4

# Cell Differentiation Induction Using Extracellular Stimulation Controlled by a Micro Device

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## 1. Introduction

The stem cell differentiation is greatly dependent on the living environment *i.e.*, the cell differentiation determined by timing, amplitude, amount and etc. of stimulation from outside of cells (Lanza & Rosenthal, 2004). If living environment of cell can be controlled artificially by using micro device, we will be able to guide the cell having specific function from stem cells. The micromachining technology allows integration of various mechanical, electrical, and chemical elements, and has produced micro devices that can manipulate chemical solution, small mechanical parts, cells and etc. (Meyer et al., 2000; Takeuchi & Shimoyama, 1999; Nakashima et al., 2010; Nakashima & Yasuda, 2009; Chen et al., 2007; Taff & Voldman, 2005; Choi & Park, 2005; Doh & Cho, 2005). Our purpose is to fabricate micro devices which can control cell differentiation and axon elongation by extracellular stimulation. The cell differentiation induction on the micro devices will be applied to a technique for restoring impaired or lost biological function and controlling differentiation of a stem cell to a specific cell. This paper presents two micro devices intended to control the induction of cell differentiation dynamically by chemical stimulation and mechanical stimulation. First, we present a chemical stimulation device consisting of a microvalve, a nano-holes array, and a chamber, which are placed very close to one another. The amount of chemical solution released from the nano-hole array can be controlled very precisely by opening and closing the microvalve. Also, we show the behavior of cells stimulated using the fabricated chemical stimulation device, *i.e.*, differentiation guidance of cells using release control of nerve growth factor (NGF) which is a protein that enhances axonal outgrowth from a cell body. Next, we present a mechanical stimulation device consisting of a chamber for cell culture and a microlinkage mechanism for applying uniaxial stretching to the microchamber. Then we show the fluorescence observation of behavior of a cell that receives stimulation by fabricated mechanical stimulation device.

## 2. Chemical stimulation device

### 2.1 Design of the chemical stimulation device

The microdevice we designed for chemical stimulation consists of a nano-hole array for NGF release, a hydrophobic passive microvalve for controlling NGF release, a microchannel

for carrying NGF and a chamber for cell culture as shown in Fig. 1(a, b) (Nakashima & Yasuda, 2005; Nakashima & Yasuda, 2009). The device is microfabricated from a SOI (Silicon on Insulator) wafer as detailed below. A chamber for cultivating a cell was fabricated on one side of the wafer, and a microchannel was fabricated on the other side. The nano-hole array was fabricated in a $SiO_2$ membrane of 0.5 μm in thickness. The inside diameter of the nano-holes must be less than 0.5 μm because the typical diameter of an axon terminal is 0.5 – 4 μm. The etching technique using FIB (Focused Ion Beam, Seiko Instruments Inc., JFIB-2300) satisfies the need for the fabrication of such small holes. Nerve cells are cultured on a $SiO_2$ diaphragm including the nano-hole array.

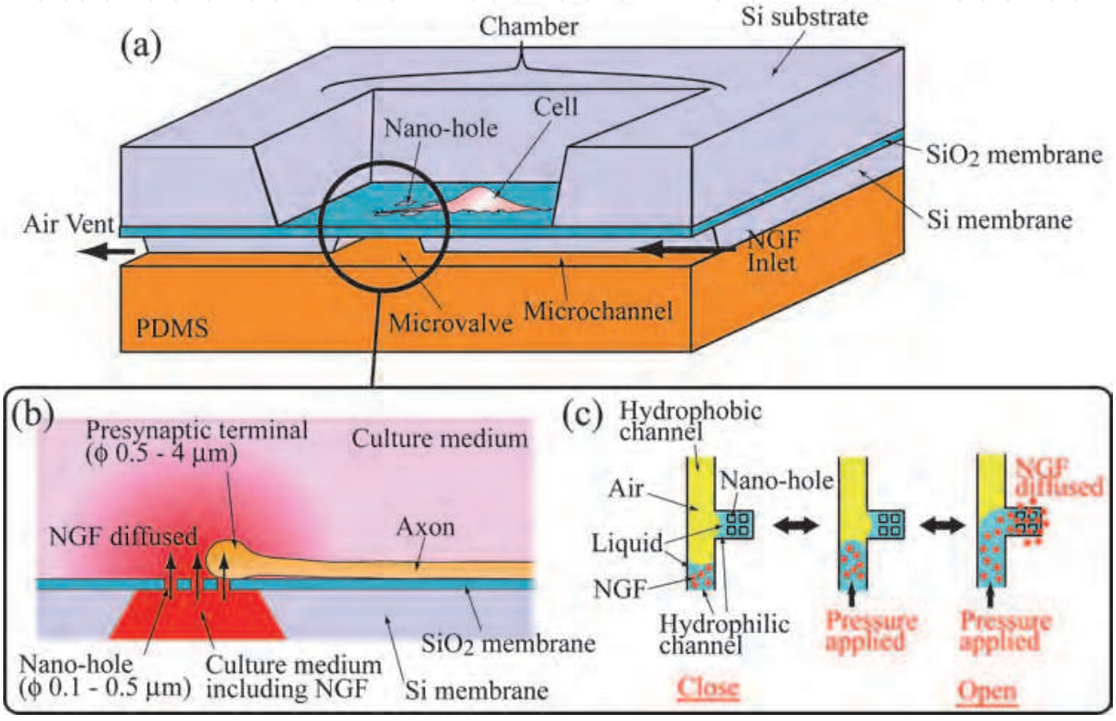


Fig. 1. Schematic of a fabricated chemical stimulation micro device.

