary neurons remaining on the upper membrane are removed along with the upper layer. To prevent the membrane from cracking due to water pressure during the overlaying of the two layers and the removal of the upper layer, approximately 60–70 microholes with a diameter of 3 μm were opened around every seeding hole of the upper layer (figure 1(d)). In this way, multiple single-neuron samples co-cultured with astrocytes are prepared simultaneously.

Figure 2 shows the fabrication processes and photographs of the upper and lower layers. Each layer was fabricated from a silicon wafer with a single-sided polished surface, crystal orientation of <100>, diameter of 50 mm, and thickness of 300 μm. The upper layer was fabricated as follows. Using plasma-enhanced chemical vapor deposition (PECVD), 1.2 μm thick and 1.5 μm thick SiN membranes were deposited on the mirror surface side and non-mirror surface side of the wafer, respectively (figure 2(a1)). A microwell window pattern was formed on the non-mirror surface side by photolithography and CF_4 plasma etching of the SiN membrane (figure 2(a2)). The wafer was immersed in a 25% tetramethylammonium hydroxide (TMAH) solution (Wako Pure Chemicals Co., Ltd., 206-15035) at 80°C, and anisotropically etched to a depth of approximately 180 μm (figure 2(a3)). A seeding hole and pressure-relief holes were patterned on the mirror surface side by photolithography and CF_4 plasma etching of the SiN membrane (figure 2(a4)). The wafer was anisotropically etched from the mirror surface side to a depth of 125 μm in an 80°C solution of a 25% TMAH containing 0.01% Triton™ X-100 (Sigma-Aldrich, T8787-50ML). This process formed concave portions for layer alignment and created a freestanding SiN membrane (figure 2(a5)). An etchant with surfactant Triton™ X-100 was used because a truncated pyramid structure formed on the back side exposes various crystal orientations of Si on its lateral ridges which would be etched by a surfactant-free etchant [19–21]. On the other hand, the lower layer was fabricated using almost the same process as the upper layer as briefly described below. After SiN membranes were deposited on both sides of a Si wafer using PECVD (figure 2(b1)), a microwell window pattern was formed on the non-mirror surface side of the wafer (figure 2(b2)), and the wafer was anisotropically etched with a TMAH solution at 80°C to a depth of 200 μm (figure 2(b3)). Then, a pattern of microholes was formed on the mirror surface side

