Master's Thesis

Gel Alignment in 3D Microfluidic Neuronal Culture Systems

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Graduate School of Seoul National University

Mechanical and Aerospace Engineering

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Abstract

Axon plays an important role in the brain mechanisms as electric signal transmission, axonal transport, and local protein synthesis. By developing of valuable microfluidic systems, researchers easily isolate, observe, and test neurites development. In 2D culture, a microfluidic culture platform is a strong tool to probe axons independently from cell bodies, and micro-patterning method pattern axonal guidance molecules to selectively control axonal growth. However, conventional 2D environment's disadvantage is not to closely mimic physiological axon growth. To understand fully how axons as well as neural networks form and operate, some 3D cell-culture models have been recently applied widely. In vitro, a lack of an appropriate method which guides axons in 3D environment prevented investigation of axonal biology and neural networks.

Here, we describe a novel method that guides axon growth by controlling 3D gel alignment. In this method, we design a microfluidic device that

maintains 3D gel environment (Matrigel) in channel. Before complete polymerization, the random structure of Matrigel is aligned by the directional diffusion and pressure of medium from top to bottom. Then, neurospheres are cultured next to the aligned gel. Neural cells are differentiated from neurospheres inside the 3D scaffold. Because the cell motility depends on the scaffold's structure, neural cells as well as their dendrites migrate and invade directionally inside the aligned gel. Neurosphere-derived cells express the fluorescent of tubulin β III-positive (Tuj-III) neurons show the data of axon alignment (80% axons grow straight, less than 30 degree). Besides, depending on the concentration of Matrigel, the alignment of axons will be formed completely or partially. For neural network formation, synapse signals can be detected on the designated areas between the directional axons and local dendrites

The axon guidance in 3D environment provides the understanding of directional controlling axon growth, supporting the formation neural network in 3D and solving the 2D culture limitation by utilizing extracellular matrix gel on axon migration. In addition to its compatibility with optics system, the platform is useful for guiding axon with light, forming neural network, making axon injury, and observing regeneration in 3D environment.

Keywords: Gel alignment, Axon's guidance, Axon alignment, 3D environment

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Figure 17. Alignment of axons in cortical cell

a. Cortical cells express their axons straightly in Tuj-1;

b. The alignment and control samples were counted and analyzed with *t-test* (10 samples for rat cells),(**$p<0.01$ and *** $p<0.001$).

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1.Introduction

Nervous system is the most important system in human body. This system includes central nervous system (CNS) and peripheral nervous system (PNS). Nervous system controls and coordinates all organs and structures by the information flowing through neurons. If these signals are disrupted or transferred discontinuously, the movement or sensation will be lost or paralyzed due to spinal cord injury. For treatment to the lesion, the prospect of replacing lost and damaged cells by neural stem cells (NSCs) in the CNS or PNS has been recently contributed to neural therapy. In the NSCs transplantation, the recovery process depends on the axon-connection's process between new axons and existing axons in the lesion. In fact, the axons from the neurons (derived from NSCs) if aligned can hasten this recovery process [1]. How can axons be generated during neurogenesis with the necessary precision and reliability?

Axon growing is affected by guidance forces in surrounding environment. In general, four types of mechanisms can guide axon simultaneously such as contact attraction, chemo-attraction, contact repulsion, and chemo-repulsion [2]. Among these types, a lot of contact attraction methods were created to control axons and dendrites in two dimensional (2D) environments. For examples, neural cells changed their morphology on the laminin coating on micro-grooves [3]. Besides, neural stem cells grew straight on graphene oxide nanoparticle alignment [4], and dorsal root ganglia were followed by aligned

poly-L-lactic acid microfibers [5]. However, these methods required complex fabrication process (nanoparticles coating), high-tech machines (photolithography or electro-spinning) and they are lack of 3D environment for cell differentiation, migration and proliferation, respectively.

The requirement of 3D environments is necessary for *in vitro* experiments. Besides, microfluidic technique has provided a good tool for culture, stimulation and observation in 3D culture. With injected hydrogel into its channels, the device performs a proper 3D scaffold platform for cell culture. In this study, we fabricated microfluidic neural culture devices and used Matrigel for the purpose of mimicking the 3D cell culture in natural hydrogel. Among of a variety of used techniques, gel alignment is a valuable method for axon guidance in 3D culture. In this study, we present a novel method for axon alignment which can be used easily and efficiently in 3D scaffold of natural biomaterial.

1.1 Neural stem cell and neurospheres

Neural stem cell is used frequently to describe cells that can generate neural tissue or be derived from the nervous system. These cells have some capacity for self-renewal and give rise to any type of specialized cells through asymmetric cell division. Potential uses of stem cells in treatment include either transplantation to recover the missing cells or the activation of endogenous cells to provide “self-repair” [2]. However, the stem cell

differentiation to special cells does not fully understand. Some potential cues monitor the cell differentiation as growth factors, media and extracellular matrix (ECM). In the 3D environment, the affects of surrounding environment are more important than in monoculture. In this case, NS cells dependent on the ECM can differentiate to neuron, astrocytes, oligodendrocytes and other types of neural cells. For enhancement the neuron differentiation, NS cells should be dissected from the embryonic brains. Recently, neurospheres is quickly emerged as the stem cell source for isolating, and understanding of NSC stem cells.

What is neurosphere? Neurosphere is three-dimensional floating spheroid cell clusters. It is heterogeneous which contains cells at various stages of differentiation, stem cells, proliferating neural progenitor cells and post mitotic neurons and glia. Transplantation of neurospheres has shown that they can survive, migrate and differentiate in various sites of the brain. For example, rat fetal hippocampus-derived neurosphere cells could be transplanted through cerebrospinal fluid [6], then integrate well into the host spinal cord tissue [7].

