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Micro Groove Structures for Directional Control of Neuronal Growth for DAI Investigation

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***Abstract*—In this study, methods for directional control of neuronal growth are proposed in order to investigate DAI in cellular level for in vitro studies. Unlike random oriented neuronal cultures, it is aimed to study the relation between applied impact and DAI on each individual cells by using directional control methods employed by straight and angled micro grooves. PC12 cells, rat adrenal medulla-derived pheochromocytoma, are used throughout the experiments.**

Keywords— PC12 cells, DAI, Microfabrication, Directional control of neuronal growth

I. INTRODUCTION

Traumatic brain injury (TBI) continues to be a leading cause of mortality and morbidity worldwide, making TBI a significant public health problem [1]. The incidence of TBI in the United States is calculated at 4,000/100,000 per year with 20/100,000 per year mortality rate [2]. In Britain, 150/100,000 persons per year survive TBI but with severe impairments [3] whereas in Japan, 50.3% of traffic accident injuries were due to TBI [4].

Additionally, increasing evidence suggests that TBI patients suffer from diffuse axonal injury (DAI), which is one of the most common pathology of TBI, associated with rapid brain deformation, stretching, inertial forces occurring as a result of traumatic incidents such as accidents, falls and assaults resulting in the stretching of neuronal axons [5-9] where primary damage to axons progressively develops into secondary cascades such as neuronal degeneration and axonal cytoskeletal disconnection [10-13].

While most common injury cascades of TBI are recognized as cytoskeletal damage, calcium influx, neurotransmitter release, and mitochondrial dysfunction, a standard treatment protocol has not yet been established [14]. Moreover, the diagnosis of DAI without through histopathological examination, especially early or exact recognition of the extent of axonal injury, still remains as a major challenge since these injuries are not promptly detectable with standard techniques such as computed tomography (CT) or magnetic resonance imaging (MRI) scans as a consequence of the microscopic and disperse nature of the axonal pathology of DAI [15-16]. Thus, investigation of injury neuromechanics is crucial in understanding neural tolerance and developing relevant therapies and/or diagnostic procedures for DAI. Applying the defined levels of physiological injury must be realized through reliable and accurate models in order to assess the extent of injury.

In this study, methods for directional control of neuronal growth are proposed in order to investigate DAI in cellular level for in vitro studies. Unlike random oriented neuronal cultures, it is aimed to study the relation between applied impact and DAI on each individual cells by using directional control methods employed by straight and angled micro grooves. PC12 cells, rat adrenal medulla-derived pheochromocytoma, are used throughout this study.

II. MATERIALS AND METHODS

A. PC12 Cells

PC12 cells, which derived from pheochromocytoma of rat adrenal medulla, are used in this study. PC12 cells are spherical cells of several tens of micro meters in diameter, when cells are cultured in the serum-containing medium without various factors. However, when PC12 cells are cultured in the medium which includes nerve growth factor (NGF), cells change into neurons which are enlarged up to about 70-100μm in diameter, compressed and elongated dendrite within several days, as shown in Fig. 1.