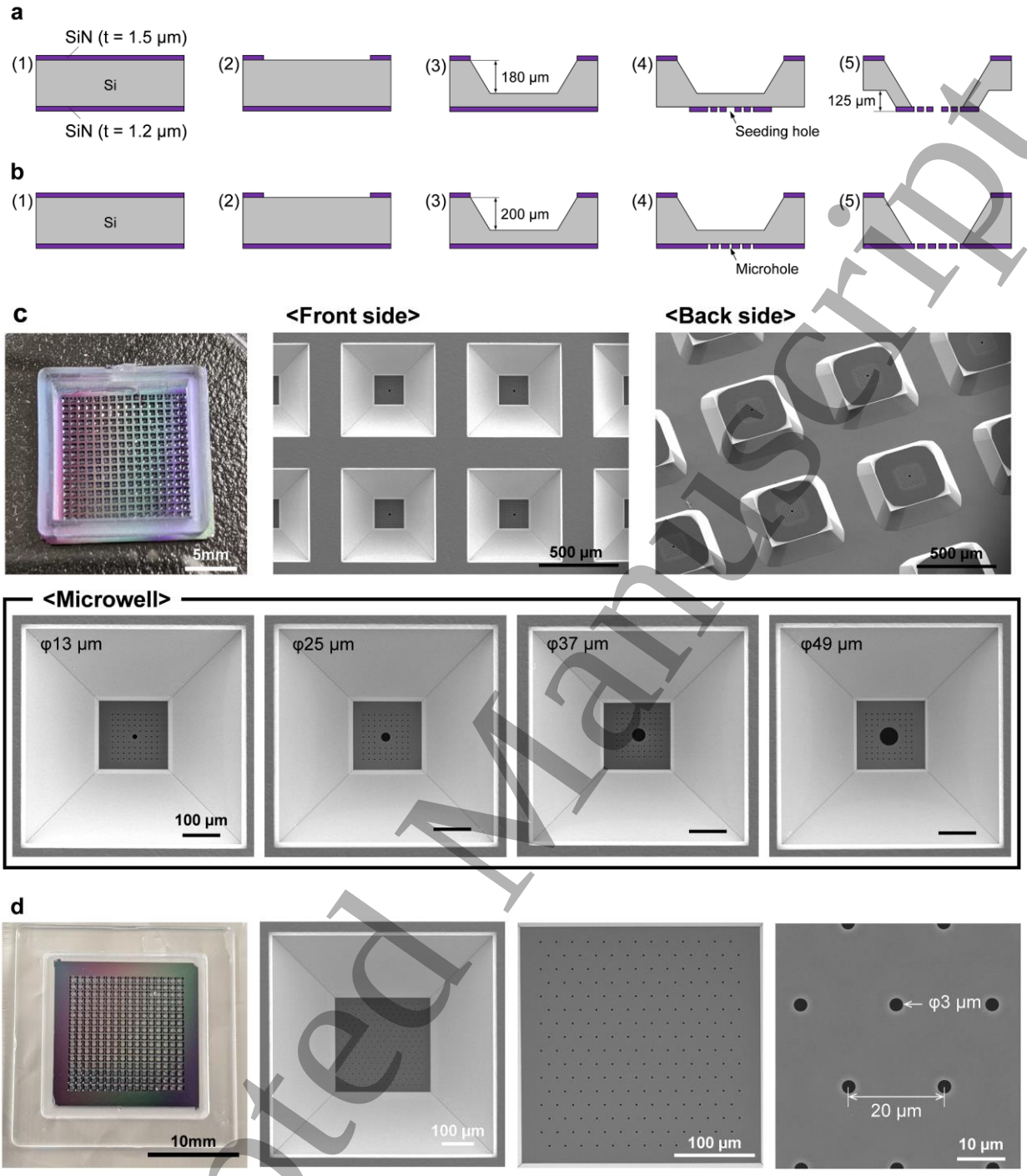

Figure 2. Fabrication processes and images of the upper and lower layers. (a) Fabrication process of the upper layer. (1) SiN membranes of 1.5 μm and 1.2 μm in thickness were deposited on the non-mirror side and the mirror side of a Si wafer using PECVD, respectively. (2) A microwell window pattern was formed on the non-mirror side. (3) Si was anisotropically etched to a depth of 180 μm using TMAH. (4) A seeding hole pattern was formed on the mirror side. (5) Si was anisotropically etched using TMAH with Triton™ X-100, and the concave portions of 125 μm in depth were formed. (b) Fabrication process of the lower layer. Almost the same process as for the upper layer was applied except that surfactant-free TMAH was used in step 5. (c) Overall photograph of the upper layer and SEM images of the microwells. The bottom membrane of the microwell was made of SiN and measures 180 × 180 μm with a thickness of 1 μm. One seeding microhole of 4 different diameters from 13 to 49 μm was opened in each membrane. (d) Overall photograph of the lower layer and SEM images of the microholes opened in the microwell bottom. The bottom membrane made of SiN measures 300 × 300 μm with a thickness of 1 μm. The diameter of the microhole is 3 μm, and the center-to-center distance between the microholes is 20 μm.

(figure 2(b4)), and the remaining Si layer was completely etched with a TMAH solution at 80°C (figure 2(b5)). Figure 2(c) and 2(d) show the photographs of the fabricated upper and lower layers, respectively. The actual diameters of the seeding holes of the upper layer were approximately 1 µm larger than the designed values due to overetching in a CF_4 plasma etching process, resulting in approximately 13 µm, 25 µm, 37 µm, and 49 µm.

2.2 Evaluation of yield of single-neuron samples

To derive the optimal condition for preparing single-neuron culture samples using a two-layered microwell-array device, we evaluated the number of neurons which adhered per well in the lower layer when the diameter of seeding holes and the seeding density were changed. In this experiment, astrocytes were not cultured on the back side of the lower layer. This is because nuclear staining is necessary to count the number of neurons, and if astrocytes are cultured, the nuclei of astrocytes are also stained, making it difficult to identify neurons.