1.2 Microfluidics and cell biology

Stem-cell behavior is exquisitely sensitive to environmental cues. And the important cues are difficult to establish, manipulate and quantify in traditional cell culture. However, the microenvironment can be controlled in

microfluidics platforms. Thus, microfluidics-lab-on-a-chip devices are known as a good tool for standard laboratory analyses, such as sample purification, labeling, detection and separation. Theses process are carried out automatically as the sample moved via microchannels to different regions of a chip

Microfluidics techniques can be used for sensitive discrimination of gene expression (and protein) at the single-cell level and they are therefore increasingly useful in stem-cell biology to understand the heterogeneity of stem-cell populations

1.3 Hydrogel and gel structure

Hydrogel is popular in 3D cell culture platform because of its extracellular matrix mimics. Thus, *in vitro* cell exhibit natural behaviors in 3D hydrogel platform. Currently, amount of hydrogels used for cells from purely natural to synthetic materials. Among these, Matrigel is an optimal matrix for NSCs because it maintains self-renewal and pluripotency of stem cells. Although the components of Matrigel have detected, its structure and properties have not understood fully. However, we can organize and observe their structure by the staining Collagen IV (a majority in Matrigel components)

1.4 Motivation and objective

Neural stem cell and their applications in stem cell transplants for spinal cord injury are objective in this study. In these previous researches, aligned

gel which achieved in different techniques can improve the recovery process. Applying microfluidic device, we can align Matrigel and build up a proper scaffold for neuron differentiation, axon guidance and neuron network.

Microfluidic device has designed with 3 channels. Two outside channels are used to culture media (the nutrient and oxygen for cells) and the middle one is injected Matrigel (for making 3D scaffold) (**Fig.1a**). Then, Matrigel is aligned inside the microchannel under the flow rate (**Fig.1b**). After that, neural cells are loading to the culture chamber. Neurons are cultured in several days and axon projections will grow along the aligned fibers of Matrigel (**Fig1.c**).

2. Methods and Materials

2.1 Microfluidic device design

In this study, we designed a new microfluidic device for creating a 3D aligned hydrogel environment. The device contained three channels, including two outside channels for culture media (media chambers), a middle one for 3D gel formation (**Fig 1a**). The gel structure of Matrigel relied on the shape of the channels, so we predicted and determined the dimensions of devices by some simulation results. Initially, we created a 3D microfluidic device in an Autodesk program **(Fig 2a**). Secondly, a variety of different dimensions were tested in a flow rate simulation program. Particularly, the height and width of the middle channels which were main dimensions were optimized carefully (a wide range from 250µm to 2000µm and a high range from 100µm to 450µm) (**Fig 2b**).

Depending on the flow rate and velocity of flow in each case, we obtained initial data (**Fig 10**) and optimized the final dimensions for fabrication. Thus, the middle channel was decided with 250µm high, 1000µm wide and 6000µm long. After that, a specific mask was produced, which was followed exactly on the device drawing.

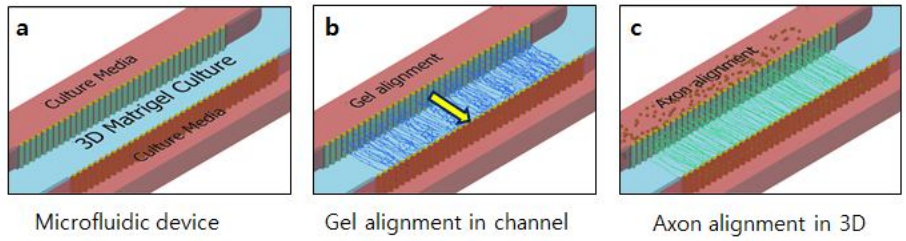

Figure 1. A experimental process for gel alignment and axon alignment

a. Microfluidic neuron culture device with 3 channels; b. The orientation of gel alignment; c. Neuron culture and axon alignment

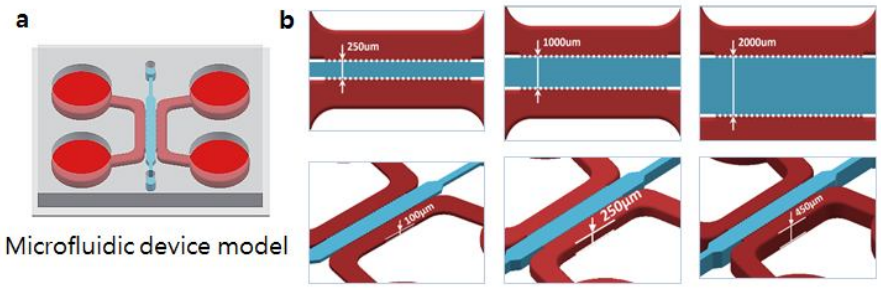

Figure 2. A 3D microfluidic device model with different dimensions

a. A view of 3D design model; b. Devices with different widths and heights.

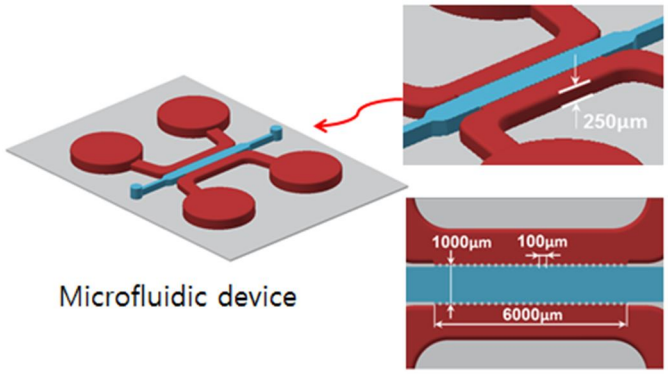

Figure 3. Optimized dimensions of drawing (height: 250um, width: 1000um)

2.1 Microfluidic neuronal culture platform fabrication

Photolithography and soft-lithography techniques are widely applied to fabricate the microfluidic devices. Firstly, photolithography is an optical means for transferring patterns onto a substrate. A photolithography process has several steps, including surface preparation, coating (spin casting), pre-bake (soft-bake), alignment, exposure, development, post-bake (hard-bake), and development. Our photolithography process had proceeded with the mask, a Silicon wafer, SU-8 100 (a negative photoresist), SU-8 developer and a photolithography machine in a clean-room. The process was occurred all below steps.

Photolithography process (for SU-8 100 with 250µm high)

- Coating: SU-8 100 was poured on the surface of a Si wafer. The velocity was 1000rpm in 5mins to obtain 250µm thickness.
- Pre-bake: The wafer was baked on a hot plate at 65^0C in 30mins then continuously baked at 95^0C in 90mins.
- Exposure: After pre-bake, the wafer and the mask were aligned on the photolithography machine and exposure at UV light in 40seconds.
- Post-bake: Following exposure, a post-bake was performed either on a hot plate or in a convention oven. The wafer was baked at 65^0C in 1min, then 95^0C in 20mins

- Develop: SU-8 resists have been optimized for use with SU8 developer. We maintain the wafer in developer in 20mins.
- Rinse and dry: Following development, the substrate should be rinsed briefly with isopropyl alcohol (IPA), then dried with a gentle stream of air or nitrogen
- After photolithography process, a master of design was created. Then, we continued the fabrication process with a soft-lithography technique. In this method, we used polydimethylsiloxane (PDMS) to make a stamp from the SU-8 master. PDMS is a two-component heat-curing system, consisted of a base part and a curing agent part. This polymer was poured on the SU-8 master and cured at 95^0C in 15mins. Then, the final step is peal off the PDMS stamp from the template.

