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Implantable neural spheroid networks utilizing a concave microwell array

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Thesis

**IMPLANTABLE NEURAL SPHEROID NETWORKS UTILIZING A CONCAVE
MICROWELL ARRAY**

by

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IMPLANTABLE NEURAL SPHEROID NETWORKS UTILIZING A CONCAVE MICROWELL ARRAY

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ABSTRACT

The goal of this study was to create pre-formed neural spheroid networks (NSN) on a polydimethyl siloxane (PDMS) concave microwell array for eventual implantation into the rat brain. Recent studies have shown that stem cells have great potential in treating various neurological insults of the central nervous system, ranging from traumatic brain and spinal cord injury, to neurodegenerative disorders. However, the use of stem cell lines in research are controversial due to the method of obtaining cells, in their formation of teratomas and degeneration into cancer cells, their non-specific differentiation, and lastly in their inability to control the location of neural connections. A novel approach to address this issue utilizes pre-formed neural networks consisting of neural spheroids on polymer scaffolds for the implantation into the rat brain. Yet, it was observed that the cylindrical shape of the wells hindered the transfer process. This study aimed to overcome the lack of neural spheroid network detachment by utilizing concave well structures, using a simple method developed in this laboratory.

Primary neurons were isolated from pregnant Sprague Dawley rats at 16 ~ 17 days of gestation. Isolated neurons were cultured in PDMS wells with a concave structure and interconnected by rounded micro channels. It was reported previously that a concave structure enabled an easier and more efficient formation of spheroids, not to mention the ease in extraction of spheroid cells. Various studies have demonstrated the effectiveness of guidance channels in promoting neurite growth. Therefore, micro channels were integrated in the micro array design, and served as a guidance conduit to enhance neurite growth, and by association, spheroid interconnection.

The primary neurons formed a spheroid structure after 3 days, upon which they began to sprout new neurites. By day 8, neurite connections peaked. Spheroid diameter underwent an initial decrease then stabilized on day 2. Various well diameters (300~700 um) and channel lengths (1.5 x diameter ~ 3 x diameter) were evaluated, with a 300 um well diameter and 450 um center-to-center channel length found to be optimal. The completed network was assessed for interconnection using calcium imaging and showed coordinated calcium signals between the neural spheroids. The network was then successfully transferred to a collagen matrigel and cultured for a week. The methodology showed an improvement in the transfer of networks, with about a 90% extraction rate. The viability of the NSN on the matrigel was assessed using a Live/Dead assay, and cells were found to have greater than 95% viability. The optimal hydrophilicity was determined for neurite extension and transfer of NSNs onto the matrigel. It was found that an incubation time between 4~6 hours was optimal.

Future studies will involve the implantation of the NSN into the rat brain. Additionally, the use of neural progenitor and stem cell lines may provide an autologous source of cells which are immunocompatible with the host. In particular, marrow stromal cells are interesting in that they may also address the ethical concerns. A long term goal is to refine the methodology and apply this research to enable studies in the treatment of patients suffering from spinal cord injury and other neurodegenerative disorders.

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ABBREVIATIONS

BSS	basal salt solution
CNS	central nervous system
DAPI	4,6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
ESC	embryonic stem cell
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
HD	Huntington's disease
hESC	human embryonic stem cell
IgG	immunoglobulin G
iPSC	induced pluripotent stem cell
MSC	bone marrow stromal cells
NIH	National Institute of Health
NSN	neural spheroid network
PBS	phosphate-buffered saline
PD	Parkinson's disease
PDMS	polydimethylsiloxane
PEI	polyethylenimine
PNIPAM	poly (n-isopropylacrylamide)
PNS	peripheral nervous system

SCI	spinal cord injury
SE	standard error
TBI	traumatic brain injury
WHO	World Health Organization

INTRODUCTION

Neural tissue engineering is an approach utilized to recover lost sections of the nervous system. Although there have been successful cases of tissue engineering approaches, such as in skin grafts, damage to the nervous system provides an intriguing challenge to biomedical researchers due to its complexity thereby presenting an ongoing challenge to successfully implement treatments for neuronal disorders. Current treatment of neuronal disorders consists predominately of management of symptoms, necessitating the need for alternative treatment methods [1]. Stem cell therapy is gaining widespread attention due to the pluripotent nature of the cells and its capability in replenishing deficient cell populations of the brain [2-4]. However, stem cell therapy is subject to several obstacles, most notably the controversy in obtaining cell samples, non-specific differentiation and formation of teratomas, and cell graft survival [5-7]. Implantable pre-formed neural networks grown in physiologically compatible scaffolds may offer a possible alternative to stem cell treatment of nervous system injury [8]. This study will begin with a brief overview of neural tissue engineering and related studies. Current causes of known nervous system insult will be inspected as well as relevant therapeutic research in the area, particularly of tissue engineering strategies. Finally, we will introduce a novel methodology to culture primary rat cortical neurons in concave microwells for prospective applications in the treatment of nervous system injury.

Tissue engineering is defined as an interdisciplinary field that combines aspects of life sciences and engineering, and applies this approach to develop strategies that

restore, maintain, or replace function of tissues or organs [9]. Neural tissue engineering is a specialized field of tissue engineering focusing on restoring function to the nervous system through grafts or implantable scaffolds to repair diseased or damaged neural tissue or cells of the central nervous system (CNS) and peripheral nervous system (PNS) [10]. For the PNS, the autologous nerve graft is considered the gold standard of treatment [11]. In the CNS however, due to the different physiological and pathological responses to neural insult, a different method of treatment is required. It is known that due to the lack of Schwann cells in the CNS, neurite damage is replaced by fibrous scar tissue mediated by glial cells [12]. Neural insult to the CNS is varied in the causes and symptoms, and can be a consequence of either traumatic brain injury (TBI), neurodegenerative disorders, or spinal cord injury (SCI). In the context of this experiment, neural insult will be limited to those that can be addressed by neural tissue engineering.

TBI is considered one of the leading causes of disability and morbidity worldwide among young adults, with an increasing number of patients suffering from TBIs due to motor vehicle accidents in low and middle income countries [13]. The aging population and increased use of firearms and explosive devices are also contributing to a higher rate of TBI. The annual incidence of mild TBI is estimated to be 10 million worldwide, depending on severity and classification schemes [14]. In the United States, it is estimated that 235,000 individuals are hospitalized for non-fatal TBI annually [15]. TBI is defined as an external injury sustained by external physical forces due to rapid acceleration/deceleration, impact, blast waves, or penetration by a projectile. Primary brain damage usually results in shearing of white-matter tracts, focal contusions,

haematomas, and microscopic damages ranging from microporation of membranes to leaky ion channels. Onset of secondary damage may develop over several hours after the injury to days, and include inflammatory responses and calcium-mediated damage [13]. Current treatment revolves around stabilizing patients, preventing further injury, and rehabilitation. However, TBI leads to long-term disability, and it is estimated that there are 3.3 million disabled patients in the US alone [16].

Parkinson's disease (PD) is a common neurologic disorder, and affects approximately 1% of individuals over 60 years [17]. PD is characterized by hallmark neuronal cell loss of dopaminergic neurons within the substantia nigra. The symptoms of PD include rigidity, tremor, and loss of postural reflexes. The exact causes are unknown, however, it is believed that the presence of Lewy bodies contribute to apoptosis of dopaminergic neurons [18]. Others have attributed PD to viral infections or environmental factors, with a small minority of cases attributed to a genetic defect [19,20]. Administration of L-Dopa is a common treatment, but is prone to numerous side effects and dopamine dysregulation syndrome in the long run [21].

Huntington's disease (HD) is another well-studied neurological disorder, and is characterized by polyglutamate expansions in the Huntingtin protein. HD is considered a heritable disease as it is caused by an autosomal dominant mutation in the Huntingtin gene. HD initially affects the GABA neurons of the basal ganglia, and exhibits symptoms of progressive physiological, motor, cognitive, and psychological deterioration [22]. The World Health Organization (WHO) estimates that 5~7 people per 100,000 in Western

countries are affected by HD [23]. There is currently no treatment, and patients eventually become completely incapacitated.

Spinal cord injury (SCI) is believed to affect over 250,000 people in the US. [24]. SCI is caused by physical trauma to the spinal cord and presents with various symptoms. SCI usually results in paralysis and downstream loss of function in specific areas, depending on which section of the spinal cord was injured. As the CNS environment prohibits regeneration of damaged neural connections, treatment options are limited, and patients usually require intense rehabilitation and experience life-long functional impairment.

The above neurological insults, and lack of current treatment options, demonstrate the need for further therapeutic research. Cell transplantation studies, especially of stem cell lines, have shown great promise in addressing these disorders. Stem cells are pluripotent, and have the capability to differentiate into any type of cell, including those of the nervous system. Embryonic stem cells (ESC) have been explored as a possible source for treating PD. Human embryonic stem cells (hESC) were differentiated into dopamine-producing midbrain neurons *in vitro* using bone marrow and aorta-gonad-mesonephros-derived stromal feeder cells [25]. Keirstead et al. demonstrated remyelination and restoration of locomotion from hESC-derived oligodendrocyte progenitor cells in a spinal cord injury model in rats [26]. Induced pluripotent stem cells (iPSC) offer an interesting alternative to ESCs. iPSCs are obtained from adult skin cells by exposing them to specific factors and restoring pluripotency [27]. Hu et al. showed

that human iPSCs followed a similar differentiation route as hESCs, but displayed poor efficiency and increased variability [28]. Research on both cell types has yet to be translated into successful clinical trials that prove their efficacy. Additionally, these cells are prone to forming teratomas and have issues with immunoincompatibility, not to mention the ethical concerns regarding the origin of hESCs [5-7, 29].

Adult stem cells have been gaining increased consideration as an autologous source of multipotent cells that address many of the ethical issues facing the use of ESCs. These cells show similarity to ESCs, but also have the advantage of ease of isolation, self-renewal, and multipotent properties [1]. Table 1 summarizes studies conducted on adult stem cells. In particular, bone marrow stromal cells (MSC) show great promise, and have been studied extensively. MSCs can be found in the stroma of the bone marrow and are a heterogeneous cell population which includes stem and progenitor cells [1]. The strongly adherent property of MSCs allows for easy isolation [30]. MSCs have been studied in the treatment of TBI. Implantation of human MSCs in rats led to improved functional benefits; however, only about 10% of MSCs transdifferentiated into neuronal cells [31]. Therefore, it was assumed that growth factors released by the MSCs contributed to functional recovery by enhancing glial, neuronal, and vascular remodeling [32]. *In vitro* studies have shown the possibility of MSCs to differentiate toward dopaminergic phenotypes [33, 34]. Yet, it is likely that MSCs confer a neuroprotective effect through release of neurotrophic factors [35]. Although MSCs have great potential, they are subject to poor cell survival and their neuroregenerative properties have yet to be fully elucidated.