Figure 1(c) shows the schematic of liquid switching principle of a microvalve using different liquid surface tensions on hydrophilic and hydrophobic channels. Liquids injected into the hydrophilic channels stop at the boundary between the hydrophilic and hydrophobic channels. The air that was initially in the hydrophobic channel separates the two liquids. When pressure is applied from the inlet, the inlet liquid will break into the hydrophobic channel and merge with the outlet liquid. If the inlet liquid includes NGF, it will diffuse into the outlet liquid. This is a hydrophobic passive microvalve which has very simple structure without any mechanically movable parts and which can be driven by low pressure (Yun et al, 2002; Lee et al, 2002).

Figure 2 shows the SEM photograph which was taken from the channel side of the device. The microchannel measures 60 μm in width and 8 μm in depth. 100 nano-holes with a square side of about 500 nm in length were opened in the $SiO_2$ membrane inside the channel. The microvalve was constructed at the T-shaped crossing of the microchannel.

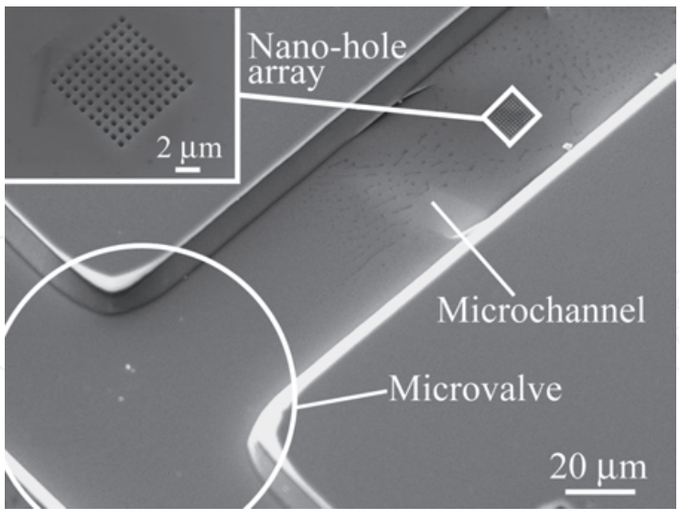


Fig. 2. SEM photographs of the fabricated device. The microchannels including a hydrophobic passive microvalve and nano-hole array.

### 2.2 Device fabrication

Figure 3 shows the fabrication process of the chemical stimulation device. $SiO_2$ films were created by thermal oxidation on both sides of a SOI wafer that consists of 350 μm thick handle Si layer, 500 nm thick $SiO_2$ layer, and 8 μm thick Si layer (Fig. 3(a)). The SOI wafer was spin-coated with photoresist ZPN-1150 (ZEON Corp.) on the front side, and the $SiO_2$ film was patterned by photolithography and wet etching. In order to fabricate the culture chamber, the thick Si layer was etched anisotropically (Fig. 3(b)). Next, the microchannel was fabricated by anisotropically etching the backside of Si layer (Fig. 3(c)). Then, the microvalve was fabricated by lift-off method of Au deposition and by creating hydrophobic SAM (self assembled monolayer), 1-octadecanethiol on the Au surface (Fig. 3(d)). The nano-holes were fabricated in a $SiO_2$ membrane by FIB (Fig. 3(e)). Finally, the culture chamber was coated by collagen, and the microchannels were covered with the PDMS sheet.

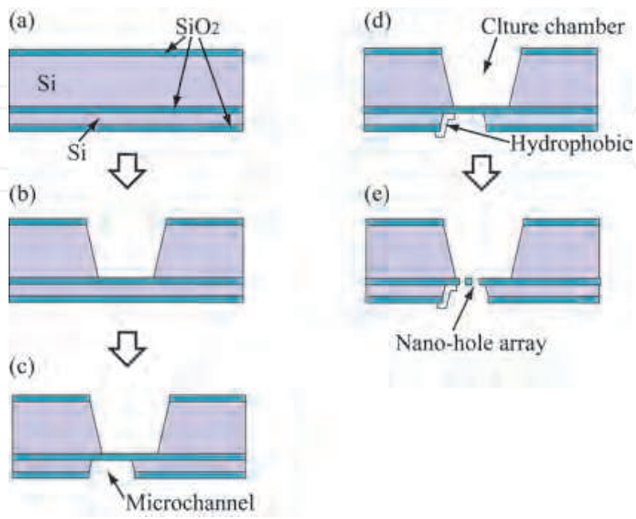


Fig. 3. Fabrication process of the chemical stimulation device.

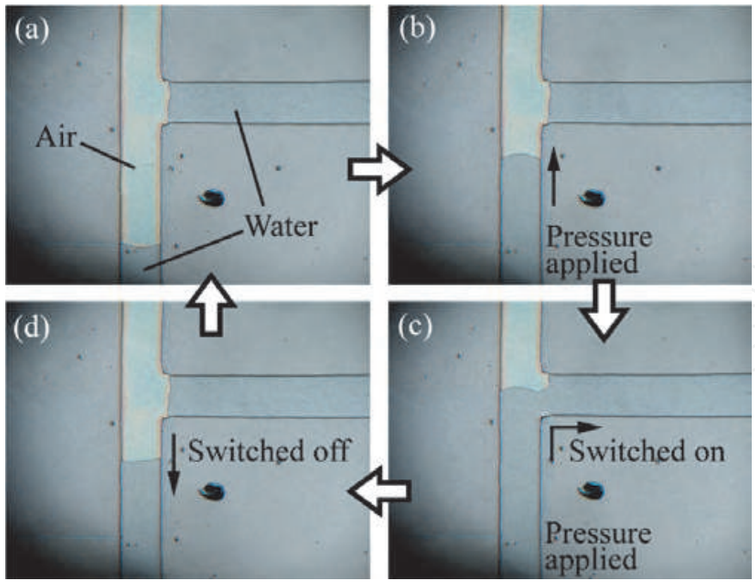


Fig. 4. Photograph of liquid switching test using the fabricated microvalve. (a) The initial state. (b) Inlet pressure was applied. (c) The valve opened. (d) The valve closed after eliminating inlet pressure.