2.2.1 Surface modification. Sterilization and surface modification of the devices and seeding of cells were all performed aseptically in a clean bench. The lower layer was sterilized by irradiation with UV light for 10 min and immersion in 80% ethanol for 10 min, and then dried while being irradiated with UV light again. After both sides of the SiN membrane of the lower layer were hydrophilized by irradiation with vacuum ultra-violet (VUV) light for 25 s, poly-D-lysine (PDL) (Sigma-Aldrich, P6407-5MG) and laminin-511 E8 fragment (Matrixome Inc., 892 012) were modified on the membrane surface by the following procedure to improve neuronal adhesion to the membrane and promote axon and dendrite growth [22, 23]. The lower layer was placed in a dish and immersed in a 0.1 mg mL^{-1} PDL solution overnight at room temperature, then rinsed with sterile water. Next, a laminin solution prepared at a coating density of 1 µg cm^{-2} was poured onto the front surface of the membrane, and the lower layer was placed in a 37°C, 5% CO_2 incubator. After 1 h, the laminin solution was replaced with a neuron culture medium. The upper layer and the PDMS frame to be mounted on the upper layer were sterilized with the same procedure performed for the lower layer. Both sides of the upper layer and the back side of the PDMS frame were treated with VUV light for 25 s to bond the PDMS frame to the front side of the upper layer. Then, the upper layer was stored in a neuron culture medium until just before use. The medium for neuron culture was prepared by adding 2% B27™ Plus Supplement (Life Technologies, A3582801), 0.25% Glutamax™-I (Life Technologies, 35050061), and 0.2% penicillin-streptomycin (Life Technologies, 15140148) to Neurobasal™ Plus medium (Gibco, A3582901).

2.2.2 Seeding of single neurons. Mouse hippocampal neurons used in the experiments were isolated by the same procedure as described in reference [24]. Brains of newborn ICR mice on postnatal day 0 (P0) were immersed in cold Hank's Balanced Saline Solution (HBSS) (Thermo Fisher Scientific, 084-08345), and a hippocampal CA3–CA1 region was obtained under a microscope. The obtained samples were isolated in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fishier Scientific, 10569-044) containing 2 U mL^{-1} papain (Worthington, PAP) at 37°C for 1 h. The cell isolation procedure was performed at the Faculty of Pharmaceutical Sciences of Fukuoka University in accordance with the animal experiment protocols (Permit Numbers: 1712128, 1912103, 2112094, and 2311081) approved by the Experimental Animal Care and Welfare Committee of Fukuoka University.

The upper layer was overlaied on top of the lower layer stored in a neuron culture medium, and the convex portions of the lower layer were inserted into the concave portions of the upper layer while the upper layer was slid back and forth, left and right. Then, neurons were seeded in the PDMS frame of the upper layer. In this experiment, three seeding densities were used as follows: low density (1,800 cells cm^{-2}), medium density (9,000 cells cm^{-2}), and high density (18,000 cells cm^{-2}). The upper and lower layers were kept overlaid in an incubator for 3–4 h, and the upper layer was removed after neurons were confirmed to have adhered to the membrane of the lower layer.

2.2.3 Immunofluorescence staining. Because single neurons die within a few days when cultured alone, the immunofluorescent staining of neurons was performed on the same day as the seeding process. The nuclear staining using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Dojin Chemical Laboratory, 340-07971) was employed because it is easier to determine the number of neurons compared to the staining of axons and dendrites. First, the lower layer was rinsed with a phosphate buffer solution (PBS) to remove medium components. Next, for cell fixation, the lower layer was immersed in 4% paraformaldehyde (PFA) PBS (Wako Pure Chemicals Co., Ltd. 161-20141) for 15 min at room temperature and rinsed with PBS at 4°C. Then, for permeabilization of cell membranes, the lower layer was treated with PBST (PBS containing 0.1% Tween 20 (Sigma-Aldrich, P2287-100ML)) for 10 min at room temperature and rinsed with PBS. Finally, the lower layer was incubated with DAPI (diluted at 1:500) for 30 min at room temperature and rinsed with PBS.