Table 1. Adult Stem Cell Therapies for Various Neural Degenerative Disorders

Disease Model	Cell Type	Cell Origin	Functional Benefits	Reference
HD	MSC	Rat Mouse Mouse Rat	Migration of MSCs to striatum Decrease striatum atrophy Reduction behavioral deficits Neuroprotective Effect	Sadan, Shemesh et al. 2008 Amin, Reza et al. 2008 Dey et al. 2010 Moraes et al. 2012
	Adult NSC	Rat Rat	Formation functional GABAergic and glutamatergic synapses Preservation striatal tissue and behavioral function	Toda et al. 2000 Roberts et al. 2006
PD	MSC	Rat Rat Mouse	Improved dopamine production Preservation dopaminergic neurons Improvement basic motor behavior	Danielyan et al. 2011 Glavaski-Joksimovic et al. 2010 Kang et al. 2011
	iPSC	Rat Rat Human	Integration and survival of cells Reduction motor asymmetry Successful differentiation into dopaminergic neurons and improved behavioral deficit in rats	Cai et al. 2010 Hargus et al. 2010 Swistowski, Zeng et al. 2010
	Adult NSC	Mouse	Reduction rotational deficit	Ziavra et al. 2012

The application of tissue engineering strategies including the use of biocompatible scaffolds offers the possibility of enhancing stem cell survival. Scaffolds in a tissue engineering context must be biodegradable and biocompatible, and must minimize immune response while conferring longevity to implanted cells [36]. Porosity is also an important factor as nutrients must be able to pass through. Biologically compatible

scaffolds have been shown to increase the viability of implanted stem cell grafts. In a TBI mouse model, MSCs seeded on a collagen gel-based scaffold, and injected in the lesion, led to a decrease in the lesion volume and improved spatial learning and sensorimotor function [37]. A biomimetic scaffold integrating laminin, and seeded with mouse ESCs resulted in improved migratory potential and process extension in a PD model [38]. Recent developments have led to a more advanced biomimetic scaffold that increased the *in vitro* viability of human MSCs in a polyethylene glycol gel [39]. In short, the combination of cell transplant therapy and tissue engineering approaches, utilizing biologically compatible scaffolds, have become the paradigm in neural tissue engineering.

The treatment of neural insult, whether from disease or physical injury, has seen vast improvements in the past decade. Ongoing research has used induced pluripotent stem cells (IPS), embryonic stem cells (ESC), and marrow stromal cells (MSC), which have provided a ground breaking way of addressing neural pathologies such as Parkinson's disease and Huntington's disease [40-43]. Stem cell research has shown great promise in treating these neurological disorders. However, they are limited in their ability to selectively create neural networks, as the neurites are prone to randomly creating undesirable neural connections [8]. Although fetal nerve grafts have been employed with some degree of success, the grafts are unable to cover long distances, not to mention the ethical concerns regarding the origin of the grafts [44-46]. It is also not clear at this point whether the stem cells form new synapses or that they stimulate the production of trophic factors and glial cells such as astrocytes or oligodendrocytes, which aid in the rejuvenation of pre-existing neural networks [47]. Kato-Negishi et al. have proposed a

novel approach in addressing this issue by creating pre-formed neural networks consisting of neurospheroids on polymer scaffolds for the implantation into the brain [8]. However, it was observed that there was a lack of neural network transfer due to the use of cylindrical wells (Figure 1). It is believed that an angled structure increases adherence of cells to the edges of the wells, and thus diminishes the capacity of neural network transfer.

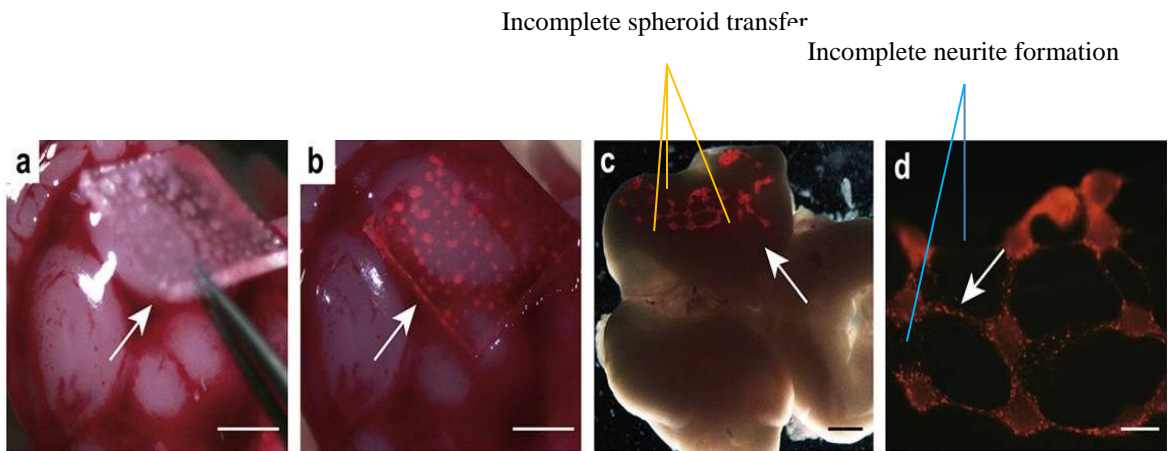


Figure 1. Lack of neural spheroid transfer and neurite formation in cylindrical wells.
(Figure amended from Kato-Negishi et al., 2010)

Previous experiments have shown that spheroids are easily dislodged from concave wells with the slightest of physical forces (Jeong; unpublished data). Also, several studies have shown that guidance channels enhance the directional sprouting of neurites [48-50]. We propose a novel method of improving transfer efficiency and neurite interconnection by using concave wells interconnected by rounded guidance channels that act as conduits for the purpose of forming neural networks. The results may lead to an improvement in the

transfer efficiency of neural networks, and also more complete neurite formation due to improved physical cues from the microchannels. This research may be applicable in transplanting neural networks into the injured brain, and further the spinal cord. Future studies will involve growing and differentiating stem cell lines in the array for eventual transplantation. Use of MSCs may provide an autologous supply of cells, while being considerably easier to isolate and addressing any ethical concerns.

SPECIFIC AIMS

This study aims to address the problem of lack of detachment of neural spheroid networks by utilizing concave microwells as opposed to cylindrical wells, using a simple technique developed by Jeong et al [51]. Neural spheroids grown in cylindrical wells have a semi-spherical shape due to the adherence of the spheroid to the well bottom. The semi-spherical shape may compromise the detachment of the NSNs due to this adherence. Previous research has shown that spheroids cultured in concave wells maintained a full spherical shape (Figure 2), and were easily dislodged, even with the slightest of physical stimuli. Research on neurite extension has shown the viability of using microchannels as physical guidance cues that enhance directional neurite formation. We hypothesize that the use of concave microwells will lead to fewer adherences of spheroids, and improve the transfer of NSNs. Additionally, the integration of rounded microchannels, which serve as a conduit for guiding neurites between the spheroids, can possibly enhance neurite interconnection. We propose that this method will improve the formation of

neurites as well as transfer of NSNs for the purpose of transplantation into the brain. Further research may allow for the culture of stem cell lines, and potential applications in cell replacement therapy for the brain and spinal cord.

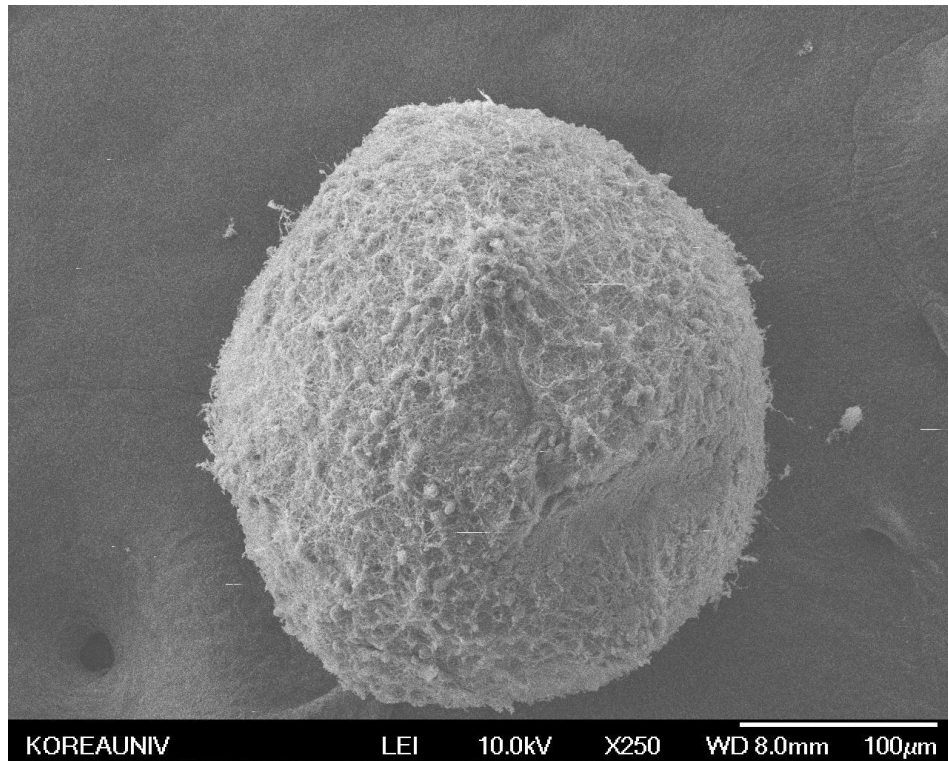


Figure 2. **SEM image of a neural spheroid** (Yoon-Jung Choi, 2011; Used with permission).

METHODS

Design of Microwell Array

A microwell array was fabricated using conventional soft lithography techniques. Briefly, a microwell array with well diameters between 300~700 μm in 200 μm increments was designed using AutoCad (Autodesk, San Rafael, California). Channel lengths had a center-to-center length of 1.5 x diameter (d), $2d$, $2.5d$, and $3d$. A photoresist film (Nepco, Seoul, S. Korea) was printed for use in the fabrication of the array. SU-8 was spin-coated onto a silicon wafer, then soft and hard baked according to manufacturer specifications. The photoresist film was aligned with the wafer and exposed to UV light at 350 nm using a MDA-400 aligner (Midas System, Daejon, S. Korea). The SU-8 underwent post-exposure baking at 65 °C for 1 minute, and 95 °C based on SU-8 thickness. The SU-8 was developed to fabricate a master mold on a silicon wafer.

Fabrication of PDMS Concave Microwells

Polydimethyl siloxane (PDMS; Sigma-Aldrich, St. Louis, MO) was mixed at a 10:1 ratio (prepolymer:curing agent) and placed in a dessicator. The PDMS prepolymer was poured onto the SU-8 mold and baked in an incubator at 80 °C for two hours (Figure 3). The dried PDMS was detached from the wafer in methanol solution. The concave structure was produced according to Jeong et al [51]. Briefly, PDMS prepolymer was poured into the wells of the base mold, and placed in a dessicator to eliminate any bubbles. Excess PDMS was raked out using a 1 mL pipette tip, and allowed to dry in the incubator at

80 °C for 2 hours (fig. 3). The surface tension of the PDMS induced a meniscus shape, and formed a concave well as the prepolymer solidified.