### 2.3 Switching test of the microvalve and chemical release tests

Figure 4 shows the photograph of the test result using deionized water as the test liquid. When water was injected into the channel, it was infiltrated into the hydrophilic channel by the capillary force and stopped at the edge of the hydrophobic channel. Inlet water was initially separated from outlet water (Fig. 4(a)). When inlet pressure was applied, inlet water began to flow through the hydrophobic channel (Fig. 4(b)) and eventually merged with outlet water (Fig. 4(c)). When inlet pressure was eliminated, water stopped flowing and separated into two fluids. At the inlet, water flowed backwards (Fig. 4(d)) and returned to the original state (Fig. 4(a)). This enabled us to open and close the microvalve with inlet pressure. Also, we confirmed that when culture medium is used as a working fluid having higher viscosity than water, the fabricated microvalve could open and close.

### 2.4 Chemical release tests

Chemical release tests were conducted using the fabricated microfluidic device. We observed diffusion of the fluorescent dye (Rhodamine B, Molecular Probes, Inc., R-648) released through the nano-hole array under the inverted microscope (Nikon Co., TE2000). Figure 5 shows experimental setup for chemical release testing. When pressure was applied from the liquid inlet, a hydrophobic microvalve will open and the fluorescent dye oozed from the nano-hole array and spread out into the deionized water that filled the chamber of the device.

Figure 6 shows that the fluorescent dye was oozing from the nano-hole array and spreading out into the deionized water by diffusion. The fluorescence intensity was measured at several positions (see Figure 6(b)) at 60 sec after the diffusion began. The dye concentration decreased by 56 % at the position of 50 μm away from the nano-hole array as shown in Figure 7.

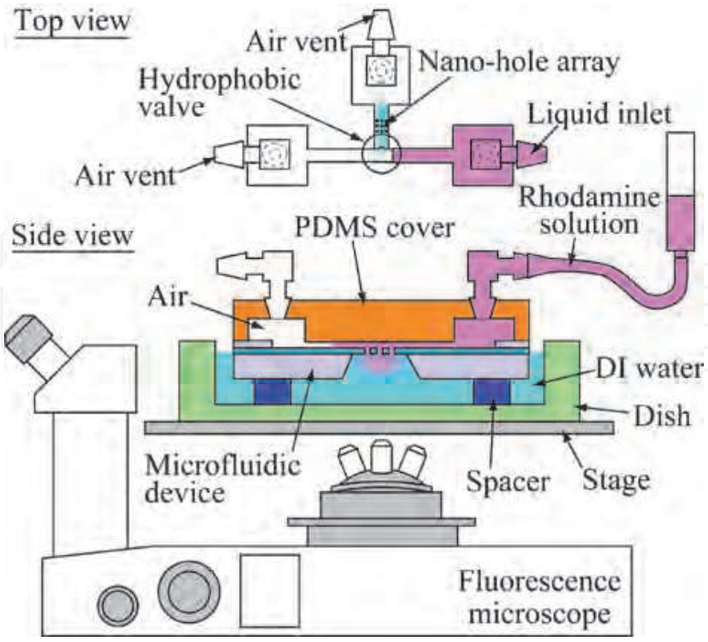


Fig. 5. Experimental setup. The fabricated microfluidic device was covered with PDMS film. Diffusion of the fluorescent dye through the nano-hole array was observed under the inverted microscope.

The variation in the fluorescence intensity synchronized with opening and closing of the microvalve as shown in Fig.8. The fluorescence intensity rose immediately after the valve opened, and decreased immediately when the valve was closed. Additionally, the fluorescence intensity changed according to the switching interval, the smaller the fluorescence intensity change. This result means that the fabricated device can control release of chemical solution by opening and closing of the valve.

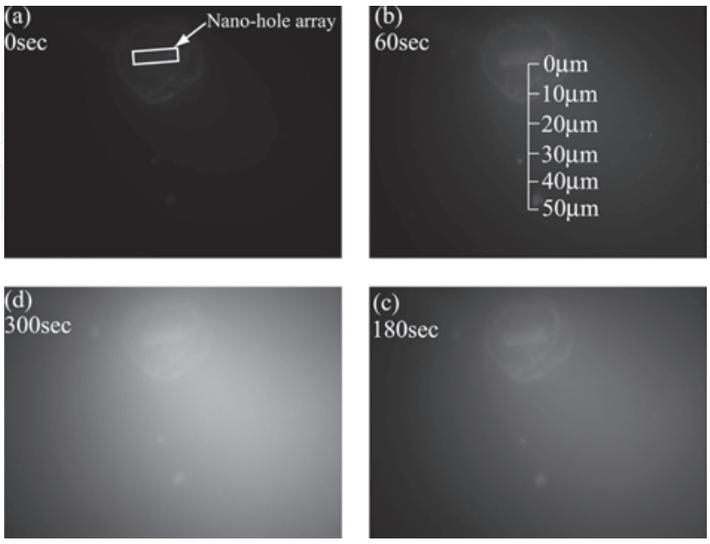


Fig. 6. Diffusion of fluorescent dye through a nano-hole array. Light gray indicates high concentration of fluorescent dye.