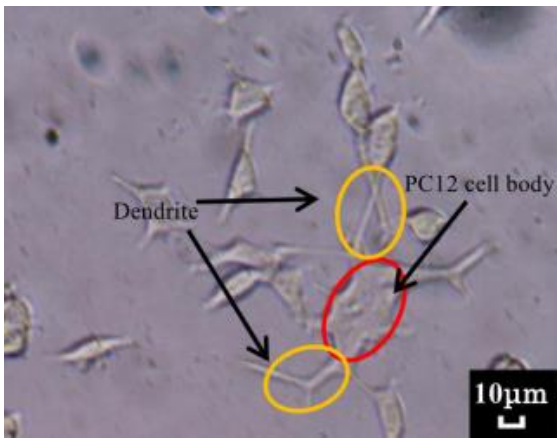


Fig. 1 *Cultured PC12 cells*

B. Microfabrication Procedure

Negative resist SU-8 (3025) (Nippon Kayaku Co., Ltd.) is coated by using spin coater on commercial Si substrate. Thickness of the resist material is determined by the rotation speed and spin time of the spin coater. By controlling these combinations, it is possible to achieve resist thickness in the range of about 5 - 50µm. This process determines the stereoscopic vision of shape. I-line light (wave length 365nm) is used for exposure of the desired patterns. After development process, developer is removed by the rinsing agent (isopropyl alcohol). Baking is done at 175 ˚C. Dimethylpolysiloxane (PDMS) resin (SYLGARD 184) (Dow Corning Toray Co., Ltd.) is poured on the resist patterns in order to transfer shape under vacuum conditions. Curing is done for 45 minutes at 65 ˚C. Micro shapes are generated by reversing of transfer the shape by resin to a mold. Peeling of PDMS is carried out soon after curing process. The diagram of the microfabrication procedure can be seen in Fig. 2.

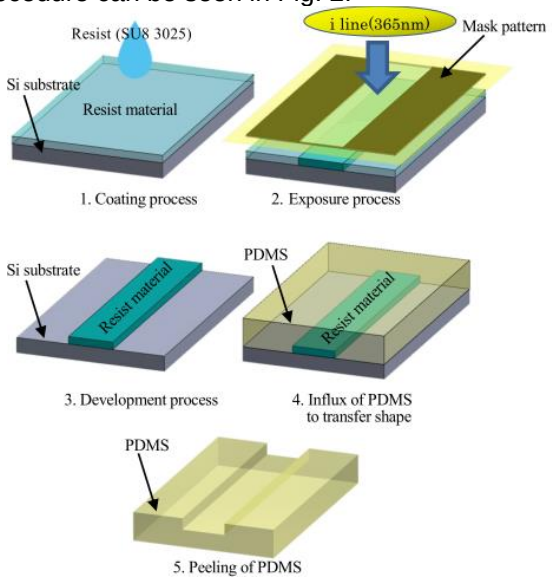


Fig. 2 *Microfabrication process*

C. Cell Culture

PDMS has hydrophobic surface property, therefore, in order to perform cell culture, plasma treatment is performed to render to PDMS surface hydrophilic. Plasma process is carried out for 10 minutes using pure O_2 gas plasma. Poly-L-Lysine (PLL) is coated on the plasma treated PDMS surface one day before cell seeding. PLL is a synthetic molecule that can be used to promote cell attachment to plastic and glass surfaces. It is useful in nerve cells and transformed cell lines for such as cell adhesion, proliferation and promoting differentiation. PC12 cells are seeded at a density of 10000 cells/cm^2 for straight micro groove designs and 5000 cells/cm^2 on angled micro groove structures. Nerve Growth Factor (NGF) is added after cell seeding. Dendrites are extended from the cells by the effect of NGF. Dendrites extend about 100 - 150µm over about 4 days. Observation of cells is carried out every day by using optical microscope. Cell extension through the micro shapes is observed. It is also evaluated whether it is possible to control the extension length in the angular direction.

III. RESULTS AND DISCUSSION

A. Straight Micro Groove Structures

PC12 cells are usually around 10µm, therefore micro groove structures are designed with the varying groove widths of 10, 20, 30, 40, 50 and 100µm with varying heights of 30 and 50µm and the length of 10mm where pitch distance between grooves is 100µm. Design specifications and actual measurements are listed in Table 1.

TABLE I. DESIGN SPECIFICATIONS AND MEASURED VALUES OF MICRO GROOVES

No		Height [µm]		Width [µm]	Measured value [µm]	
1	7	50	30	100	95.9	96.0
2	8			50	45.2	46.8
3	9			40	36.3	37.2
4	10			30	27.1	26.5
5	11			20	22.0	21.0
6	12			10	10.2	10.2

Angle of the growth direction, growth length and the number of dendrites in one micro groove are measured by using the image analysis software, Image J. Measurement results for each micro groove width and height can be seen in Fig.3.