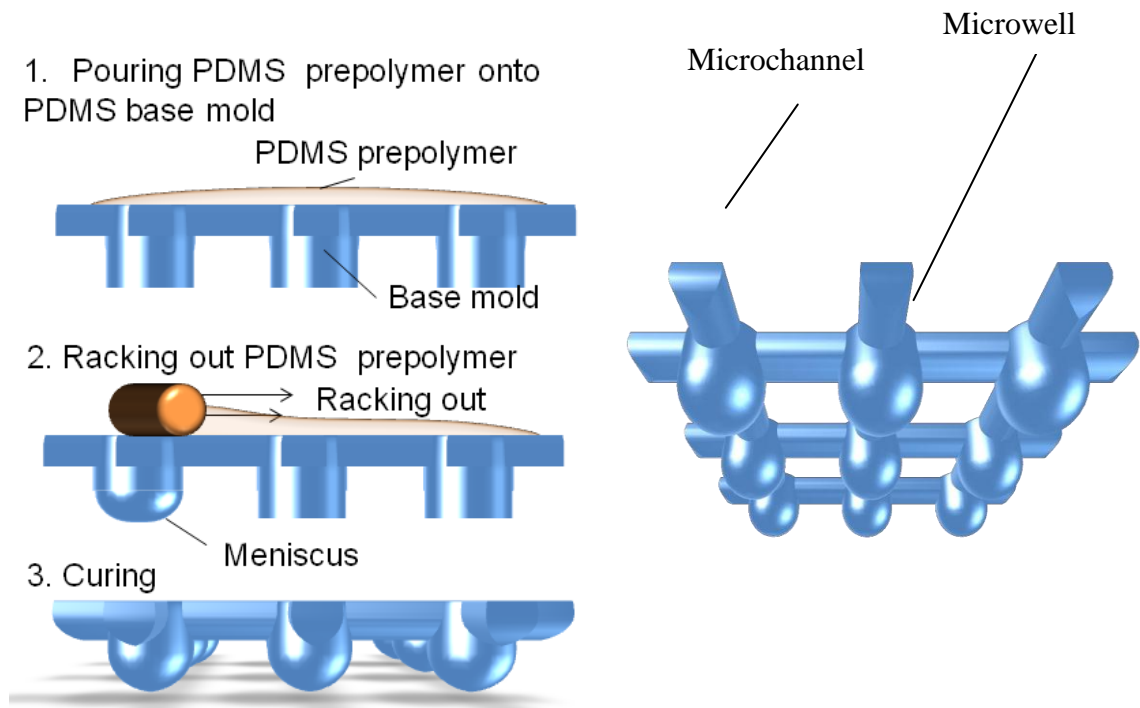


Figure 3. Schematic diagram of concave well fabrication. PDMS prepolymer was poured onto an initial layer of base PDMS cylindrical mold; excess PDMS was raked out using a cylindrical apparatus and formed a meniscus shape due to the surface tension of PDMS.

Scanning Electron Microscopy (SEM)

Microwell arrays were mounted onto a specimen stub with double-sided graphite tape, sputter-coated with palladium alloy, and observed under a scanning electron microscope (JEOL Ltd, Tokyo, Japan) (Figure 4).

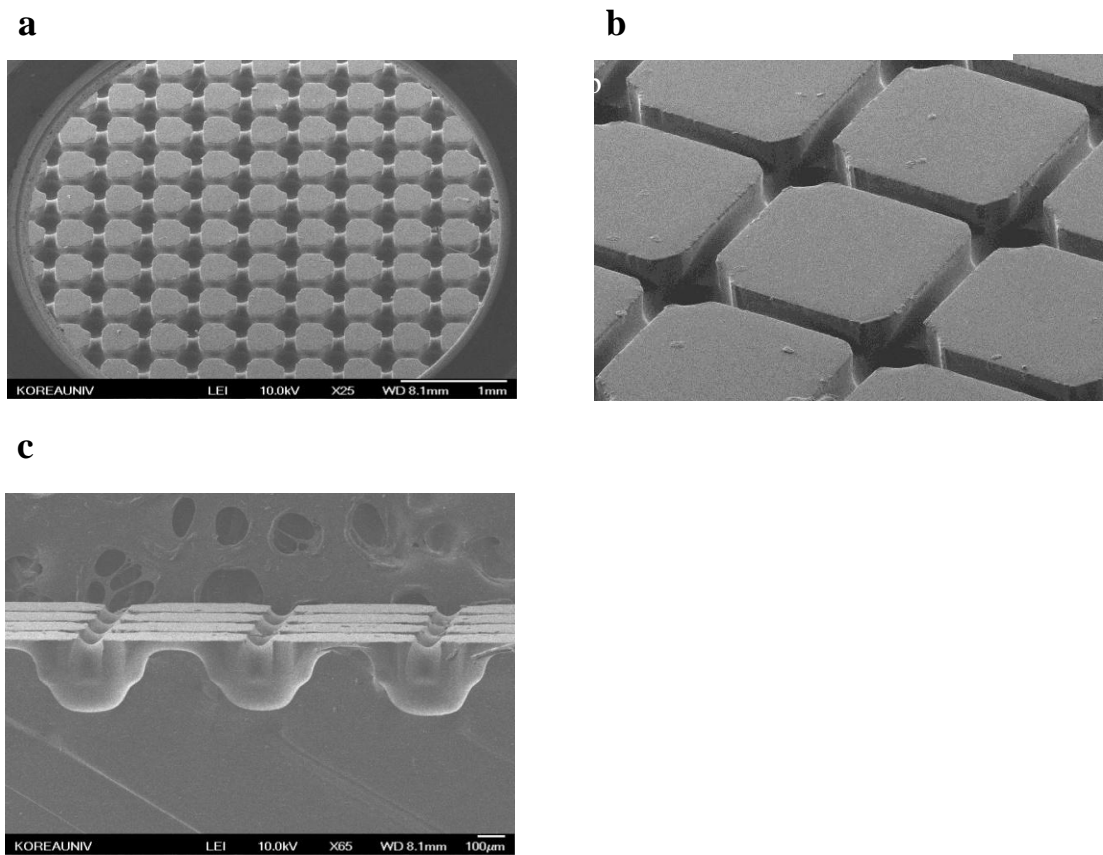


Figure 4. SEM images of PDMS concave microwell array. a) shows a top view of the wells b) close-up of wells at 75x; c) front view of wells showing concave shape

Isolation of Primary Neurons

Primary neurons were prepared as previously described [52]. Briefly, pregnant Sprague-Dawley rats (DBL, Seoul, South Korea) at 16~17 days of gestation were euthanized using CO₂. The cortices of the fetuses were isolated using a pair of microscissors and suspended in cold Hank's buffered saline solution (HBSS). The mixture was trypsinized with TrypSE (Invitrogen) at a ratio of 3:1 (HBSS:Trypsin) for 15 minutes in a water bath

at 37 °C. The mixture was washed twice with HBSS then finally washed with bare neurobasal media (Gibco). The cortices were fragmented using a pipette and passed through a 0.22 um nylon filter (Gibco). Cells were cultured in neural basal media supplemented with B-27 (Gibco), PenStrep (Gibco), and glutamate (Gibco). All procedures adhered to the standards of the Institutional Review Board of Korea University.

Creation of Neural Spheroids

Cell viability was assessed using trypan blue (Invitrogen), and seeded at a density of 5.0×10^6 cells/mL in the concave wells. Cells generally had a viability of 62%. The cells were allowed to settle into the wells in an incubator at 37 °C, 5% CO₂, and 80% humidity for 30 minutes. Excess cells were washed away with bare media (Figure 5). Nerve growth factor (Prospec) was added at a concentration of 1 ng/mL. After the final wash, seeded cells were observed under an inverted phase contrast microscope (EVOS, AMG, USA). Cells were incubated at 37 °C, 5% CO₂, and 80% humidity for 13 days. Media was changed every two days.

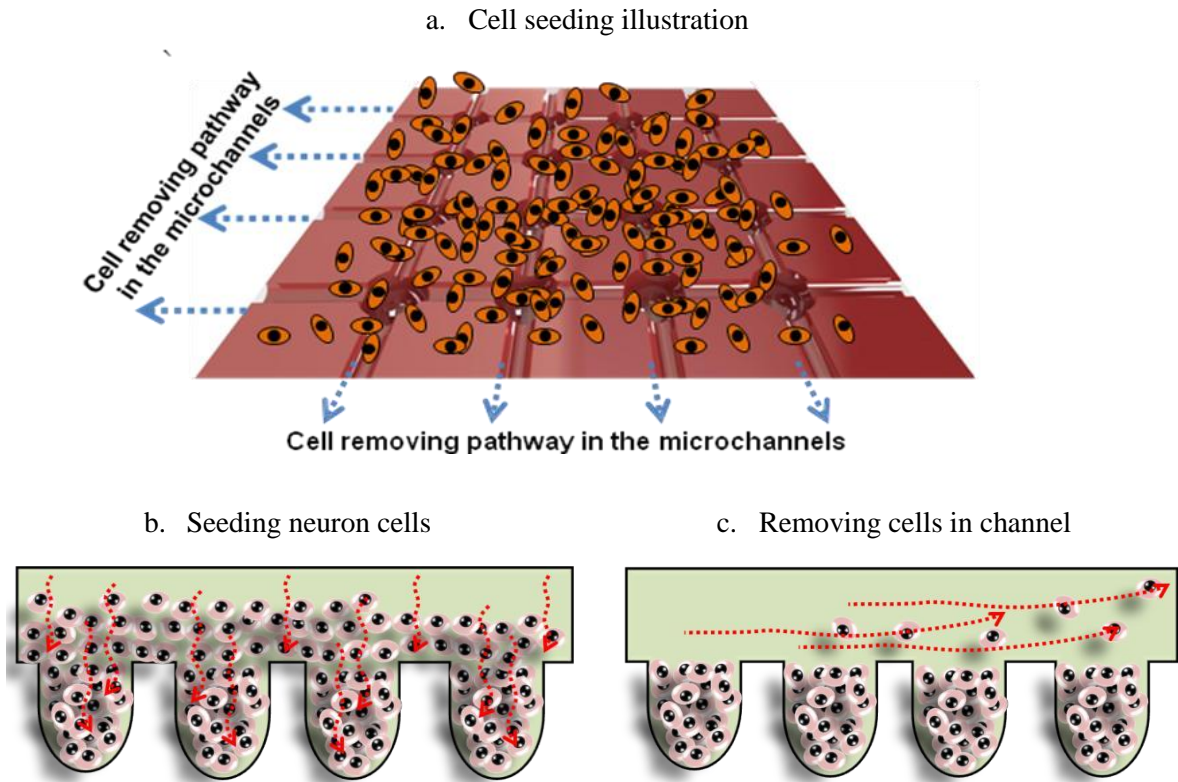


Figure 5. Schematic diagram of cell seeding. (a) Cells were seeded at a density of 5.0×10^6 cells/mL and incubated for 30 mins. (b) and (c) Excess cells that did not settle in the wells were washed out with media; cells within the wells were not washed away.