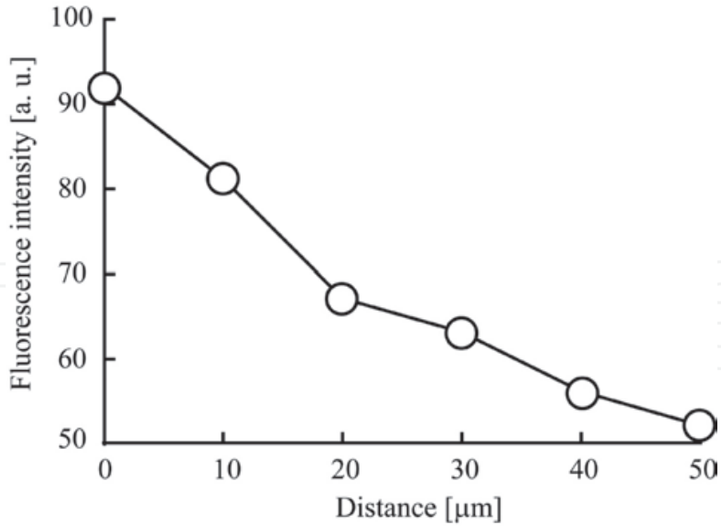


Fig. 7. Relationship between the fluorescence intensity and distance from the nano-hole at 60 sec after the diffusion began.

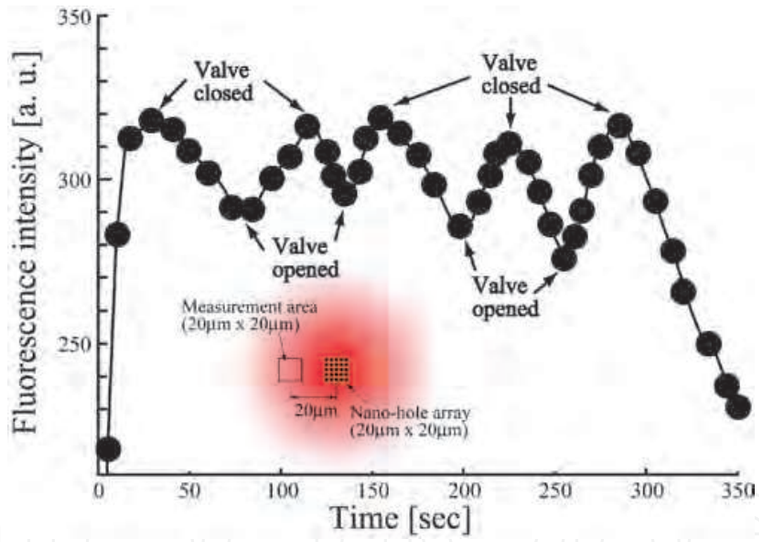


Fig. 8. Variation in the fluorescence intensity after the microvalve is opened and closed repeatedly.

### 2.5 Cell differentiation induction test by chemical stimulation

PC 12 (adrenal pheochromocytoma) cells were used in the experiments and prepared as follows. The cells were maintained in culture medium (RPMI1640, SIGMA-ALDRICH Corp.) supplemented with 10 % fetal bovine serum and 100 ng/ml nerve growth factor (NGF). The cells were cultured in 5 % $CO_2$ at 37 °C for 3 days to start their differentiation and make them sensitive to NGF stimulation. Then, these cells were collected by pipetting, and cultivated without NGF on the cell culture chamber of the fabricated device in 5 % $CO_2$ at 37 °C for 30 min. Finally, the cells were stimulated chemically by releasing NGF through the nano-hole array on the device.

We confirmed whether cell differentiation can be controlled on the device or not. Figure 9 shows experimental procedure for cell differentiation induction. Growth of cells stimulated by NGF is observed under a microscope from the chamber side. The inlet culture medium containing NGF and the culture medium in the cell culture chamber are initially separated by a closed hydrophobic microvalve. When pressure is applied from the inlet, the inlet liquid and the chamber liquid are connected by opening the microvalve. After that, NGF diffused into the chamber liquid and is released into the chamber through the nano-hole array. Thus, the cells in the chamber are stimulated by NGF.

We conducted the experiment that was NGF stimulation controlled by periodic switching of the microvalve using the fabricated microdevice. We observed the growth of cultured cells while the microvalve was switched at 1Hz under the inverted microscope. As shown in Fig.10, the lower left cell that adhered to the device surface elongated the axon toward nano-holes as time passed. On the other hand, because the right side cell did not adhere to the device surface at first, it could not begin to differentiate. However, after it succeeded in adhering to the device surface 2 hours after the original stimulus was applied, the right side cell also demonstrated our intended movement manner, *i.e.* it elongated the axon toward the nano-holes as time passed. From this result, we conclude that we can guide the cell differentiation and the axon elongation by the fabricated microdevice.