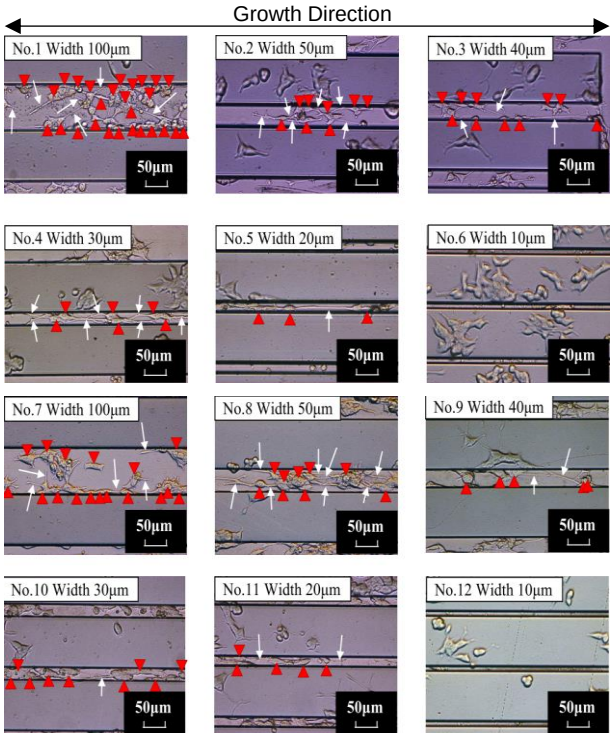


Fig. 3 *Measurements (No.1-No. 6: 50µm, No. 7-No. 12: 30µm groove height) [Arrows: Dendrites, Triangles: Cell bodies]*

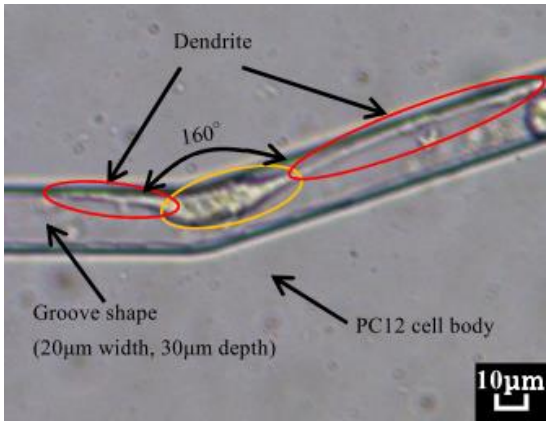
Experiments show that it is difficult to observe the growth rate of the dendrites at the early stages of the

cell culture due to the short dendrite length as well as the later stages of the cell culture due to the increased cell density. Moreover, it is observed that the growth rate of the cells differs individually and the number of cells are different in each groove. However, qualitative trends are still be able to be confirmed. It is also confirmed that time of cell density increase depends on the width and depth of the micro grooves and 10µm groove width is not suitable for PC12 cell culture. Longest elongation is observed with groove width of 20µm, shown in Fig. 3, Frame 5, with the smallest growth angle. However the effect of groove height of 50 and 30µm is not observed throughout the experiments. Results suggested that the smaller the growth direction angle the more probability for the dendrite elongation. Also, dendrites tend to elongate longer if there are no other cells in the elongation direction. Therefore, it is thought to be better to decrease the cell density for the next experiments in order to achieve longer dendrite elongation.

B. Angled Micro Groove Structures

Results from the straight micro groove experiments showed that 20µm groove width is the most suitable however cell density needs to be decreased in order to improve the dendrite growth length. Therefore, 5000 cells/cm^2 cell density used throughout the cell culture for the angled micro groove experiments. Experiments are carried out by using micro grooves with 20µm width, 30µm depth and angles ranging from 20-160 degrees with 10 degrees increments.

It is observed that the direction control of dendrite growth can be successfully achieved by using angled micro grooves and examples for 160 and 120˚ angles are shown in Fig. 4.