Matrigel Transfer

Matrigel (Invitrogen) was added to the wells after removal of media. A rounded glass slide was placed as a cover. The wells were placed upright and incubated at 37 °C for 16 hours, while immersed in supplemented media. The slide was carefully removed with a small volume of media and placed in a petri dish and observed under a microscope.

Live/Dead Assay

Cell viability was assessed with the Live/Dead assay. Briefly, cells were incubated with ethidium/calcein (Live/Dead Assay; Invitrogen) in PBS for 30 minutes at 37 °C, 5% CO₂ and 80% humidity. Cells were washed with PBS three times then observed under a fluorescent microscope (Axio Observer.A1. Carl Zeiss, Inc).

Calcium Imaging

Calcium fluxes were measured as previously described [8]. Briefly, 1 mM Fluo-4/AM (Invitrogen) in DMSO was added to cells in a basal salt solution (BSS) consisting of 20 mM HEPES-NaOH (pH 7.4), 1.8 mM CaCl₂, 5.4 mM KCl, 5.5 mM glucose, and 130 mM NaCl. Cells were incubated for 60 mins at 37 °C for one hour. Cells were washed three times with BSS, then observed under an inverted fluorescent microscope (Axio Observer.A1. Carl Zeiss, Inc). Images were captured at 50 ms intervals for 100 frames.

Immunofluorescent Imaging

Cells were fixed with 4% paraformaldehyde in 0.1% Triton X-100 in PBS solution. Cells were rinsed with 0.1% FBS in PBS solution three times, then incubated overnight at 4 °C with primary rabbit polyclonal anti-neurofilament antibodies (Abcam). The cells were rinsed and incubated at 4°C for 90 min with Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated anti-rabbit IgG (Invitrogen) secondary antibodies, as appropriate. The cells were then incubated with DAPI (4,6-diamidino-2-phenylindole), and confocal microscopic images were obtained (Olympus, Japan).

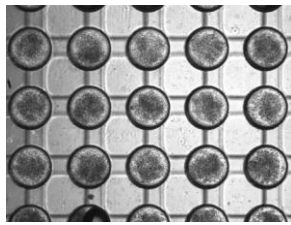
Statistical Analysis

All statistical analyses were performed using SPSS (IBM, New York, USA). Values are indicated as the mean \pm SE (standard error of the mean). A student T-test was used to measure statistical significance of experimental groups, where $p < 0.05$ was considered as statistically significant.

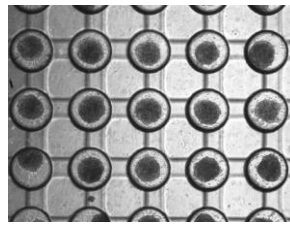
RESULTS

Spheroid Formation and Neurite Interconnection

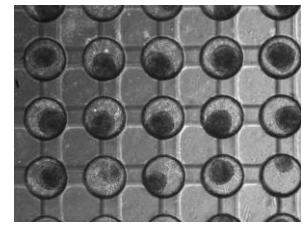
The optimal level of spheroid formation and neurite interconnection was assessed utilizing the concave microwell array. Well diameters were 300 μm , 500 μm , and 700 μm . Channel length was varied between 1.5d to 3d in 0.5 increments, where d is the diameter of the wells, and length was determined by the distance from the center of each well to the next well. Previous research has shown that the concave microwell array is an efficient platform for culturing cell spheroids of uniform size and shape [51]. Characteristics of spheroids, including the size, shape, and viability was assessed over a 13 day period. Figure 6 shows phase contrast images of spheroid formation in the control group (cylindrical wells) with a 300 μm diameter, 450 μm channel length (center-to-center) microwell array over a 13 day period. There was a lack of neurite extension in the cylindrical wells after 13 days. Spheroids grown in concave wells, with definite neurite growth interconnecting the wells are shown in Figure 7. Figure 8 compares the neurite formation in the 300 μm concave wells with varying microchannel length at day 13. Neurites seem to sprout from day 3, and peak at day 8, after which there is little change. It was found that a smaller well diameter and channel length generally led to the more efficient formation of spheroids and interconnection of neurites. Therefore, the 300 μm diameter well with 450 μm channel length was found to possess the most optimal dimensions for culturing neural spheroid networks.



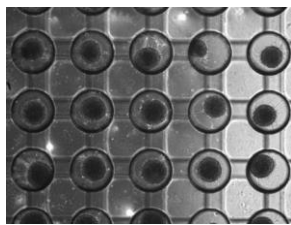
Day 0



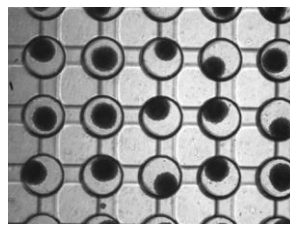
Day 2



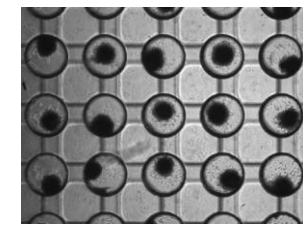
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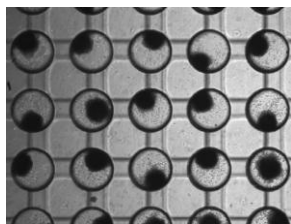
Day 6



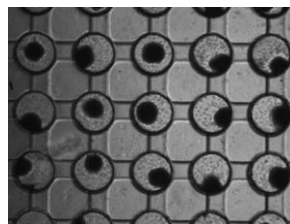
Day 8



Day 10

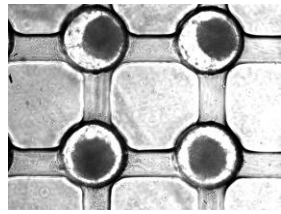


Day 12

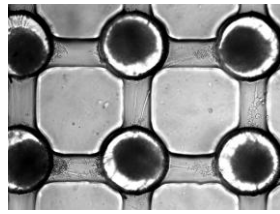


Day 13

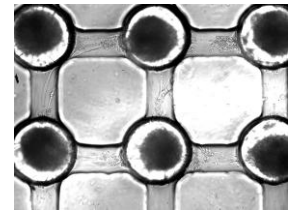
Figure 6. Spheroid formation in 300 μm diameter cylindrical microwell array over 13 days. Microchannel length was 450 μm (center-center).



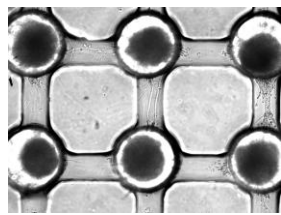
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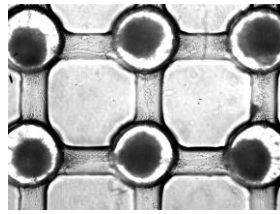
Day 5



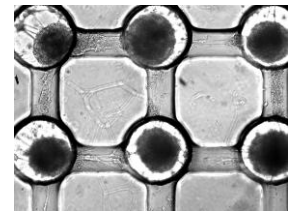
Day 6



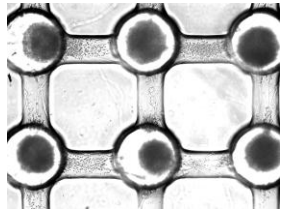
Day 7



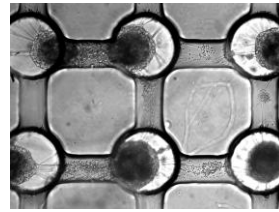
Day 8



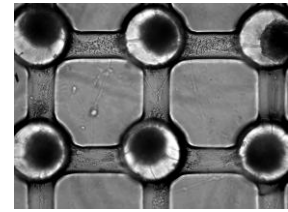
Day 9



Day 11

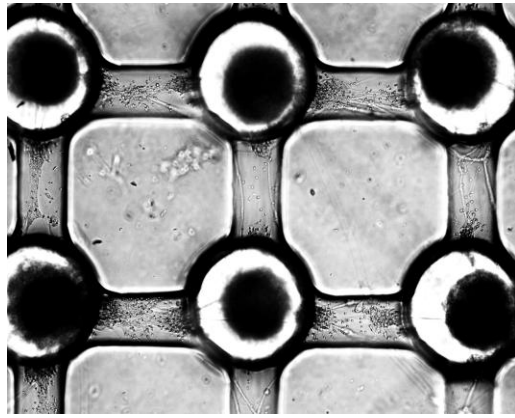


Day 12

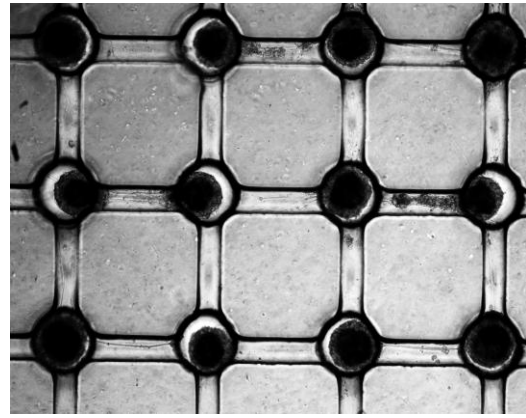


Day 13

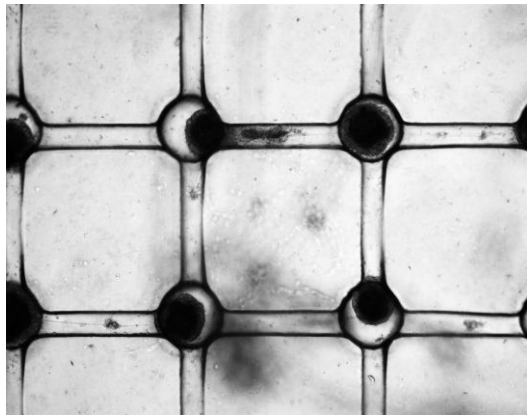
Figure 7. Spheroid formation in 300 μm diameter concave microwell array over 13 days. Microchannel length was 450 μm (center-center).



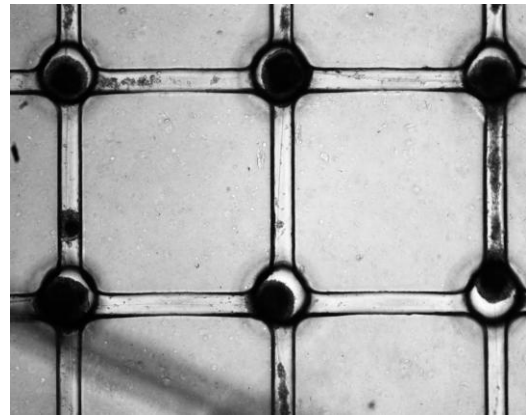
a) 450 um channel



b) 600 um channel



c) 750 um channel



d) 900 um channel

Figure 8. Comparison of neurite formation in 300 um concave microwell arrays with varying channel length at day 13. Channels were a) 450 um, b) 600 um, c) 750 um, and d) 900 um.