Finally, a PDMS stamp was treated in the plasma preparation chamber for 15seconds before bonding with the glass coverslip. The plasma preparation will incorporate oxygen atoms in the PDMS surface, leaving it hydrophilic. Thus, this process enhanced the bond stronger and created a PDMS device. In figure 4, a device model and its product were shown and compare to a coin (less than 5cm) (**Fig 4.b**)

2.2 Reduced Growth Factor Matrigel

Matrigel is an assortment of extracellular matrix (ECM) proteins that have been extracted from Englebreth-Holm-Swarm tumors in mice. This gel is

applied for the growth of human embryonic stem cells; mimic the ECM in cancer and stem cell culture (an optimal matrix for culture of stem cells because of its ability to maintain self renewal and pluripotency). Matrigel has 1851 total proteins identifications. Among of these proteins, Laminin (800,000 Da), Collagen IV (540,000 Da) and Enactin (158,000 Da) are main components. It also has amount of growth factors as basic fibroblast growth factor (b-FGF), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and nerve growth factor (NGF). However, reduced growth factor (RGF) Matrigel is used in our study because of elimination of growth factor effect. The concentration of growth factors is reduced in RGF Matrigel. (**Table 1**)

Protein	**Percentage**	**Growth Factor**	**Concentration**
Laminin	61 %	bFGF	0-0.1pg/ml
Collagen IV	30 %	EGF	<0.5ng/ml
Entactin	7 %	IGF-1	5ng/ml
Heparim sulfate proteoglycan	Not available	PDGF	<5ng/ml
		NGF	<0.2ng/ml
		TGF-β	1.7ng/ml

Table 1 *Matrigel™ growth factor reduced composition*

Physical properties of Matrigel are listed below

- ✓ Elastic modulus : 440±250Pa
- ✓ Viscosity : 10-15cP
- ✓ Concentration : 10-12mg/ml
- ✓ Conductivity : 1380mS/m
- ✓ Surface coverage: 6-10ug/cm^2
- ✓ Store at -20^{0}C (long time) or 2-8^{0}C (short time)
- ✓ Pre-polymer temp. 4^{0}C
- ✓ Make gel within 5mins at 20^{0}C and completed gel in 30mins at 24-37^{0}C

2.2 Gel alignment method

In this study, we made a novel method for gel alignment in microfluidic devices. Matrigel reduced growth factor was applied for neural stem cell culture in these systems. Initially, for gel preparation, we thawed and maintained a Matrigel vial at pre-polymer temperature point (4^{0}C) by the ice. A sterilized device, bonding with glass coverslip, was also cooled down on ice before gel injection (**Fig 5d**). We injected Matrigel (10 microliters per channel) slowly into the channel and up-standed them in 5 minutes before adding media into the chamber (**Fig 5e~g**). During the gel time, from the pre-polymer point to gelation point, we incubated a vertical device constantly in incubator at 37^{0}C. After 1 hour incubator, we used this model for cell loading (**Fig 5h**).

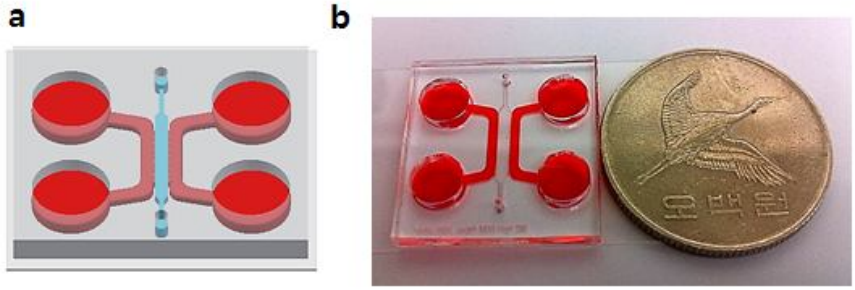

Figure 4. A microfluidic device model and a real device.

a. A 3D microfluidic device model in the design; b. A real device with a coin

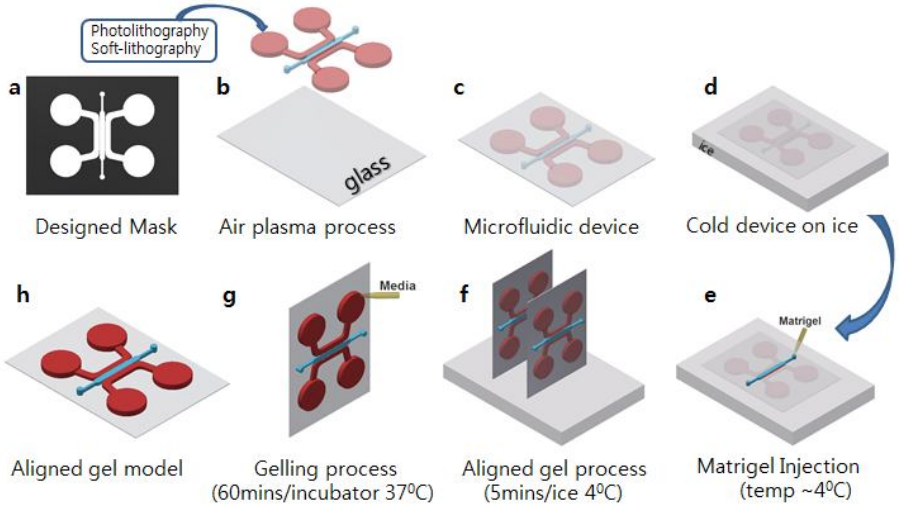

Figure 5. A novel method for gel alignment in microfluidic device

a~c. Photolithography and soft-lithography process; d~h. Gel alignment method

2.3 Cell culture

Neural stem cells (NSCs) are widely applied for neural cell differentiation and axon projections. Recently, neurospheres were considered as the stem cell sources for transplantation and *in vitro* neurogenic cells researches. In this study, we harvest NSCs derived mouse embryo brains (TP12.5) in the neo-cortex region (**Fig 6a**). Neurosphere culture media has main components of DMEM, B27 (1X), N2 supplement (1X) and epithelial growth factor (EGF) (20ng/ml) and basic fibroblast growth factor (b-FGF) (10ng/ml).