2.3 Co-culture of astrocytes and single neurons

Co-culture of astrocytes and single neurons on both sides of a SiN membrane of the lower layer was performed by applying the optimal condition of seeding hole diameter and seeding density which was obtained in section 2.2. Then, immunofluorescence staining was carried out to confirm the

adhesion of both cells to the membrane and evaluate neuronal activity. This experiment demonstrated the effectiveness of the two-layered microwell-array device.

2.3.1 Surface modification. Co-culture of astrocytes and neurons requires a long incubation period of several weeks or more. For this reason, it is important to immobilize laminin more tightly on the SiN membrane of the lower layer in order to ensure stable adhesion of cells to the membrane surface. Therefore, (3-aminopropyl)triethoxysilane (APTES) (Sigma-Aldrich, A3648-100ML) and glutaraldehyde (Wako Pure Chemicals Co., Ltd., 079-00533) were used for covalent modification of laminin (laminin-511 E8 fragment) [25, 26]. After formation of hydroxy groups on the surface of the membrane with VUV light irradiation, APTES molecules were covalently bonded to the hydroxy groups by silane coupling treatment. Glutaraldehyde, which has formyl groups at both ends, was used as a cross-linker between the amino terminus of APTES and the amino group of laminin. The bonds between these molecules are all covalent, which means that the bonds are stable over the long term. The surface modification of the lower layer was carried out as follows. First, a PDMS frame for holding an astrocyte suspension was attached to the back side (astrocyte culture side) of the inverted lower layer. Next, the lower layer was immersed in a 10% APTES solution for 2 h and rinsed with 99.5% ethanol and MilliQ water. The lower layer was then immersed in a 2.5% glutaraldehyde solution for 1 h and rinsed with PBS. Finally, the lower layer was immersed in a laminin solution adjusted to a modification density of 1 μg cm^{-2} and placed in an incubator at 37°C, 5% CO_2 for 1 h. No surface modification was applied to the upper layer.

2.3.2 Cell seeding. Mouse cerebral cortical astrocytes were also isolated at Fukuoka University according to the aforementioned protocol [24]. Brains of newborn (P0) ICR mice were immersed in cold HBSS, and cerebral cortices were obtained under a microscope. The sampled cerebral cortices were dispersed with 0.05% trypsin-EDTA solution (Wako Pure Chemicals Co., Ltd., 204-16935), plated in culture flasks, and grown in a 37°C, 5% CO_2 incubator for 2 weeks. Before seeding astrocytes to the device, cells (microglia and granule cells) other than astrocytes were removed by tapping the flasks strongly several times and washing with PBS.

After astrocytes were isolated and extracted by dispersion treatment with Accumax (Innovative Cell Technologies, Inc., AM105), they were seeded at a density of 20,000 cells cm^{-2} in the PDMS frame of the inverted lower layer. The medium for culturing astrocytes was prepared by adding 10% fetal bovine serum (Life Technologies, 10270106), 0.5% penicillin/streptomycin, and 0.1% MITO+ Serum Extender (Corning, 355006) to DMEM (Gibco, 10569010). The PDMS frame was removed the next day after the astrocytes were seeded. On the 5th day of astrocyte culturing, the lower layer was returned to its original front-to-back orientation, and the upper layer was overlaid on top of it. Then, neurons were seeded at the low density of 1,800 cells cm^{-2} in the PDMS frame of the upper layer.