Change in Diameter of Spheroid over Time

To assess the change in spheroid formation over a 13 day period, spheroid diameters were measured for the three varying well sizes using ImageJ [53]. Figure 9 depicts the changes in spheroid diameter for 300, 500, and 700 um wells over a 13 day period. The

spheroid diameter in the 300 μm concave wells started off around $256 \pm 4.1 \mu\text{m}$ and stabilized by day 2 with a diameter of $210.2 \pm 3.7 \mu\text{m}$. In contrast, the spheroids in the 500 and 700 μm concave wells stabilized after 6 days, with a decrease from initial values of 384.5 ± 3.7 and $622.2 \pm 0.8 \mu\text{m}$, to 306.1 ± 3.6 and $350.8 \pm 0.8 \mu\text{m}$ respectively.

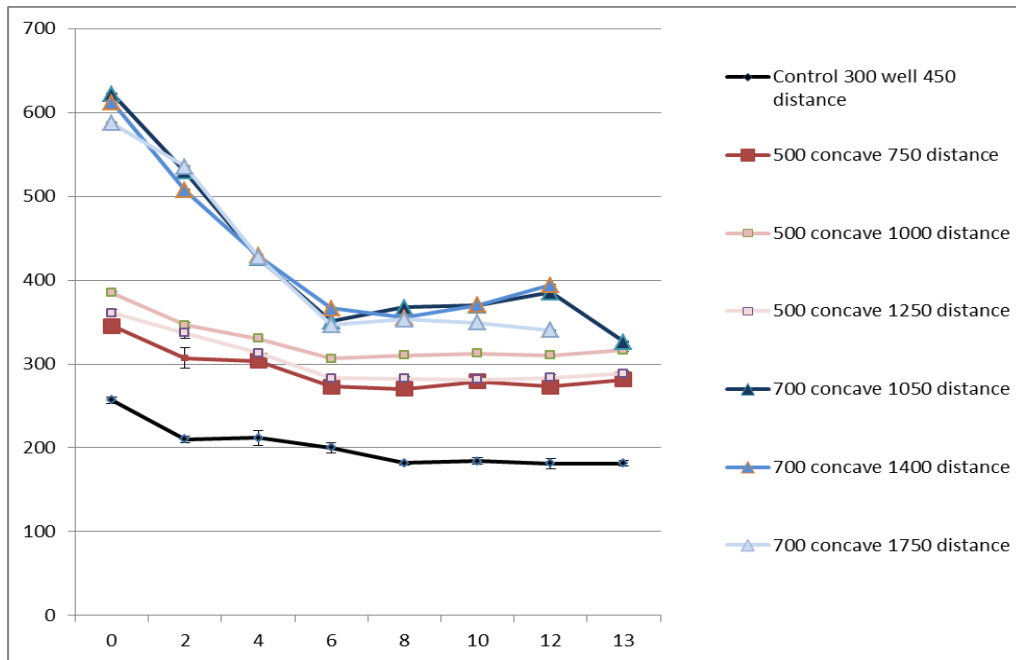


Figure 9. Change in spheroid diameter over 13 days for 3 different well sizes. Bars represent standard error. The distance is the length of the microchannels. N= 3 each for each well size group.

Assessment of Neurite Formation

Neurite formation was characterized by immunofluorescent staining to determine the extent of neurite interconnection. Figure 10 shows two different NSN groups: (b) and (d). Neurites were stained with anti-neural filament (MAP2) and is displayed in green, while blue is a DAPI stain of the nucleus. Neurites formed between the spheroids in the

concave wells. In contrast, there was no neurite interconnection in the cylindrical wells

(c).

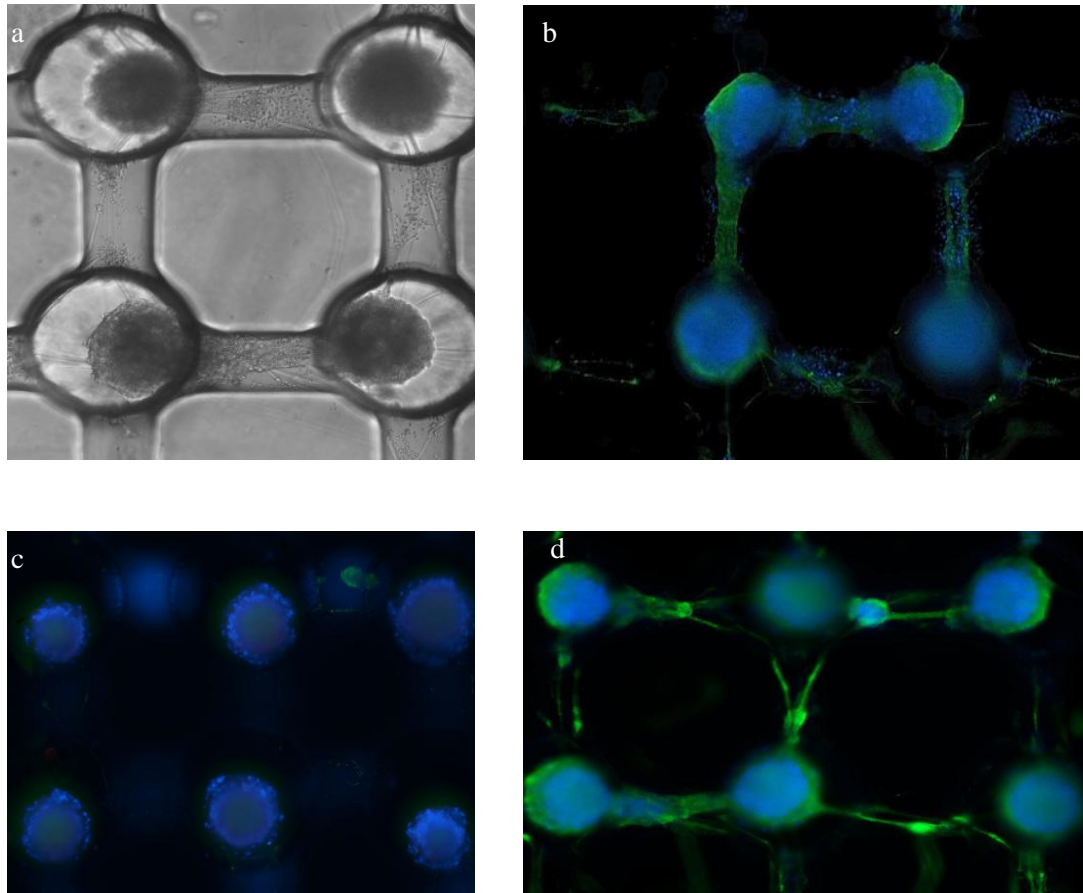


Figure 10. Immunofluorescent Images of NSN. a) phase contrast image of a NSN; b) confocal image of same NSN; c) fluorescent image NSN in cylindrical wells; d) fluorescent image of NSN in concave wells.

Calcium Imaging

In order to determine the degree of interconnection of the neurites in the NSN, we performed a calcium imaging experiment to measure the calcium flux. Figure 11 shows a group of four neural spheroids interconnected by neurites. We used a pseudocolor scheme in Metamorph software (Molecular Devices) to better visualize the fluxes.

Fluorescence intensity of the calcium spikes follow the color-coded bar, where red characterizes the highest intensity and purple lowest. Spheres 1 and 2 showed coordinated calcium fluxes as did spheres 3 and 4 (Figure 11 (a) and (b)). Intensity profiles were plotted on a graph; spheres 1 and 2 showed similar profiles, while spheres 3 and 4 showed coordination, indicating neurite interconnection

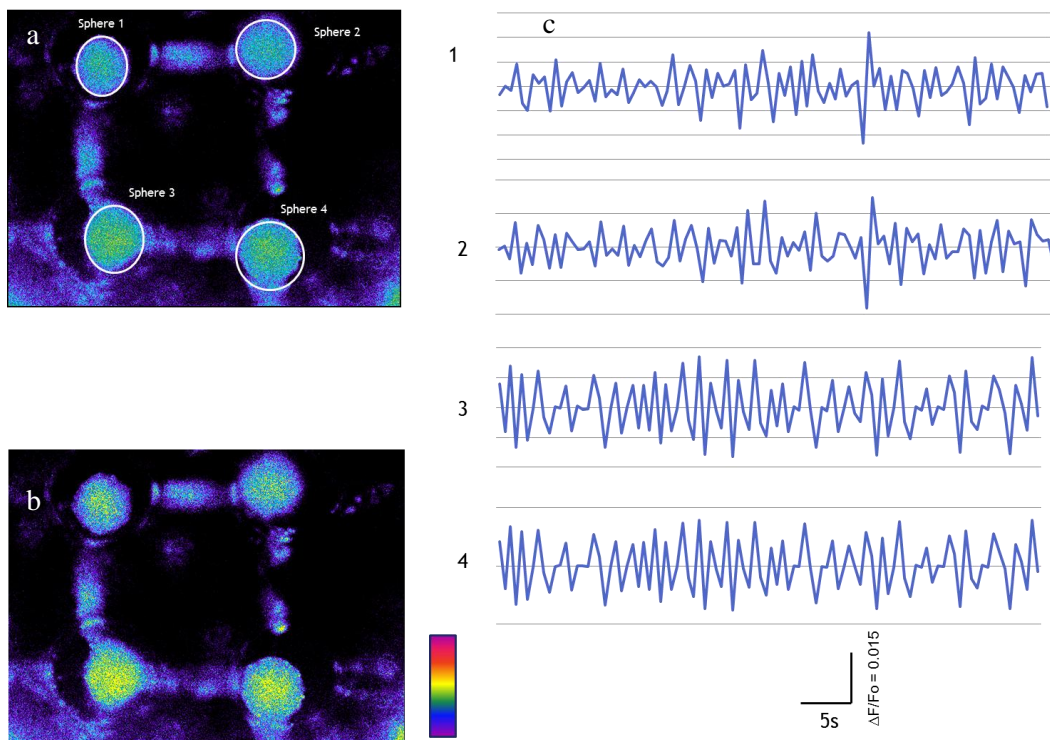


Figure 11. Calcium imaging of a NSN. $\Delta F/F_0 = 0.015$; 5s. a) a pseudocolor scheme of 4 spheroids b) increased fluorescent intensity of spheroids 3 and 4 c) a graph plot of fluorescent intensity of the spheroids. Sphere 1 and 2, 3 and 4 showed coordinated Ca fluxes. $\Delta F/F_0$ is the momentary fluorescent intensity and 5s is the total duration of the recording; image capture time interval was 50 ms for 100 frames. The vertical color bar represents fluorescent intensity.

Matrigel Transfer

In order to determine the ideal conditions for transfer of NSNs onto the matrigel, we varied the incubation time and orientation – up-right or upside-down –, as well as the residual media in the wells, of the NSN in the concave microwells. Figure 12 compares the different conditional settings and the resulting transfer of NSNs onto the matrigel. It was found that NSN transfer onto the matrigel occurred more readily when the wells were oriented up-right with a small amount of media. An incubation time exceeding 16 hours was found to be optimal. Figure13 shows a Live/Dead assay of a successful transfer of a NSN onto the matrigel after 16 hours. The spheroids maintained a high viability after their transfer onto the matrigel (green) and maintained a grid-like configuration. A small degree of necrosis (red) was visible in the middle of the spheroids.