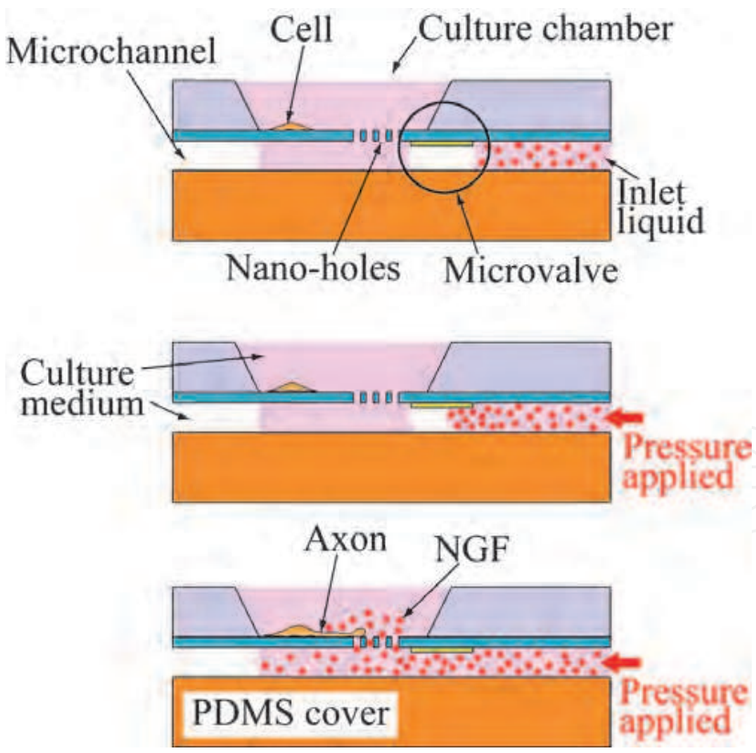


Fig. 9. Experimental procedure for cell differentiation induction using fabricated microdevice.

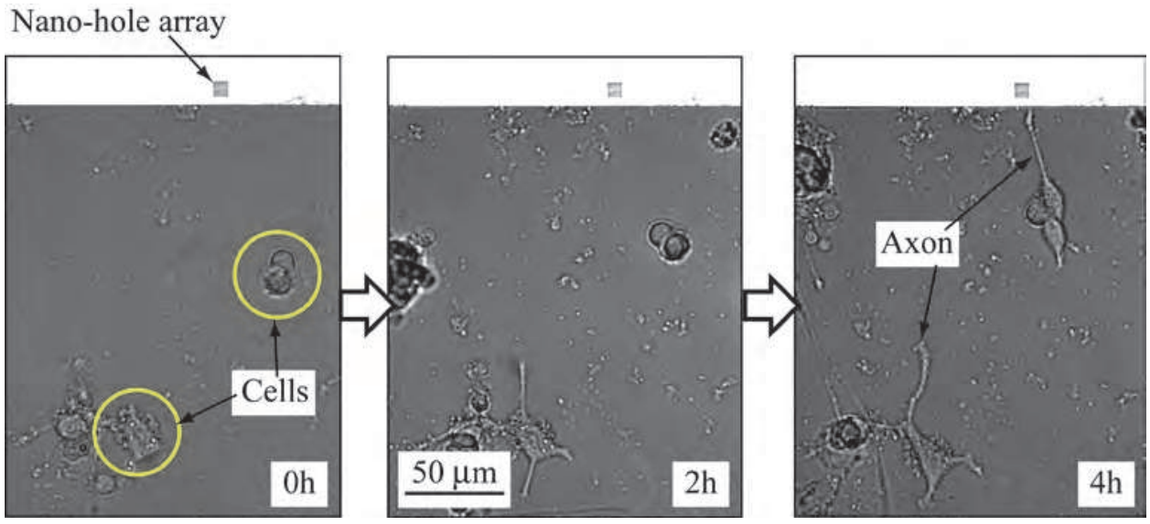


Fig. 10. Cell differentiation guidance on the fabricated microfluidic device. The axons elongated toward the nano-holes according to NGF stimulation from the nano-holes.

## 3. Mechanical stimulation device

### 3.1 Design of the mechanical stimulation device

The mechanical stimulation device developed in the present study is fabricated on a glass coverslip and 6 devices are fabricated on one coverslip. This coverslip with the microdevice is bonded to the bottom of a $\phi$ = 35 mm culture dish with a 14 mm hole and used in the experiment. With this device, an inverted microscope and an oil-immersion objective lens can be used for the observation. The schematic of a mechanical stimulation device is shown in Fig. 11. The size of each device is 2 mm square. This device consists of one microchamber made of PDMS, one pair of stretching arms by which the microchamber is uniaxially stretched, the journal bearings that support the stretching arms, and the slider that drives the stretching arms (Sato et al., 2010). The slider is pushed by a microneedle connected to the micromanipulator that drives the pair of stretching arms. Finally, sliding movement is converted into rotation movement via the journal bearing, and the microchamber is stretched from each side.

Figure 12 shows the SEM photograph of the fabricated device. A micro three-dimensional structure was fabricated by multiple exposures to SU-8. The dimension error in the link mechanism was measured as approximately 5 μm. The clearance in the journal bearing and slider was designed to be 10 μm to allow the dimension error arise in the fabrication process, therefore, the journal bearing, the most important element in the link mechanism, was successfully fabricated. It was also confirmed that the slider was successfully fabricated and that all of the link mechanisms were movable structures. The chamber has a thin film stretching part (stretching substrate) in the center of the chamber. The size of the stretching substrate is 400 x 100 μm and the thickness is 10 μm. The stretching substrate is supported by the surrounding support frame with the size of 50 μm width and 50 μm thickness. The microchamber also has a binding part with stretching arms at both the ends of the support frame. In the fabrication process, six microchambers are molded on one glass coverslip at once. The excess part is cut and removed to obtain the chamber shape.