2.3.3 Immunofluorescence staining. Immunofluorescence staining was carried out on the 7th and 14th days after the beginning of co-culturing. On day 7, β-III tubulin in neurons and glial fibrillary acidic protein (GFAP) in astrocytes were stained to confirm that neurons and astrocytes had adhered to both sides of the SiN membrane. On day 14, β-III tubulin in neurons and vesicular glutamate transporter 1 (VGLUT1) localized at synapses were stained to confirm synapse formation in neurons. The two experiments for fluorescence double staining were performed by combining β-III tubulin antibody with GFAP antibody and β-III tubulin antibody with VGLUT1 antibody using the following procedure. First, the lower layer was rinsed with PBS, immersed in 4% PFA PBS for 15 min at room temperature for cell fixation, and rinsed again with PBS at 4°C. Next, blocking was performed with PBST solution containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, A7030) and 22.52 mg mL^{-1} glycine (Wako Pure Chemicals Co., Ltd., 075-00731). The lower layer was then incubated overnight at 4°C with the following primary antibodies and rinsed with PBS: anti-GFAP antibody (Abcam, ab7260, rabbit polyclonal, diluted at 1:1000), anti-β-III tubulin antibody (Abcam, ab7751, mouse monoclonal, diluted at 1:1000), and anti-VGLUT1 antibody (Synaptic Systems GmbH, 135303, rabbit polyclonal, diluted at 1:6800). Finally, the lower layer was incubated at room temperature for 1.5 h with the following Alexa Fluor dye-labeled secondary antibodies corresponding to the primary antibodies, and then rinsed with PBS: Alexa Fluor 488 (Abcam, ab150077, diluted at 1:500) for GFAP, Alexa Fluor 594 (Abcam, ab150116, diluted at 1:700) for β-III tubulin, and Alexa Fluor 488 (Abcam, ab150081, diluted at 1:1000) for VGLUT1.

3. Results and Discussion

3.1 Evaluation of yield of single-neuron samples

To confirm that neurons were adherent to the well bottoms of the lower layer, the well bottom surfaces were observed under a phase-contrast microscope at about 3 h after seeding of neurons (figure 3(a)). Then, nuclei of neurons adhering to the well bottoms were stained with DAPI, and the number of nuclei per well was manually counted. Because the position of the well bottoms could not be identified in the fluorescence images, the bright-field images of the wells were merged with the fluorescence images (figure 3(b)). The relationships between the number of neurons per well and the number of wells having each cell count at 3 different seeding densities