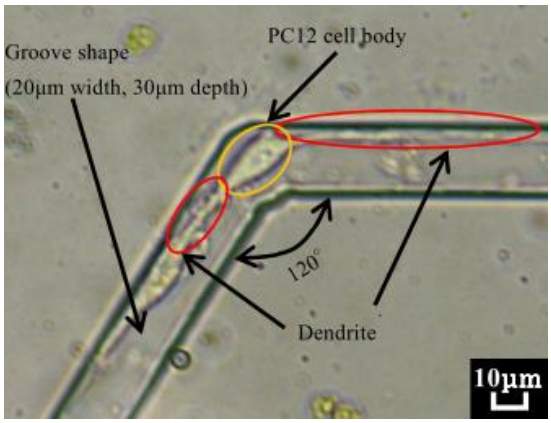
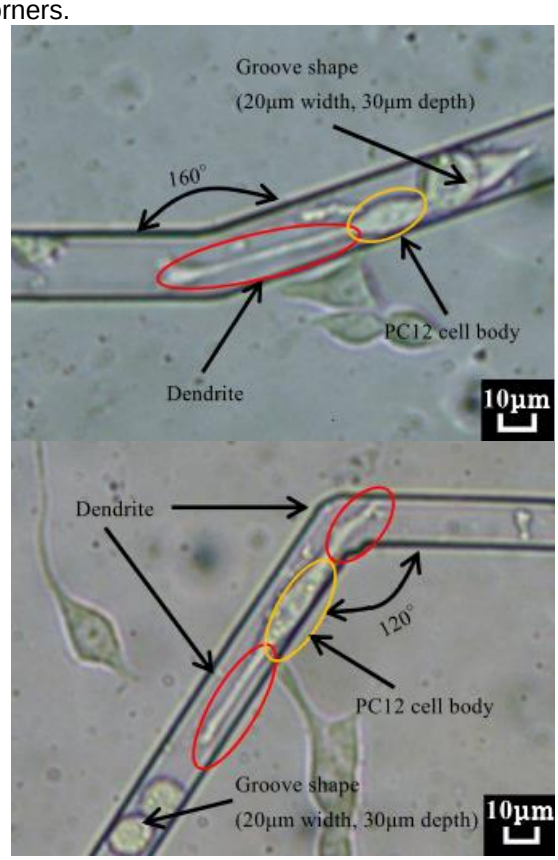


Fig. 4 *Dendrite growth in 160 (upper) and 120˚ angled (lower) micro grooves*

However, it is also seen that if the cell body is not placed at the angled corner of the micro grooves, the successful dendrite elongation may not be achieved. Fig. 5 shows some examples of elongation obstructions with 160, 120 and 80˚ angled grooves caused by cell bodies placed in the grooves not at the corners.



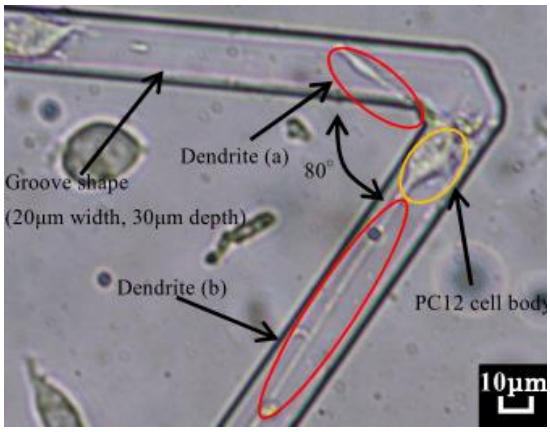


Fig. 5 *Examples for elongation obstructions*

As can be seen in Fig. 5, for any angle range, results suggest that dendrite elongation may be obstructed by the micro groove walls when cell body is not aligned at the angle corner of the micro groove structure.

IV. CONCLUSION

The straight micro groove configurations have been successful in controlling the linear elongation of dendrites. It is observed that PC12 cell bodies in the micro groove structures can deform into elliptical shapes making it possible to control the growth direction. However, it is also seen that in angled micro groove configurations, the elongation of dendrites depends on the position of the cell body. Since it is confirmed that cell body needs to be contained at the angled corner of the micro groove structure to ensure elongation, future work will focus on that point. For this purpose, it is planned to fabricate new angled micro groove structures with a deeper pocket at the corner part for accurate positioning of the cell body for future experiments.

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