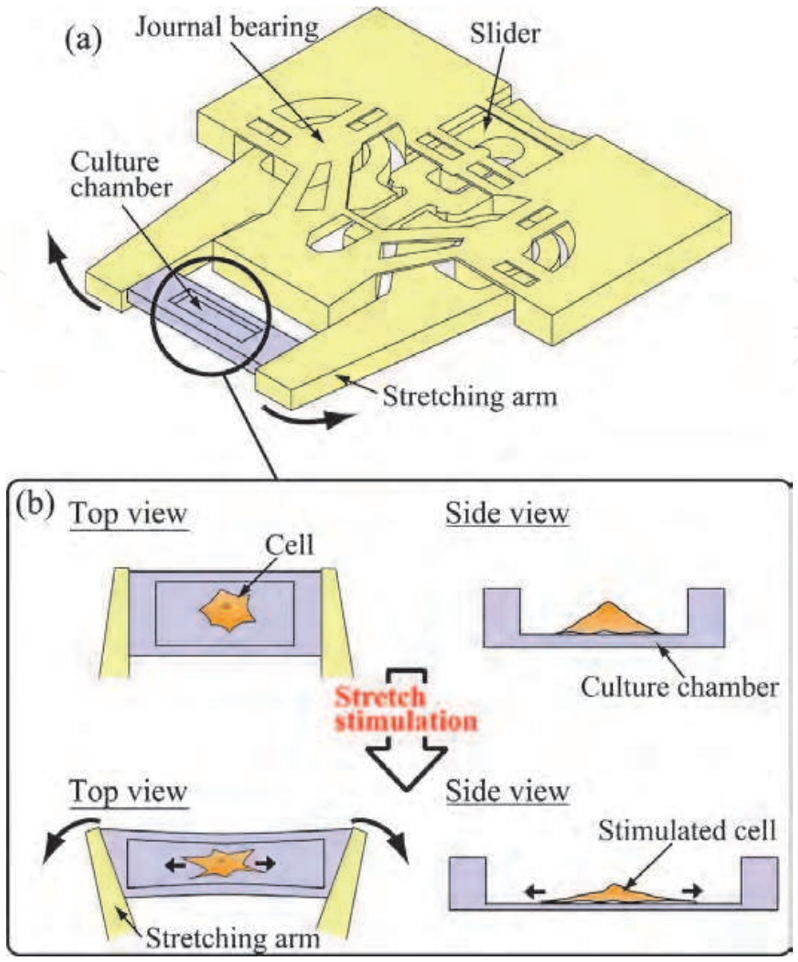


Fig. 11. Schematic of a fabricated mechanical stimulation micro device.

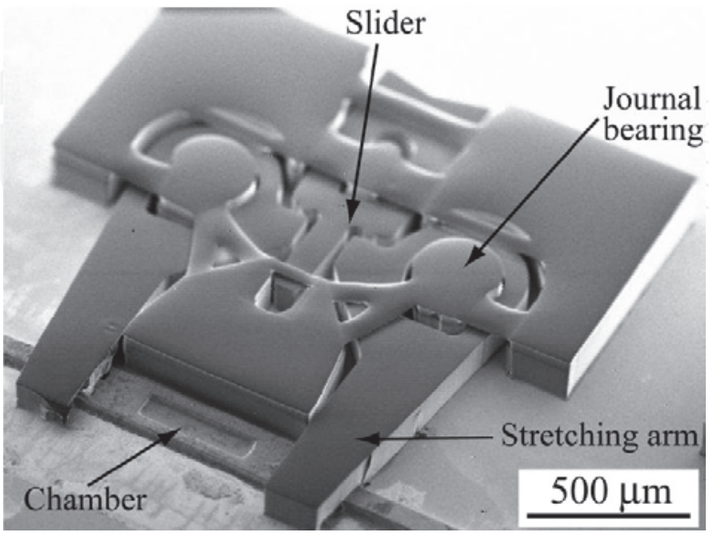


Fig. 12. SEM photograph of the fabricated mechanical stimulation micro device.

### 3.2 Device fabrication

The fabrication process of the device consists of two processes. One is the micromolding of silicone elastomer (PDMS) to fabricate the microchamber. The other is the patterning of thick photoresist to fabricate the link mechanism. The microchamber is made of transparent PDMS enable the observation using the inverted microscope. Although PDMS is generally used in the experiment of applying stretching deformation as a mechanical stimulus to cells (Costa et al., 2002), its chemical stability makes it difficult to shape by a microfabrication technique such as etching. Therefore, we adopted micromolding to fabricate the microchamber.

The fabrication process is shown in Fig. 13, and an outline of the process is described below. In the first step of the fabrication process of the microchamber, an original model of the microchamber for molding is prepared. The photoresist (SU-8, Kayaku MicroChem Co., Ltd) is spin-coated to 10 μm thickness (Fig. 13(a, b)), exposed and developed to pattern the stretching substrate (Fig. 13(c)). A second layer of SU-8 with 50 μm thickness is spin-coated onto the first layer (Fig. 13(d)), exposed and developed to pattern the support frame (Fig. 13(e)). By these two steps, an original model of the microchamber is obtained. In the next step, following the fabrication of the original model, the fluorine polymer is sprayed onto the original model to form very thin layer as mold-releasing agents (Fig. 13(f)). PDMS (KE-106, Shin-etsu Silicone) is casted on the original model to obtain a mold transcribed with the shape of the original model (Fig. 13(g)). The obtained mold is again sprayed with fluorine polymer, and punched to form the gate for molding (Fig. 13(h, i)). Finally, the microchamber made of PDMS is molded by using the obtained mold. The link mechanism is fabricated by the multilayer SU-8-based MEMS process (Foulds, I.G. & Parameswaran, M, 2006). This fabrication process includes multiple exposures to obtain movable three-dimensional microstructures.