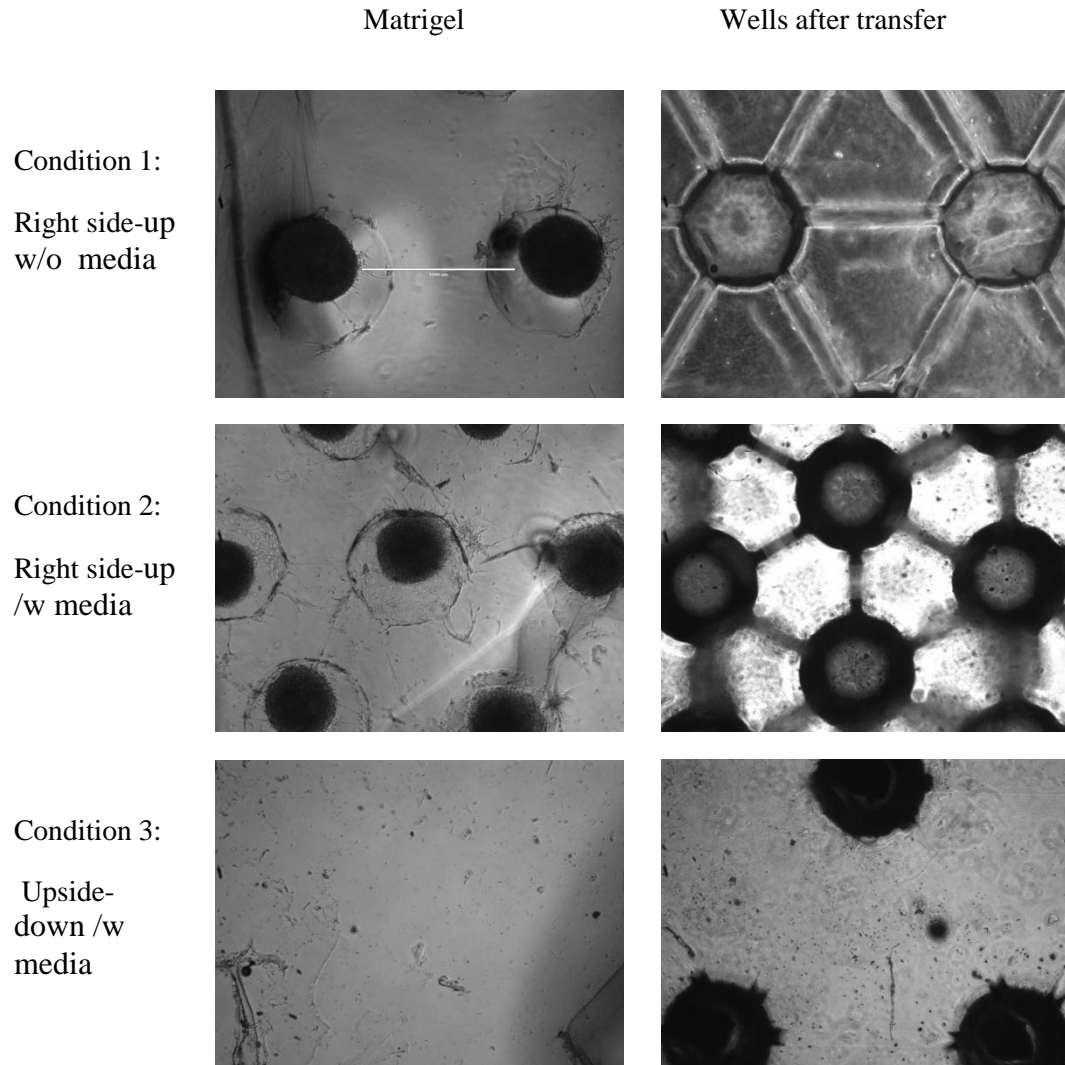
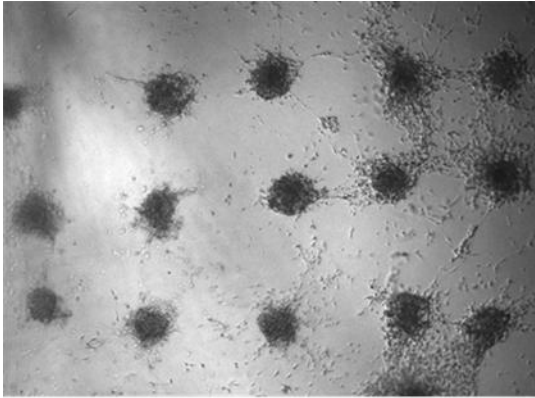
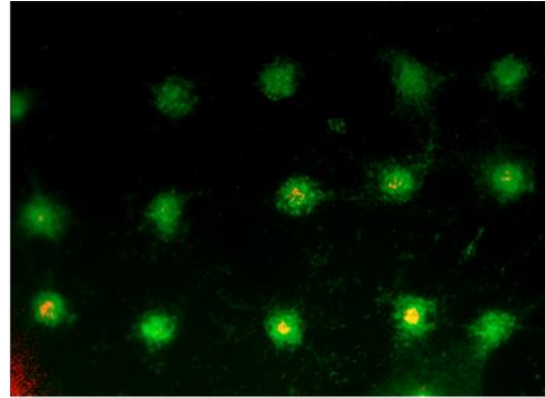


Figure 12. Varying conditional settings of matrigel transfer of NSNs. Right side-up orientation showed the best transfer, while upside-down orientation resulted in lack of transfer. Further, the presence of a small amount of media improved transfer rates.



Phase contrast image of NSN



Fluorescent image of NSN

Figure 13. Live/dead image of successful NSN transfer onto matrigel.
Green represents live cells while red indicates necrosis.

Optimal Hydrophilicity for Neurite Extension and Matrigel Transfer

It is known that cell adherence is a necessity in order for neurites to extend their processes. Rogers et al. achieved cell adherence through the coating of extracellular matrix (ECM) proteins laminin and fibronectin, which have become the common ECM material to enable cell adherence on hydrophobic surfaces [54]. However, doing so would hamper the transfer of the NSN onto matrigel. Since the surface of PDMS is normally hydrophobic, we attempted to control the neurite extension and transfer by applying oxygen plasma onto the surface of the wells to induce a hydrophilic charge. A previous study has elucidated the characteristics of hydrophilic charge on the surface of PDMS [55]. Hydrophilicity was controlled by incubating the wells at 80 °C for different periods of time to hasten hydrophobic recovery. Incubation time varied from 30 minutes to 7 hours, with the control having no exposure to oxygen plasma. Figure 14 shows a comparison of wells at day 0 with varying hydrophilicity. An incubation time of 30

minutes showed the highest degree of cell adherence, with a decreasing amount of cells adhering with higher incubation time. The control showed the best spheroid detachment, while maintaining the original configuration; neurite formation was minimal. Stronger hydrophilicity (3 hours incubation time) showed moderate detachment; however, the spheroid shape was flattened due to strong adherence of cells to the substrate. The 7 hour incubation demonstrated the ideal combination of neurite interconnection and spheroid detachment. Figures 14-16 show the wells and matrigel after transfer for the control, 3 hour incubation, and 7 hour incubation groups. Figure 17 summarizes the three groups in terms of neurite extension, detachment, and maintenance of original shape. One of the wells in the 7 hour group was lost after 8 days due to contamination.

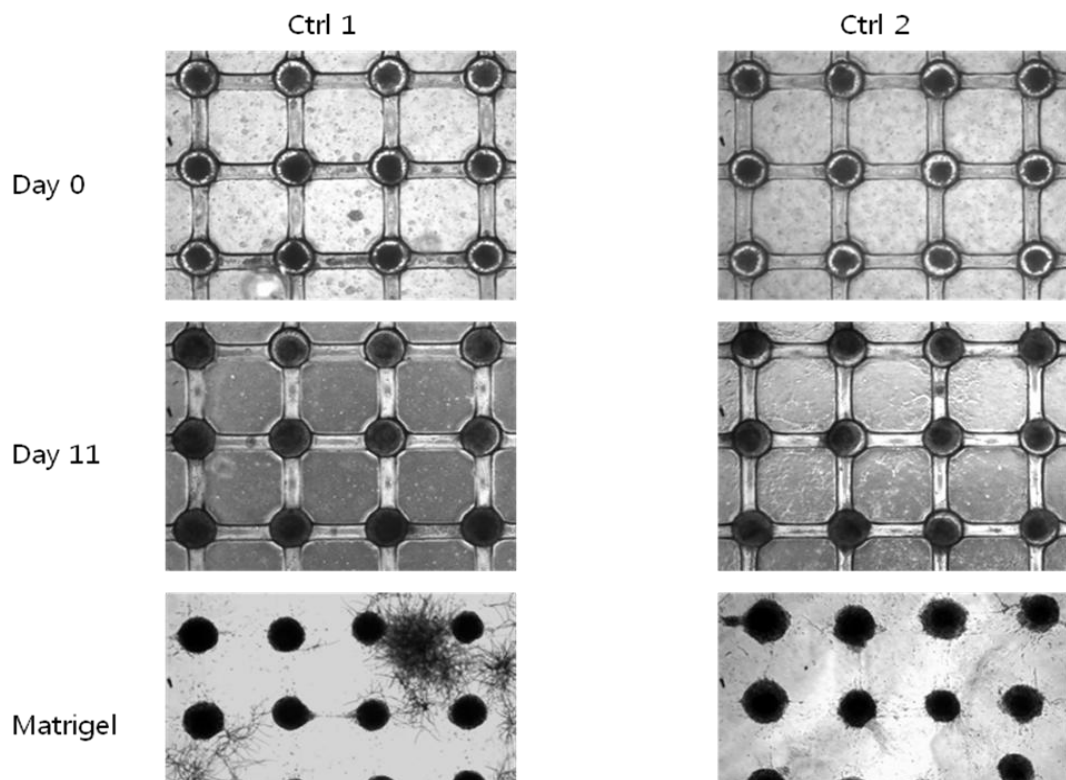


Figure 14. Phase contrast images of 2 wells of the control group. The first row is an image at day 0; 2nd row is at day 11; and the last row is after transfer onto matrigel.