After 2 days, neurosphere formations occur and form around 200 um diameter at day 5. In micro-well hydrogel plates, uniform size neurospheres are collected at day 7 (average diameter 250um) (**Fig 7 a**). Collected neurospheres are injected into channel in the media without both EGF and b-FGF for cell differentiation. Both glial cells and neurons are differentiated by these neurospheres. In gel alignment, only axon can go inside, follow the gel structure and reach to the gel boundary.

Besides, primary cells derived the rat embryo brain (TP16) were used for single cell application (**Fig 6b**). After dissection, single cells are cultured in channel with neural basal media (NBM), glutamax (2mM) and B27 (2%). After 1 day culture in microfluidic device, axon projections can be observed and counted. All axons are marked with Tuj-3 antibody and counted under confocal microscope (**Fig 8a,b**).

2.4 Gel fixation and Immunofluorescence staining

Gel fixation is applied for observing gel structure without label by fluorescent. Gel alignment samples are sucked out media completely and wash twice with phosphate-buffered saline (PBS) in 5 minutes. We fix Matrigel by different concentrations of ethanol solvent (50%, 70% and 99%). Each concentration is maintained in 5 minutes at room temperature. Dehydrate very quickly through 99% alcohol. The structure of gel is capturing by Nikon Inverted Microscope.

After cell culture experiments, the gels were fixed in 4% formaldehyde in PBS for 10 min, and then permeabilized in 0.5% Triton X-100 in PBS for 5 min. The blocking buffer was incubated in 15min. Then, the samples were incubated with the antibody mouse monoclonal Tuj 1 (Neuron-specific class III beta-tubulin) (1:100) for neuron marker, antibody glial fibrillary acidic protein (GFAP) (1:80) for astrocyte marker and DAPI for nucleic. The structure of Matrigel was stained with anti collagen IV (1:100) and anti laminin (1:80). The cells and gel fibers were analyzed using an Olympus FV1000 confocal laser scanning microscope.

2.5 Images acquisition and data analysis

Neurite outgrowth is a basic proof of neural cell viability. The outgrowth was analyzed with a different interference contrast (DIC) microscope during the cell culture time (after 1, 3, 5, 7, and 14 DIV). Fluorescent images have

been capture with the Olympus confocal microscope using Tujβ-3, GFAP and DAPI. Using the analyzer of ImageJ software, we measure and count the axon outgrowth and cell available on each sample. For alignment measurement, we used the Curve align detection method and Hugh transform detection (an application based on Matlab software). Depend on these detections, we can measure the angles and calculate the different data between control samples and align samples. The results of comparison are tested with t-test analysis.

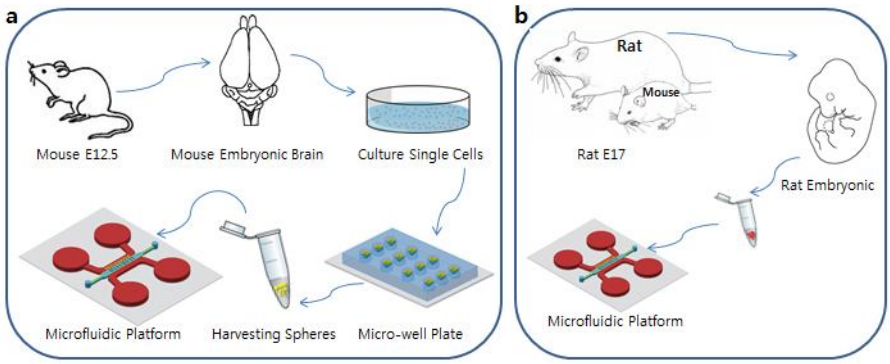

Figure 6. Different Cell Culture Assays

a. Mouse neurosphere assay **b.** Rat primary cell assay

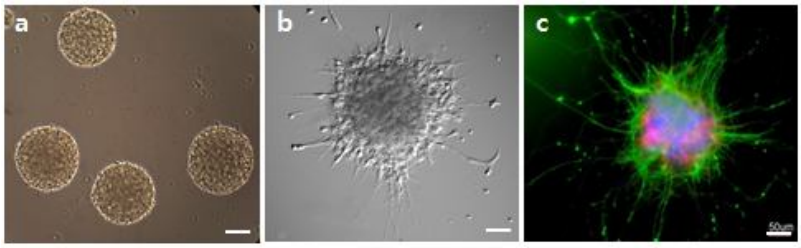

Figure 7. Uniform Neurosphere Culture and Neural Cells Differentiation

a. Uniform neurosphere after 5 days culture in micro-well plates; b. Neural cells differentiation captured by DIC microscope; c. Neurosphere label

a.

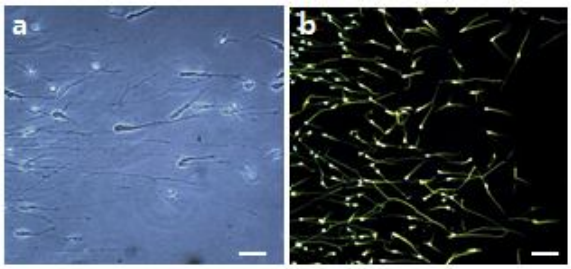

Figure 8. Cortical Cells Culture and Axon Growinga. a.Uniform neurosphere in micro-well plates; b. Neural cells differentiation (DIC microscope)

3. Results

3.1 Microfluidic device design

As we mentioned above, we obtained the flow rate in the microfluidic channel when using a flow simulation program. As a result of simulation, the flow was laminar flow on the center region and they flowed unidirectional on the edge regions. So, we determined to limit the reasonable results on the center of the channel (4000µm per 6000 µm length) (**Fig 9**).

Following the mention in figure 2b, we changed and simulated different dimensions of the width and the height of the channel. The graph expressed the maximum velocity (mm/s) of each dimensions on three conditions (water; Matrigel; Matrigel and water). In case of water, the value was increased very fast because of its low viscosity. In contrast to Matrigel, their values were not different too much because of higher viscosity of Matrigel (more than 10 times to water). However, the combination of water and Matrigel gave us the reliable tendency for flow appearance in the device. According to the results, we had the increased tendency of velocity when the height was increased. However, the tendency of flow was decreased when we increased the width lager. (**Fig 10**),

With the simulation results and the valuable fabrication ability, we determined the height and width of the channel (250µm high and 1000µm wide).