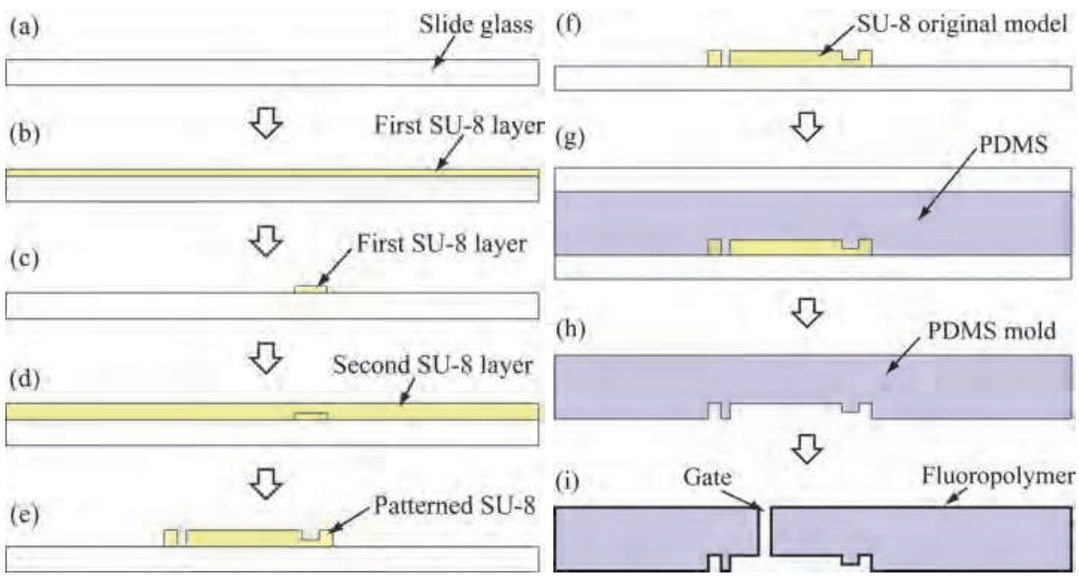


Fig. 13. Fabrication process of the original model and the micromold for the fabricated microchamber.

The fabrication process chart is shown in Fig. 14, and an outline of the process is described below. First, Ge is sputtered onto the glass coverslip and patterned as the sacrificial layer by which the microchamber (Fig. 14(j)), stretching arms and slider part are rendered movable. The PDMS microchamber is molded on the Ge-patterned coverslip (Fig. 14(l-n)). The first layer of SU-8 is spin-coated to 100 μm thickness (Fig. 14(o)), and exposed using the photomask of the link mechanism pattern (Fig. 14(p). In addition, without development, the second layer of SU-8 is spin-coated to 50 μm thickness on the first layer (Fig. 14(q)). The second exposure is applied to pattern the axis of the journal bearing (Fig. 14(r)). Finally, the surface exposure technique is applied to fabricate the stopper of the journal bearing (Fig. 14(s)). After the curing and the development of the entire device structure (Fig. 14(t)), the Ge sacrificial layer is removed and the link mechanism is completed (Fig. 14(u)).