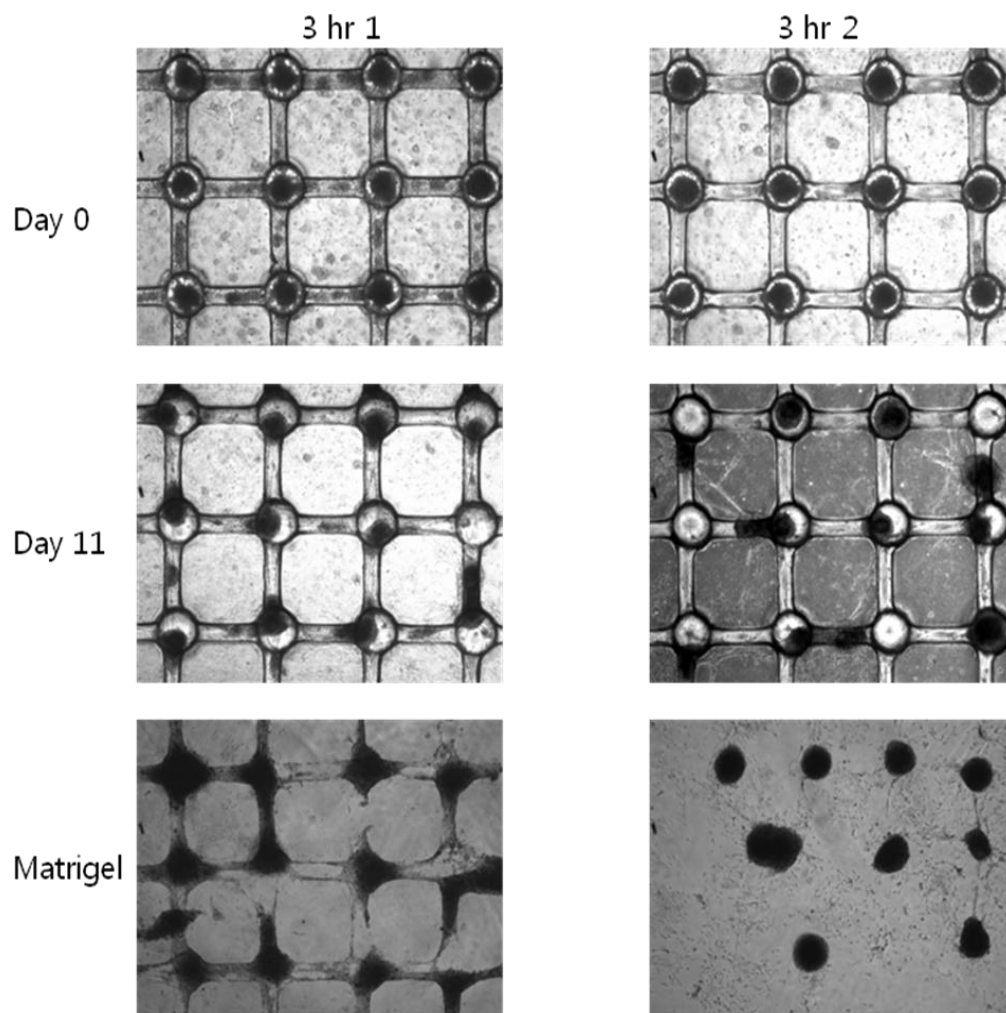


Figure 15. Phase contrast images of 2 wells after 3 hours of incubation.
 The first row is an image at day 0; 2nd row is at day 11; and the last row is after transfer onto matrigel.

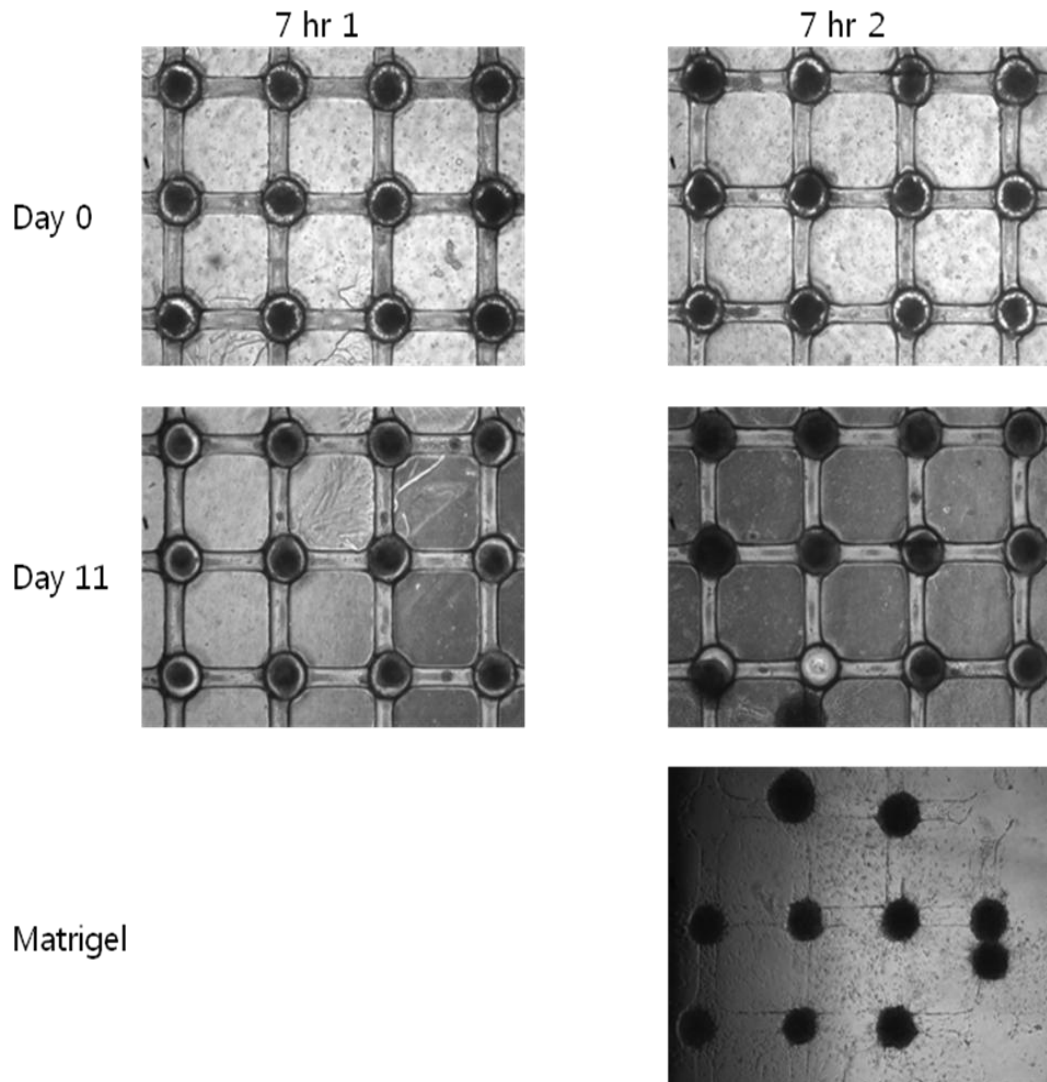


Figure 16. Phase contrast images of 2 wells after 7 hours of incubation. The first row is an image at day 0; 2nd row is at day 11; and the last row is after transfer onto matrigel.

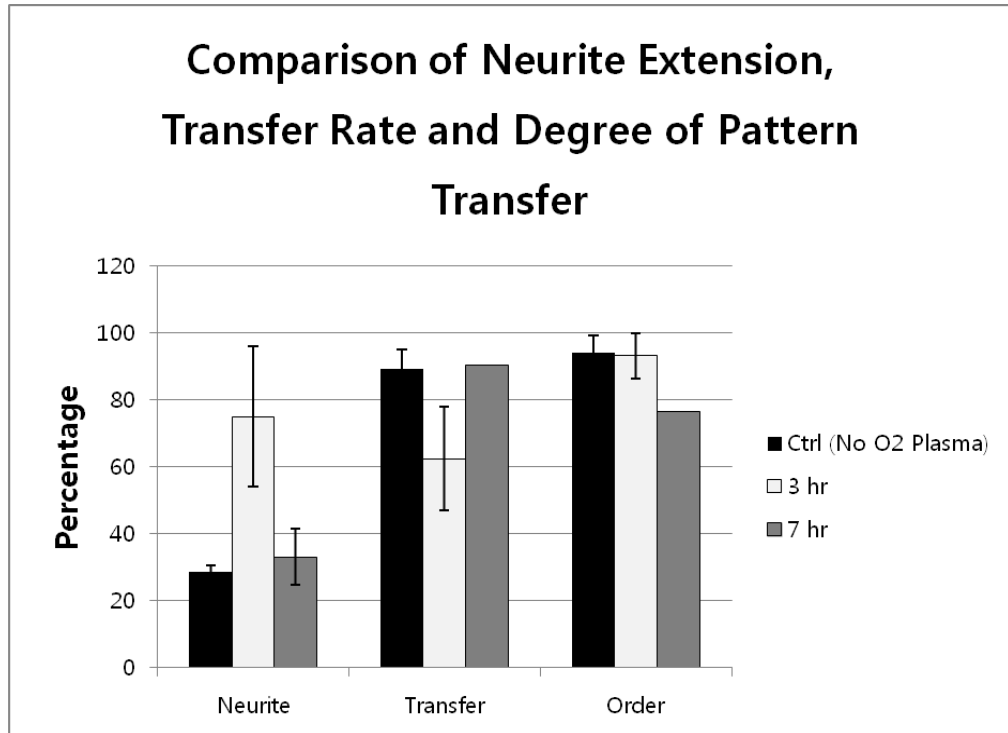


Figure 17. A bar graph summarizing the neurite extension, transfer rate, and pattern retainment of the 3 groups. Bars represent standard error; n =2 for each group.

DISCUSSION

In this study, we demonstrated the feasibility of forming neural spheroid networks in a concave microwell array for eventual implantation into the rat brain. Cell spheroids are conventional 3-dimensional cell culture models, which in contrast to 2-dimensional cell cultures, more accurately mimic the *in vivo* environment [56-58]. In the native cellular environment, 3-dimensional cell–cell and cell–ECM interactions are pivotal in creating a communication network that maintains the specificity and homeostasis of the tissue [59]. Previously, Jeong et al. have demonstrated the viability of using concave microwells to produce homogeneous spheroid-shaped embryoid bodies. It was also noticed that spheroids were easily dislodged from the concave wells with the slightest of physical stimuli (Jeong GS, unpublished data). Therefore, it was a goal of this study to culture homogeneously-sized neural spheroids in a concave microwell array that might allow for convenient extraction.

Kato-Negishi et al. demonstrated the neural stamping method by culturing NSNs in a cylindrical well array, and transferred the NSNs onto various substrates. We noticed that there was a lack of spheroid transfer, as well as incomplete neurite formation (Figure 1). We suspected that cylindrical wells used by the aforementioned researchers had definite edges to which the spheroids would attach to, thus hindering the detachment and transfer process of NSNs. In order to solve the problem of incomplete neurite connections, we studied previous research on microchannels and its effects on neurite growth [48-50]. It was shown that the implementation of microchannels acted as a physical guidance cue

that encouraged the growth of neurites along the channels. Therefore, we designed concave well structures with interconnecting rounded microchannels. Various attempts were made to optimize the diameter and distance of the microchannels. It was found that smaller wells and shorter microchannel distances improved the formation of spheroids and neurite interconnection, corroborating the results from Kato-Negishi. It is believed that the proximity of the shorter microchannels allowed for better interconnection between the spheroids through a shorter distance required for biochemical cell signaling. As a result, we used the wells with a 300 μm diameter and a microchannel length of 150 μm (450 μm center-center). Although Kato-Negishi's group found that a 100 μm diameter with a 100 μm distance between wells was optimal, our group was able to achieve 300 μm spheroids with minimal central necrosis. Additionally, the integration of microchannels allowed for robust neurite formation, even in distances that exceeded 100 μm .