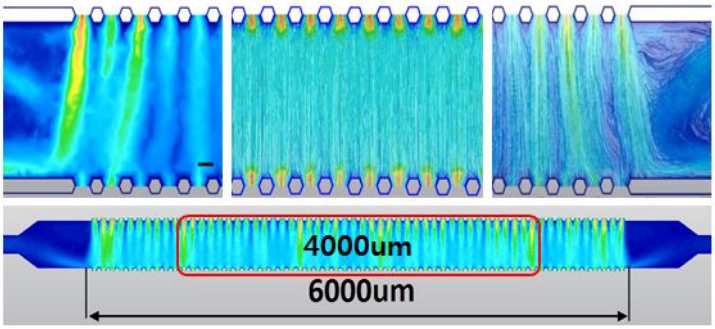

Figure 9. 3D flow rate simulation results (the difference between the edge and the middle of a channel)

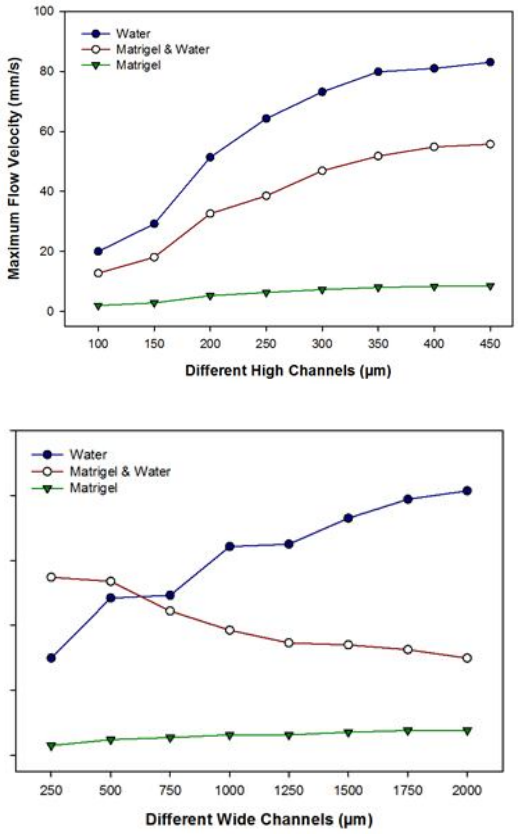

Figure 10. Maximum flow's velocity in different width and height

3.2 Cell culture

In vitro, axon is generated from primary cell, cell lines or neurospheres. However, neurosphere has become popular as the reliable NSCs source for transplantations *in vivo* because of their self renewal and pluripotency abilities. For this reasons, we culture neurospheres and use axon derived-neurosphere as guided objects. Neurospheres are cultured from the mouse embryonic (E12.5) in the neurosphere media with FGF and EGF. After 4 days culture, average neurosphere size is 250~300um. Neurospheres are loaded into the aligned gel samples and culture in the differentiation media (without EGF and FGF) for neuron differentiation. Axon derived-neurosphere appears and grows after 1 day culture in device. After 5 days culture, axons grow fully in the gel then are stained for cell images analysis.

Other neurons derived cortical cells are harvested from the rat embryonic (E17). The differences of cell sources as single or cluster of cells is not different for axon alignment in this method. We apply both different types of cells for axon alignment.

3.2 Gel alignment

Aligned Collagen fibers have previous presented in some methods related to both flow and magnetic field [7]. Using the effect of directional flow, we achieved the alignment of Matrigel during its gelation time. After gel injection, both random gel (**Fig. 4a**) and aligned gel (**Fig. 4d**) samples are treated for

structure detection. Gel structures are expressed in two detective methods. Firstly, we wash the samples with Ethanol solution (different concentration 50%, 70% and 99%) and maintain the structure in Ethanol 99%. Dehydrated gel images are captured by differential interference contrast (DIC) microscope show the total structure of Matrigel. Gel maintains in the random orientation (**Fig. 4b)** and aligned directions **(Fig. 4e**). Besides, Collagen IV antibody is also used for gel Collagen detection, and then the sample is captured with 488mm wavelength. Fluorescent images of Collagen IV express partially structure of Matrigel (**Fig. 4c**) and aligned gel (**Fig. 4f**)

Finally, image processing is applied for angle detection and measurement. Curve align method allow us to quickly and automatically quantify the alignment of periodic structures. Beside, Hough transform technique is concerned with the identifying positions of arbitrary shapes. Using these methods, we can compare the different structure of random orientations and aligned orientations. The graph shows the wide distribution of random Matrigel structure (Fig.5 a).It totally differs with the high concentration of vertical lines in the gel alignment (Fig. 5b). We get the same results in the Matrigel structure (washed Ethanol) and Collagen IV structure (stained Collagen IV antibody).

3.3 Axon alignment

After harvesting uniform neurospheres, we loaded them to Matrigel channels. At 24h after seeding, the cells projected their axons in Matrigel. After 5 days culture, the cells were fixed, labeled and mounted on different samples. Axons are expressed in Tuj-1 antibody and DAPI for their nucleic. Aligned growth and extension of axons from neurosphere-derived neurons were measured and showed in the compass plot. The angle and amount of axons were presented differently on the graph. While the distribution of angles is similar in entire range (-90^0 to 90^0) which less than 10%, the aligned distribution (ranged from -10^0 to 10^0) is more than 20%. The high concentration of small angles (around 10^0 degree) elucidates the alignment of axons in the aligned gel. Finally, the date exhibits very well aligned and well extended axons. The alignment of axons is compared to the alignment of Matrigel structure. Without alignment, the gel fibers and axons orient randomly. Only on the aligned platform, the observation of alignment is elucidated in the range in $\pm 10^0$ degree.

3.4 Axon alignment with different cell types

We confirm the alignment of gel can be useful for the other cell types. In initial cases, the neurospheres which derived mouse embryonic brain (TP12.5) are loaded to the aligned Matrigel. Axons are projected by the neurosphere-derived neurons are counted and measured after 12days culture. Alignment

average values are compared to the random orientations (**Fig 7a, b**). Besides, with the fluorescent dye, we can recognize the differentiation of the glial cells which also migrate along the orientation. The neurospheres have given the potential ability to proliferation, migration and differentiation within the aligned gel. We predicted that inside the aligned gel, the neuron differentiation can be enhanced, and the migration of glial cells also changed by the aligned fibers.