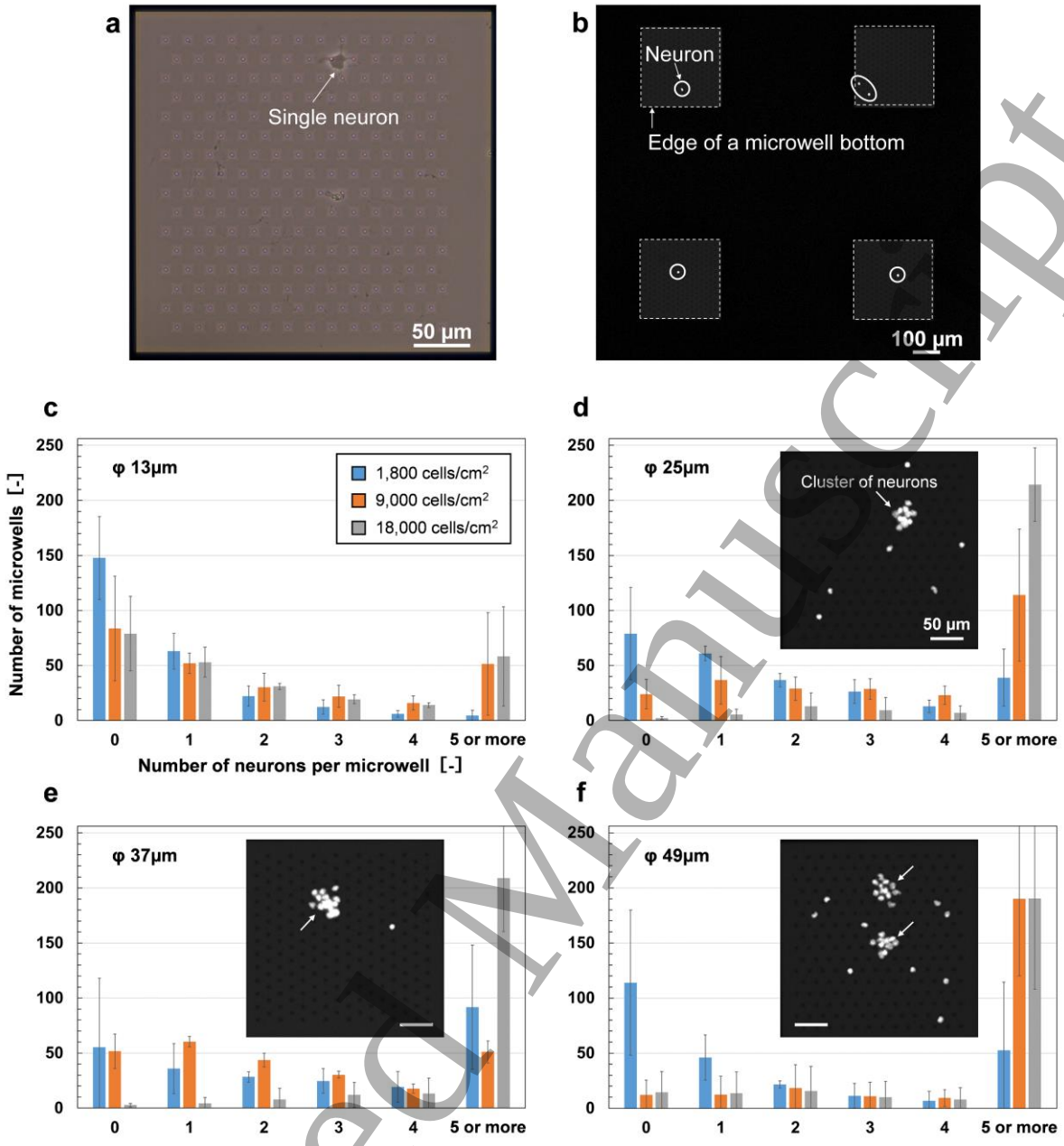

Figure 3. Images and counts of obtained single-neuron samples. (a) Phase-contrast image of a single neuron in a microwell. At about 3 h after seeding, neurons were observed to have adhered to the well bottoms. (b) Fluorescence image of neurons adhering to the bottoms of 4 wells. After the nuclei of neurons were stained with DAPI, the bright-field image and the fluorescence image were obtained and merged to identify the position of each well and count the number of neurons per well. Neurons are circled in white in the figure. (c)–(f) The number of neurons per well versus the number of wells having each cell count. The average value of 3 experiments is plotted, and the standard deviation is indicated by an error bar. Fluorescence images inserted in the graphs show clustered neurons.

are shown in figures 3(c–f) for each seeding hole diameter. Three experiments were carried out for each seeding density, and the average numbers of wells are plotted in the graphs. Error bars indicate standard deviations. Overall, while the low-density seeding condition resulted in a high number of empty wells with no neurons, the high-density seeding condition resulted in a decrease in empty wells and an increase in wells with 5 or more neurons. Under the high-density seeding condition with the seeding hole diameters other than 13 μm, adhesion of 5 or more neurons occurred at a high probability. Also, under the low-density seeding condition with the seeding holes of 13 or 25 μm diameter, single neurons were obtained in approximately 60 wells out of the total 256. In particular, the yield of single-neuron samples at the seeding