It must be noted that there were other differences in our protocol compared to Kato-Negishi's group. We used neural basal media supplemented with B-27 and nerve growth factor (NGF) as opposed to using Dulbecco's modified eagle media (DMEM) supplemented with F-12 and B-27. Neural basal media is known to suppress the proliferation of glial cells, necessitating the need for an external source of biochemical growth factors, hence the use of NGF. In contrast, Kato-Negishi's group relied on the proliferation of glial cells as a source of NGF, but was unable to achieve successful NSNs in wells that exceeded 100 μm . We suspect that minimal growth of glial cells and a higher concentration of neurons permitted for a more efficient supply of nutrients to

primary neurons within the neural spheroids, allowing for larger well diameters with minimal central necrosis. Additionally, the protocol was able to forego the use of a supplement (F-12) without compromising the viability of the neural spheroids.

In order to visualize the neurites, we performed immunofluorescent imaging using fluorescent and confocal microscopy. Cultures were stained with an antibody for neurofilament, which stains the microtubules within the neurites. It was confirmed that neurites originated from the spheroids and interconnected with adjacent cells. Indeed, they interconnected robustly in the absence of glial cell proliferation, which is in stark contrast to Kato-Negishi's group. It has been previously mentioned that glial cells are a prerequisite to axonal and dendritic extension [60-63]. It is possible that NGF acted as a mitogen on the glial cells, although the media (Neural Basal media) contains anti-mitotic factors that inhibit glial cell proliferation. We believe that the dose of NGF used (1 ng/mL) may have been sufficient to overcome the inhibitory effects of the media. A previous study has shown that growth factors can have a mitogenic effect on Müller glial cells of the retina [64]. Diaz-Cintra et al. found that NGF promoted the maturation of neurons and glial cells in a fetal graft [65]. Further experiments will have to be conducted to confirm this.

Calcium imaging, which is a common method of visualizing transient biochemical fluxes, was used to assess the degree of neurite interconnection between the spheroids within the network. As noted in Kato-Negishi's study, the spheroids exhibited spontaneous bursts of calcium fluxes of an oscillatory nature, confirming the presence of functional synapses. This was captured by detecting the fluorescent intensity of the bursts

by a high speed camera, and plotting the temporal intensity on a graph. Adjacent spheroids showed coordinated burst of calcium fluxes, which shows that they were interconnected with each other.

Although a primary goal of this study was to improve the growth of neurites, preliminary experiments resulted in inconsistencies. Whereas the very first experiment resulted in robust neurite growth, successive experiments failed to replicate the previous results. It is known that neurons require some degree of adhesion for successful neurite growth as the outer membrane consists of a slightly hydrophilic charge [66]. PDMS is a hydrophobic polymer, and in general does not encourage the growth of neurites. The use of oxygen plasma is a common method of bonding PDMS units. Oxygen plasma was also used in this experiment in the fabrication of wells, particularly in bonding PDMS perimeters for usable wells. Therefore, we hypothesized that the inconsistencies in the neurite growth from the preliminary experiments resulted from residual hydrophilic charge conferred by the oxygen plasma. Consequently, we conducted an experiment to determine the optimal amount of hydrophilic charge that allows for maximal neurite growth. We subjected the surface of the microwell array to oxygen plasma for 60 s, then incubated the wells at 80 °C to control the amount of hydrophilicity. Kim et al. have shown that PDMS exposed to oxygen plasma slowly recovers its hydrophobic state in air. This process of recovery was found to accelerate at higher temperatures. It was found that 3 hours had too much of a hydrophilic charge as to inhibit the transfer process of the NSNs onto the matrigel, as well as flattened spheroids that were elongated in shape due to stronger adherence. However, the 3 hour group did have the best formation of neurites,

confirming the importance of cell adherence. The 7 hour group displayed little difference from the control group, which was not exposed to oxygen plasma, and therefore had a completely hydrophobic surface. We surmised that the optimal level may lie between 3 and 7 hours, and incubated the rest of the experimental group for 5 hours, which generally resulted in adequate neurite growth.

As the surface of the brain is very delicate, we considered possible ways to avoid the direct application of PDMS onto the brain surface. Considering that Kato-Negishi's group incubated the NSNs on various substrates for up to 24 hours, we viewed this method as impractical due to the amount of time required and possibility of damaging the surface of the brain. Kato-Negishi's group also treated the surface of the substrates with polyethylenimine (PEI) to enhance the transfer of the NSN. Again, we considered this as infeasible during implantation. It is not mentioned in Kato-negishi's paper as to the use of PEI during the implantation process onto the rat brain. It is entirely possible that they ignored this step, which may account for the lack of NSN transfer. As an alternative, we considered the possibility of using a hydrogel intermediate.

The current paradigm for neural tissue engineering relies on scaffolding materials to support the implanted cells. Previously, hydrogel scaffolds have been shown to improve the viability of implanted cells while being biocompatible [67]. Therefore, we tried two hydrogels: collagen type I gel and matrigel. Collagen I hydrogel was found to be too soft and transfer was not successful due to the inability of the gel to capture the spheroids. In contrast, matrigel, which consists of collagen type IV, was found to be an efficient medium to transfer the NSNs onto. Matrigel is slightly more firm than collagen

type I, and was able to effectively capture the spheroids while maintaining the grid-like configuration of the NSN. It was also noticed that the orientation of the wells after addition of matrigel had a significant effect on the transfer rates. When the wells were placed upside-down, transfer was significantly impeded. We suspect that the spheroids do not settle to the ground entirely, but are slightly adrift due to a lower density than the media. When placed upside-down, the spheroids tended to float and would be closer to the well bottom than the interface between the matrigel and residual media. The loose configuration of the spheroids is noticeable when handling the wells for observation, where the occasional shaking motion of the hand may dislodge the spheroids from the wells. Therefore, a right side-up configuration and an incubation time of 16 hours was sufficient to maximize transfer of NSNs onto the matrigel.

Additional experiments will have to be conducted in concave wells with a 100 um diameter and 100 um interconnecting microchannels to make a more accurate comparison. Continuing problems with the fabrication of such dimensions, mostly due to the two-layer structure required to integrate the microchannels, resulted in difficulty in aligning the two layers, and prevented the effective fabrication of the 100 um concave microwell array. This is more a matter of inexperience than the actual difficulty in fabricating such a structure. Further efforts will be made to perfect the procedure as to enable a more definitive comparison of the two methods. However, it should be noted that we achieved a fair degree of success using the 300 um concave wells, with improved neurite formation and transfer efficiency while using a different media (neural basal media); not to mention the omission of any F-12 supplement. Future studies will include the application of the

matrigel/NSN onto the rat cortical brain and growth of stem cell lines, particularly MSCs, to differentiate into neurons. The presence of glial cells within the spheroids will have to be detected to ascertain the effects of glial cell proliferation on neural spheroids. Additional scaffolding materials may be explored, especially of smart thermal responsive material such as poly (n-isopropylacrylamide; PNIPAM), which may allow the transfer of intact NSNs without an intermediate hydrogel component.

LIST OF JOURNAL ABBREVIATIONS

Adv. Cancer Res.	Advanced Cancer Research
Adv. Healthcare Mater.	Advanced Healthcare Materials
Annu. Rev. Biomed. Eng.	Annual Review of Biomedical Engineering
Biotechnol. J	Biotechnology Journal
Hum. Mol. Genet.	Human Molecular Genetics
J Biomed Mater Res	Journal of Biomedical Materials Research
J.Cereb.Blood Flow Metab.	Journal of Cerebral Blood Flow & Metabolism
JCI	Journal of Clinical Investigation
J Head Trauma Rehabil.	Journal of Head Trauma Rehabilitation
J. Neural Eng.	Journal of Neural Engineering
J Neurosci.	Journal of Neural Science
J Neurosci Methods	Journal of Neural Science Methods
J Neurosci Res	Journal of Neural Science Research
J Neurosurg	Journal of Neurosurgery
J Neurotrauma	Journal of Neurotrauma
J. of Cell Science	Journal of Cell Science
J Psychiatry Neurosci	Journal of Psychiatry & Neuroscience
J Tissue Eng Regen Med	Journal of Tissue Engineering and Regenerative Medicine
Lancet Neurol.	Lancet Neurology
Nat. Med.	Nature Medicine

Nat Neurosci	Nature Neuroscience
Neurobiol Dis	Neurobiology of Disease
Neurol Res	Neurology Research
Neurosci Lett	Neuroscience Letters
Philos Trans R Soc Lond Sci.	Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences
Proc Natl Acad Sci	Proceedings of the National Academy of Sciences
Surg. Annu	Surgery Annual

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EDUCATION

2007-Present	Boston University School of Medicine	Boston, USA
	<ul style="list-style-type: none">• M.A. in Medical Sciences, 2013	
2005-2007	Curry College	Milton, USA
	<ul style="list-style-type: none">• B.A. Biology, 2007	
2003 - 2004	University of Massachusetts	Amherst, USA
1998 – 1999	University of Massachusetts	Amherst, USA
1995 - 1998	Pickering College (High School)	Newmarket, Canada

WORK EXPERIENCE

2011-Present	Researcher (Korea University)	Seoul, Korea
	<ul style="list-style-type: none">• Researched neural spheroid networks in a biomedical engineering lab.• Published second author in Advanced Materials• Publish first author in Biomaterials (In progress)	

2010-2011	Lab Technician (Seoul National University)	Seoul, Korea
	<ul style="list-style-type: none"> • Learned immunology lab techniques. 	
2009-2010	Researcher	Boston, USA
	<ul style="list-style-type: none"> • Researched mitochondria in a molecular medicine lab. 	
2005-2006	Milton Hospital Volunteer	Milton, USA
April, 2003 –	ASE Korea (Formerly Motorola Korea)	Paju, Korea
July 2003	<ul style="list-style-type: none"> • Intern test systems engineer 	
January 2001 -	Korean Augmentation to the US Army	Tongduchon,
March 2003	(KATUSA)	Korea
	<ul style="list-style-type: none"> • Served in the 2nd Infantry Division, 1st Brigade Equal Opportunity Office working as a translator, interpreter, and Equal Opportunity Representative. • Played a crucial role as a Non-Commissioned Officer in charge of maintaining equality among soldiers within the brigade. • Awarded the Army Achievement Medal (AAM) and Army Commendation Medal (ARCOM) for services rendered to country and US military. 	

LEADERSHIP ROLES

2011-2012	Led a project on neural spheroid networks as a researcher.	Seoul, Korea
2002-2003	Played a critical role as a non-commissioned officer in the KATUSA program.	Tongduchon, Korea
1996	Pickering College student proctor.	Newmarket, Canada

SKILLS AND OTHER INFORMATION

- Languages**
- Strong written and oral communication skills in English (Lived in the U.S. and Canada for over 16 years).
- Computer Skills**
- Highly proficient with computerized office software such as MS Word, Excel, PowerPoint, experience in designing Web Pages on the Internet, and mcaro-level programming on Metamorph.
- Interests**
- Sports, Science Fiction Novels, Technology, Medicine, Computers, and Traveling.