Furthermore, the cortical cells which were dissected from the rat embryonic brain (TP16) also express the alignment results after culture in the aligned Matrigel. In our study, the primary cells which specialized to neurons grow axons faster than neurosphere cells can do. In our results, we achieved the full alignment after only 12days culture. The comparison to random Matrigel is likely similar to the neurosphere experiments.

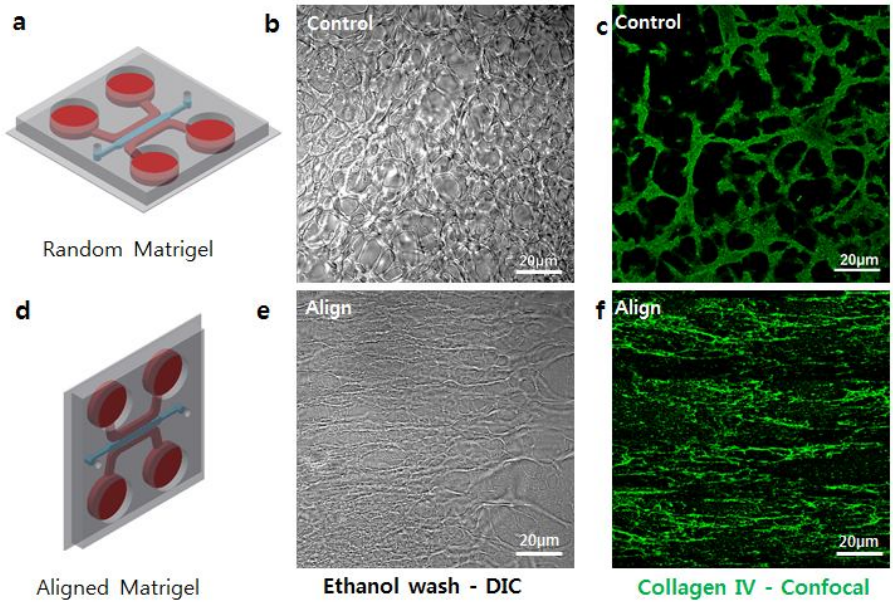

Figure 11. Gel alignment in microfluidic device

a.Random gel b. Aligned gel (without label and Collagen IV label)

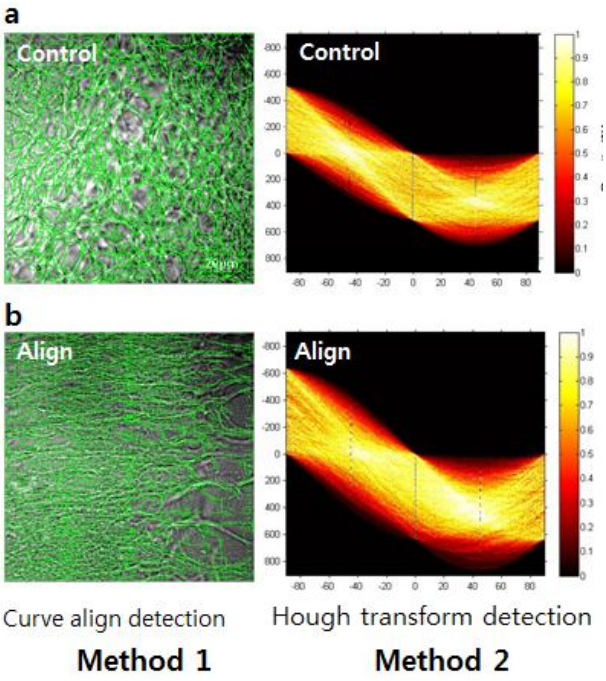

Figure 12. Image analyses using different detections

a.Random structure b. Aligned structure (with curve line detection method and Hough transform method and analyzed results)

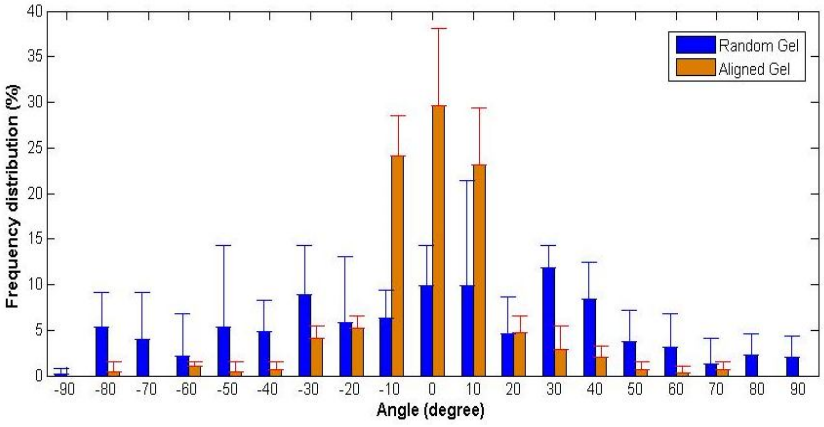

Figure 13. A correlative graph of random gel and aligned gel

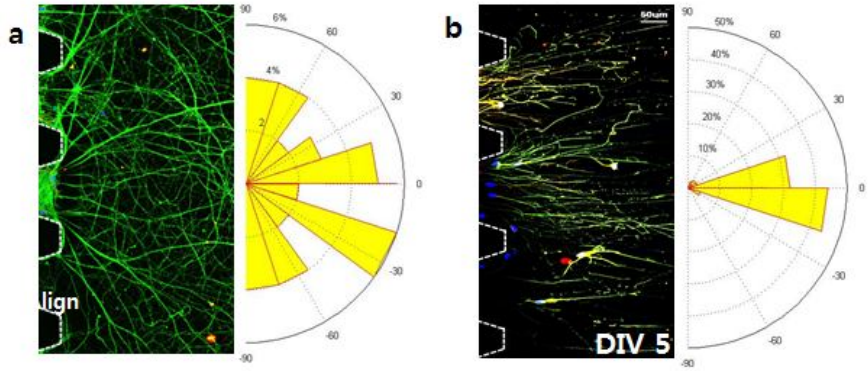

Figure 14. Axon growth and extension in Matrigel; a.Random growth of axons in no-aligned gel; b. Axon alignment within the gel alignment