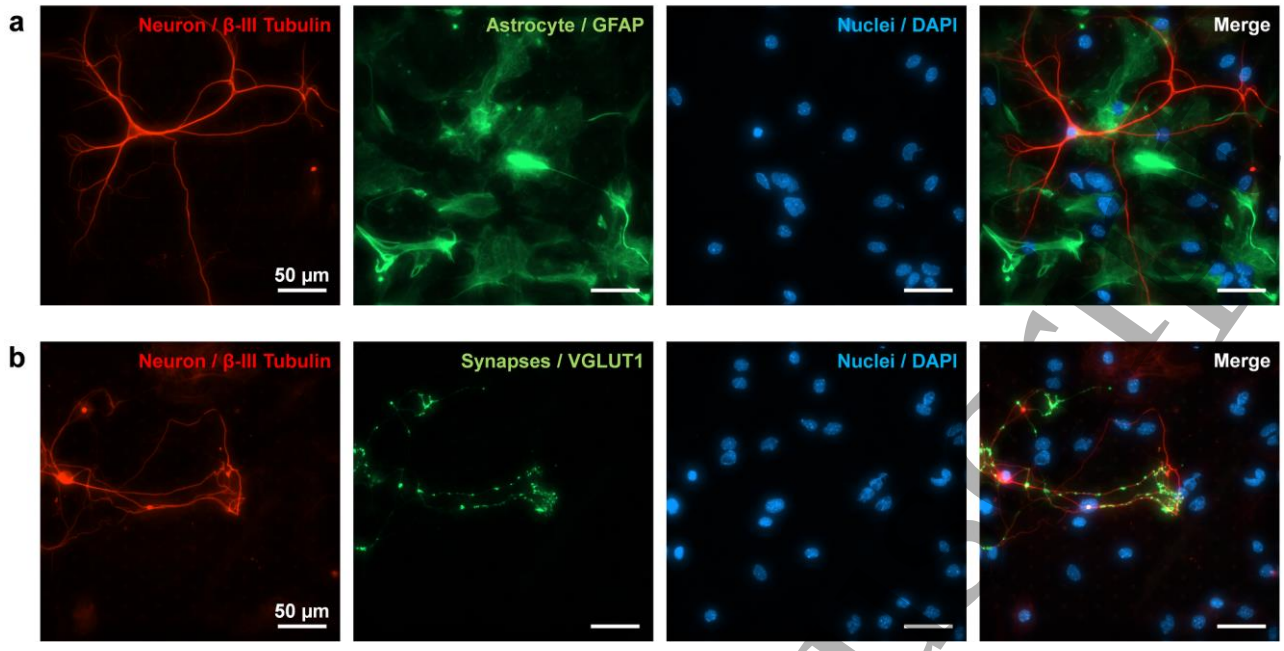

Figure 4. Fluorescence images of co-cultured astrocytes and single neurons. (a) Fluorescence images of neurons and astrocytes on day 7 of co-culture (red: single neuron, green: astrocytes, blue: nuclei of a single neuron and astrocytes). (b) Fluorescence images of neurons on day 14 of co-culture (red: neurites, green: VGLUT1 (synapses), blue: nuclei of a single neuron and astrocytes). The existence of astrocytes was confirmed by nuclear staining with DAPI. The extent of each image in (a) and (b) corresponds to the entire bottom surface of the microwell.

hole diameter of 13 μm exceeded 20% (approximately 50 wells) for all seeding conditions from low to high density. These results indicate that the seeding hole diameter of 13 μm can be used to prepare single-neuron samples without being affected by differences in seeding density.

Neurons are very delicate cells, and their cell membranes are easily damaged. For this reason, neurons should be gently manipulated by minimizing the number of pipetting operations to break cellular clusters and disperse cells. This makes it difficult to break all cellular clusters and completely disperse cells, resulting in a non-uniform density of cells in a suspension. Therefore, when a cell suspension is sampled from a tube, the actual number of neurons is likely to be different from the desired number of neurons. In fact, as shown in the photographs of figure 3(d–f), clusters of neurons were frequently observed to adhere to the wells when the seeding hole diameter was 25 μm or larger. In particular, multiple clusters were observed in the wells when the seeding hole diameter was 49 μm. Considering these various error factors due to cell manipulation, the hole diameter of 13 μm is most appropriate for reliably seeding single neurons.

3.2 Co-culture of astrocytes and single neurons

Single neurons seeded through the holes of the upper layer were co-cultured with astrocytes back-to-back on the SiN membrane of the lower layer. On the 7th day after the beginning of co-culturing, neurons and astrocytes were fluorescently stained to confirm their morphologies. As shown in figure 4(a), a single neuron (red) and astrocytes (green) were observed to adhere to both sides of the membrane. We also observed outgrowth of dendrites and axons from single neurons and confirmed that axons did not pass through the microholes to the back side of the membrane. The reason for this is inferred as follows. Prior to seeding neurons, astrocytes were cultured on the membrane and grown until confluent. In the process, astrocytes flattened and spread across the surface of the membrane, covering the membrane so that there was almost no space between neighboring astrocytes. Thus, it is likely that almost all microholes were sealed with astrocytes when neurons were seeded. Therefore, there is very little chance that axons will pass through microholes and connect with neighboring neurons. Even if axons pass through microholes, whether axons have traversed between wells can be confirmed by fluorescence observation of the back side of the membrane, so it is possible to remove such neurons from analysis.