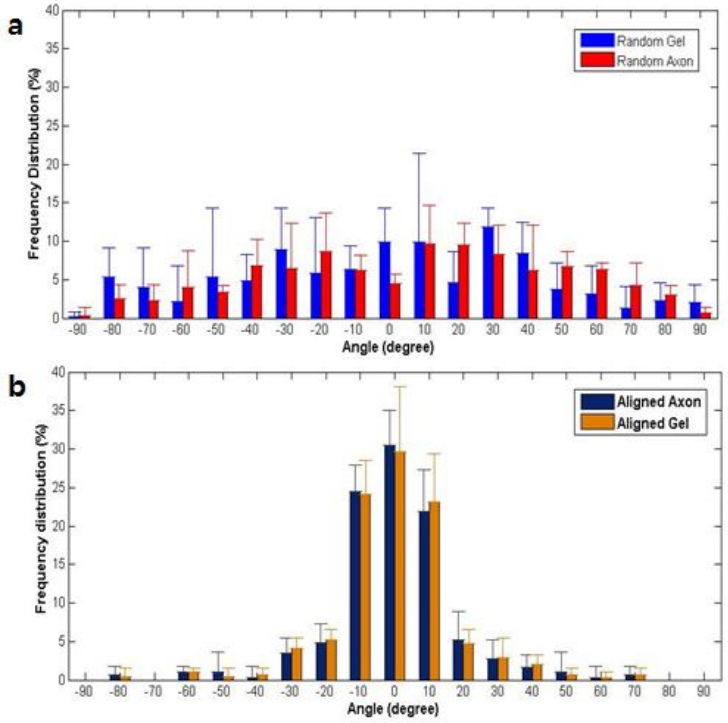

Figure 15. a. A correlative graph of random gel and random axon; b A correlation of aligned gel and aligned axon

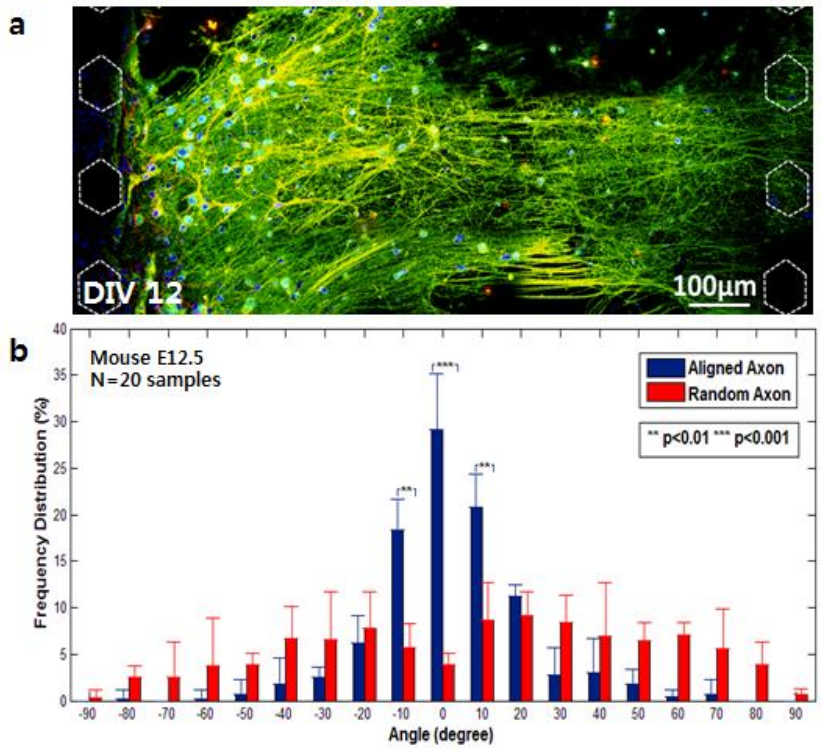

Figure 16. Alignment of axons in neurosphere

a.Aligned axons derived mouse neurospheres expressed in Tuj-1, glial cells were expressed in GFAP; b. The alignment and control samples were counted and analyzed with *t-test* (20 samples for mouse cells),(**p<0.01 and *** p<0.001).

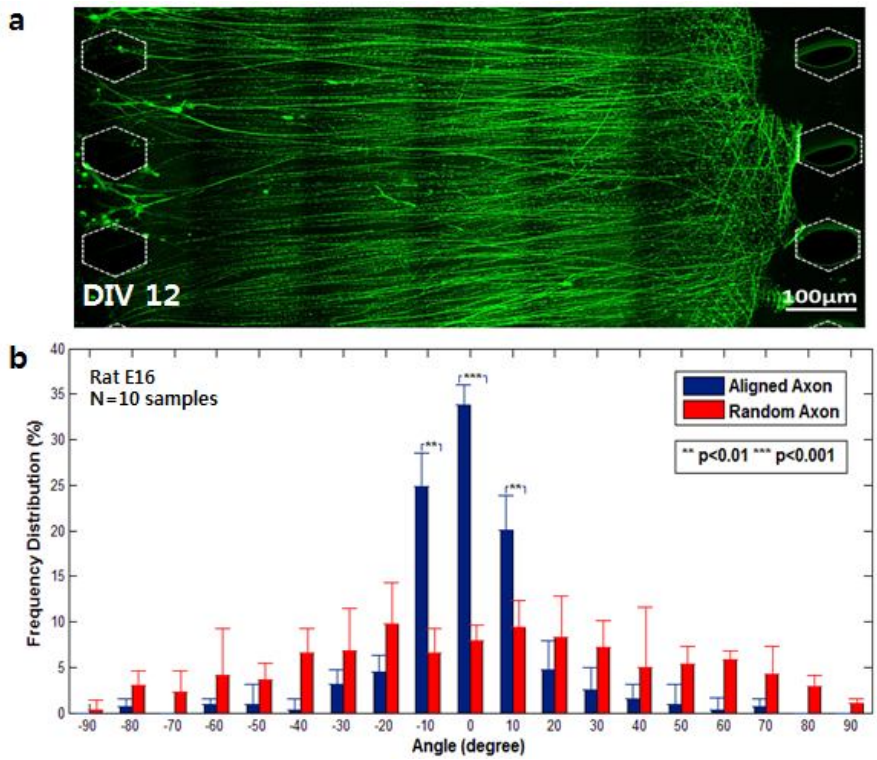

Figure 17. Alignment of axons in cortical cell

a. Cortical cells express their axons straightly in Tuj-1;b The alignment and control samples were counted and analyzed with *t-test* (10 samples for rat cells),(**p<0.01 and *** p<0.001).