On the 14th day after the beginning of co-culturing, β-III tubulin in neurons and VGLUT1 localized at synapses were fluorescently stained to confirm that neurons maintained their activity. As shown in figure 4(b), a single neuron elongated its neurites (red), and a large number of light spots indicating VGLUT1 (green) were observed along the neurites, confirming that many autapses were formed. Also, the staining of nuclei (blue) of neurons and astrocytes with DAPI

indicates that the adhesion of astrocytes to the membrane was maintained. These results suggest that good cell-to-cell communication between astrocytes and single neurons was achieved through microholes of the membrane.

As mentioned above, when the yield of single neuron samples was evaluated, i.e., immediately after neuron seeding, single neuron samples were obtained in an average of 60 wells out of 256 wells. However, the number of single neurons observed at the bottom of the wells decreased to 25 on day 7 and then to 10 on day 14. This means that the survival rate at day 14 was as low as 16.6%. This result is mainly attributed to two causes. The first was because single neurons climbed the oblique sidewalls of the wells and could no longer be observed at the bottom of the wells. In the fluorescence observation performed on day 7, such migration of single neurons was observed in many wells. The second cause is that although the cell bodies of single neurons remained at the bottom of the wells, their axons elongated over the sidewalls of the wells and connected with neurons in neighboring wells, which means that such samples can no longer be considered as single neurons. This problem was mainly confirmed in the fluorescence observation on day 14. Both of these problems can be solved by making the well sidewalls non-cell-adhesive surfaces. In the future, a surface modification method for this purpose will be discussed.

4. Conclusion

To prepare multiple single-neuron samples, we developed a novel 16 × 16 microwell-array device which consists of an upper layer for seeding single neurons and a lower layer for co-culturing single neurons and multiple astrocytes. When neurons are seeded into the microwells of the upper layer, a single neuron passes through a microhole opened in a SiN membrane of each well bottom. Then, the single neuron drops onto a microporous SiN membrane in the corresponding well of the lower layer, and will be co-cultured with multiple astrocytes which were preliminarily cultured on the back surface of the membrane.

By counting the number of neurons which adhered to the well bottoms of the lower layer, we evaluated yields of single-neuron samples for 4 different seeding hole diameters and 3 different seeding densities. The results showed that when the seeding hole diameter was 13 μm, the yields exceeded 20% at all seeding densities. Considering the large variation in actual seeding density, it was found that the seeding through microholes of 13 μm in diameter can reproducibly produce multiple single-neuron samples. Next, single neurons were co-cultured with astrocytes back-to-back on the SiN membrane, and the single neurons were found to develop neurites and express synapses. This indicates that cell-to-cell interactions through the microholes contribute to maintaining the activity of single neurons. All these results demonstrate that the two-layered microwell-array device is useful for preparing multiple single-neuron samples.

In addition, our microwell-array device is applicable not only to optical functional analysis, such as calcium imaging, but also to electrophysiological analysis using the patch clamp technique which is often used in the microisland culture method. Note that in patch clamping, the angle of a glass pipette should be carefully adjusted because the sidewall of a well may get in the way of approaching the pipette tip to a target neuron at the bottom of the well.

A major advantage of our device is that single-neuron samples can be prepared by simple pipetting used in normal culture operation, without a pump and tubing connection as required in conventional microfluidic devices. The operation of connecting tubing is cumbersome, creating problems such as liquid leakage and air bubbles, and increasing the amount of reagent. In addition, pressure-driven pumping is likely to cause serious damage to delicate neurons. Our device can prevent these problems, which means that it has high practical utility.

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Data availability statement

All data that support the findings of this study are included within the article.

Conflict of interest

The authors report no conflicts of interest.

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