4. Discussion

The design of micro-channel has an important role in the dimension of aligned gel. So, we used the simulation to calculate approximately the proper dimension of the channel. Depending on the properties of Matrigel and the pressure made by the media, we have a tendency of the affect of height and width on the flow rate inside the channel. The dimensions are determined by the proper value of this tendency. Because the flow rate is changed by the turbulent at the edge of channels, we eliminate the axon directions in these regions on the results.

In previous papers, the authors succeed alignment of Collagen (the main component of Matrigel) but the alignment of Matrigel has not achieved yet. Because of the typical properties of gel, Matrigel depends on the temperature for polymerization. So, it is hard to control the alignment with Matrigel. This work describes the alignment of 3D matrices of Matrigel in microfluidic channels. Polymerizing aligned Matrigel fibers were observed and measured both in Ethanol method and Collagen IV staining. In microchannels with widths 1000um and height 350um, the average fiber orientation is within 20^0 (from -10^0 to 10^0). As channel size increased, the degree of alignment should

be concerned to the polymerization time of total volume of Matrigel. When neurospheres or cortical cells were cultured on the aligned matrix, there were the evidences of axon alignment along the fiber orientation direction. The cell behaviors are demonstrated that relying on the structure of ECM.

While it is clear that Collagen IV orientation followed by the directional flow of media, the data also recommend that other components of Matrigel also are aligned in dehydrated Matrigel images. Although the mechanism of alignment was unclear, we likely mention the presence of media flow. For orientation, two normal contributing factors are flow and geometric restriction of the channel wall. However, in the case of Matrigel, the polymerization time is an important factor which has occurred only few minutes. In this study, we maintain the pre-polymer point of Matrigel at 4^0C by cooling down the device on ice. During this time, the devices are titled at 90^0 degree for creating the flow rate from the media channels. Because the flow rate appearance is the reason of alignment, the anisotropic structure will be transformed to isotropic structure. The laminar flows conduct all the regions simultaneously from the center to the edges of the channels, but it disappears in the rest of channels which no laminar flow. This is supported that the aligned fibers in the center of channel is more aligned then near the edges due to the turbulent flow. In all cases studied, alignment at the center of the channel is statistically indistinguishable form the edges of channel.

5. Conclusions

The alignment of ECM plays an important role in the guidance of axons derived neural stem cells. We have presented a simple method to align Matrigel (one of natural ECM) in microfluidic devices. This method obtained alignment that is as good as other methods, with no specialized machine and no other materials. The result achieved in wide area of thousands of microns and can be enhanced in multi-channels to mimicking the brain order. The neurospheres are cultured in the aligned gel can project axons straight perfectly. Besides, these stem cells type has previous transplanted to the spinal cord lesion and integrate well with exiting cells. Our result has been believed that the alignment relying on the gel structure is hastened the recovery of the injury. Furthermore, the alignment will be applying to the cell signaling, migration, and differentiation for tissue engineering.

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Abstract (Korean)

축삭(axon)은 전기 신호 전달과 축삭 수송 그리고 단백질 합성과 같은 뇌의 기전에 매우 중요한 역할을 한다. 연구자들은 새로운 미세 유체 시스템(microfluidic system)의 개발로 보다 쉽게 신경돌기를 분리하고 관측과 실험을 할 수 있게 되었다. 기존의 2 차원 배양에서, 미세 유체 배양 플랫폼은 축삭을 세포체에서 분리하여 연구하는데 유용하다고 알려져 있고, 또한 미세 페턴 방식()(micro-patterning method)은 선택적으로 축삭의 성장을 유도하는데 사용한다. 하지만, 기존의 2 차원 배양은 생리학적인 축삭의 성장을 재현할 수 없다는데 한계가 있다. 축삭과 신경회로 (neuronal network)의 형성과 기작(function)을 완전하게 이해하기 위해서 3 차원 세포 배양 모델(model)이 최근에 활발히 연구 중이다. 그러나 축삭의 성장 방향을 유도할 적절한 in vitro 방법의 개발이 충분히 이루어지지 않아 3 차원 축삭의 성장과 신경회로의 연구를 제한하고 있다.

우리는 이번 연구를 통해 3 차원 겔(gel)을 활용해 축삭의 성장을 제어할 수 있는 새로운 방법을 제시하려 한다. 이를 위해 우리는 Matrigel 3 차원 환경을 만들어 줄 새로운 미세 유체 장비를 제작하였다. 고분자화가

완전히 진행되기 이전에, Matrigel 의 구조는 아래로 향하는 배지의 확산과 압력에 의해 균일하게 배열된다. 후에는 신경구(neurosphere)가 배양된다. 신경 세포들은 3 차원 스캐폴드(scaffold)에서 신경구로부터 분화되어 만들어진다. 세포 운동성은 스캐폴드에 전적으로 의존하기 때문에 신경세포 뿐만 아니라 축삭돌기(dendrite)들은 특정한 방향으로 이동하고 침투한다. 신경구로부터 분화된 세포들은 tubuline beta 3-positive (Tuj-3)의 형광을 나타내고, 이를 활용해 우리는 축삭의 배열을 확인하였다 (80%의 축삭들이 30 도 이내의 각도로 곧게 자란 것을 확인하였다). 게다가, Matrigel 의 농도에 따라 축삭의 배열은 완전하게 또는 부분적으로 형성하는 것을 알아내었다. 신경회로의 형성을 확인하기 위해 축삭과 축삭돌기 사이의 시냅스(synapse) 신호들을 측정하였다.

우리의 3 차원 축삭 형성의 유도 방법은 3 가지의 새로운 이점을 준다. (1) 축삭 성장의 방향 조절, (2) 3 차원 신경 회로의 형성과 (3 축삭 이동을 위한 ECM 겔의 활용을 통해 기존의 2 차원 배양의 한계를 극복했다. 추가로 광학 시스템을 활용하면 우리는 축삭에 상처가 회복되는 과정 또한 관찰할 수 있다.

Keyword: gel alignment, axon’s guidance, axon alignment, 3D environment,

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