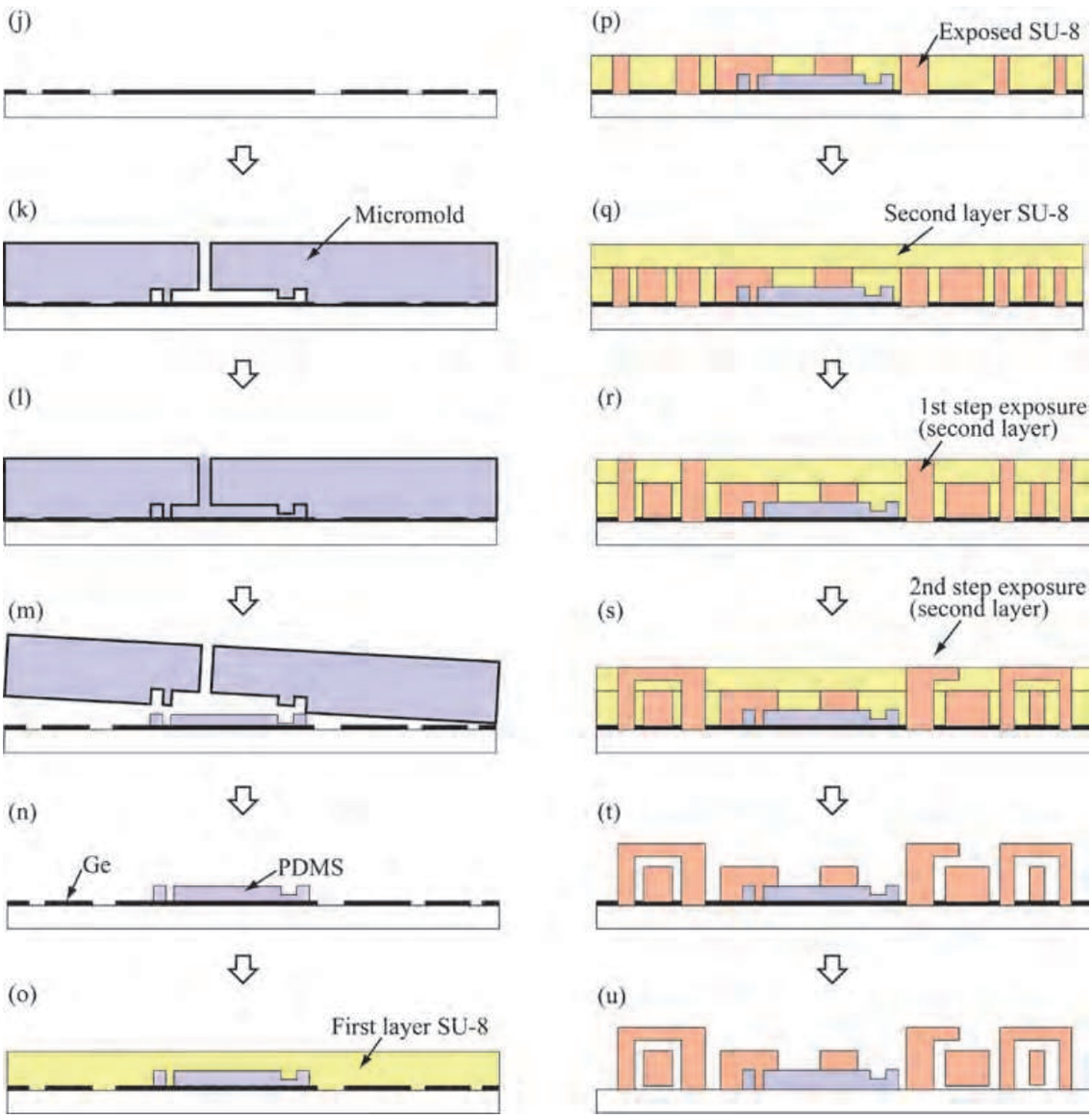


Fig. 14. Fabrication process of the microlink mechanism for mechanical stimulation device.

### 3.3 Operation test of mechanical stimulation device

The fabricated device on the glass coverslip was immersed in DI (deionized) water and tested. Figure 15 shows the image during the operation test using an inverted phase-contrast microscope (Olympus Co., CKX-41). Figure 15(a) shows the initial state of the device. Figure 15(b) shows the state after the slider was pushed by tweezers and the microchamber was stretched from both ends in the directions to the right and lift in the figure. It was shown that the fabricated device was actually able to work under observation using the inverted optical microscope.

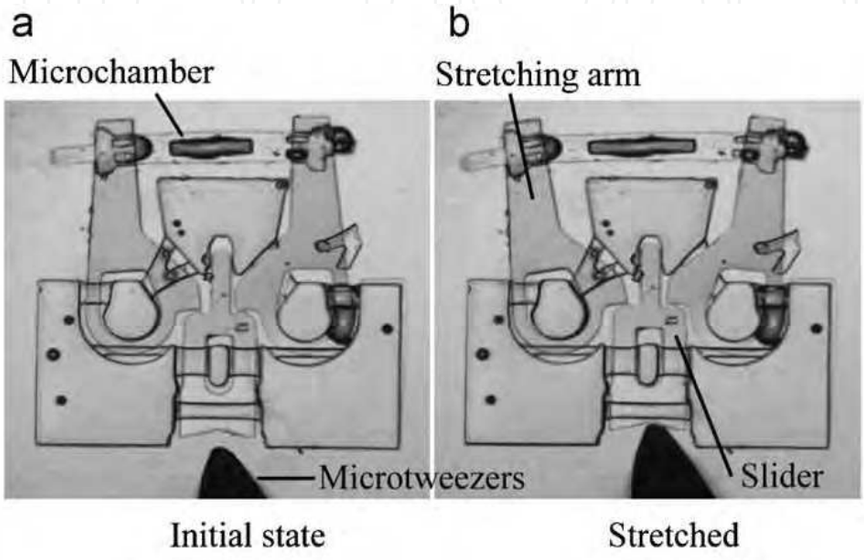


Fig. 15. Operating image of mechanical stimulation device. (a) The initial state. (b) The stretched state.

### 3.4 Cell stretching test by the mechanical stimulation device

We carried out the biocompatibility test by cell culture on the fabricated mechanical stimulation device. The cells were incubated using D-MEM with 10 % FBS (Fetal Bovine Serum), and in 37 °C, humidified 95 % air-5 % $CO_2$ atmosphere. In the preparation for the test, cultured cells were seeded into the dish with the built-in cell-stretching microdevices, and incubated for 8h. After the preincubation, fluorescent indictor dye Fluo 3 was loaded to the cell as the observation marker by incubating the cell in the opti-MEM containing 12 μM Fluo 3-AM (Dojindo) for 30 min. After loading the indicator dye, the cells were rinsed twice with PBS (phosphate buffered saline), and used for the test in normal culture medium. The epifluorescent inverted microscope was used for observation with a x 60 oil immersion objective lens.

Figure 16 shows the time lapse image of the stimulated single cell cultured on the chamber. For the sequential stretching operation, Fig. 16(b) shows an image immediately before the operation where we define t = 0.0 s. Cells are located slightly above the center in the figure. Two particles slightly below the center are dust particles of fluorescence dye. Figure 16(c) shows an image immediately after the stretching operation, t = 0.1 s. Stretching operation was continued to t = 0.7 s (Fig. 16(f)) and the amount of maximum nominal tensile strain in stretching axis was roughly estimated as 13 % by measuring the displacement of dust

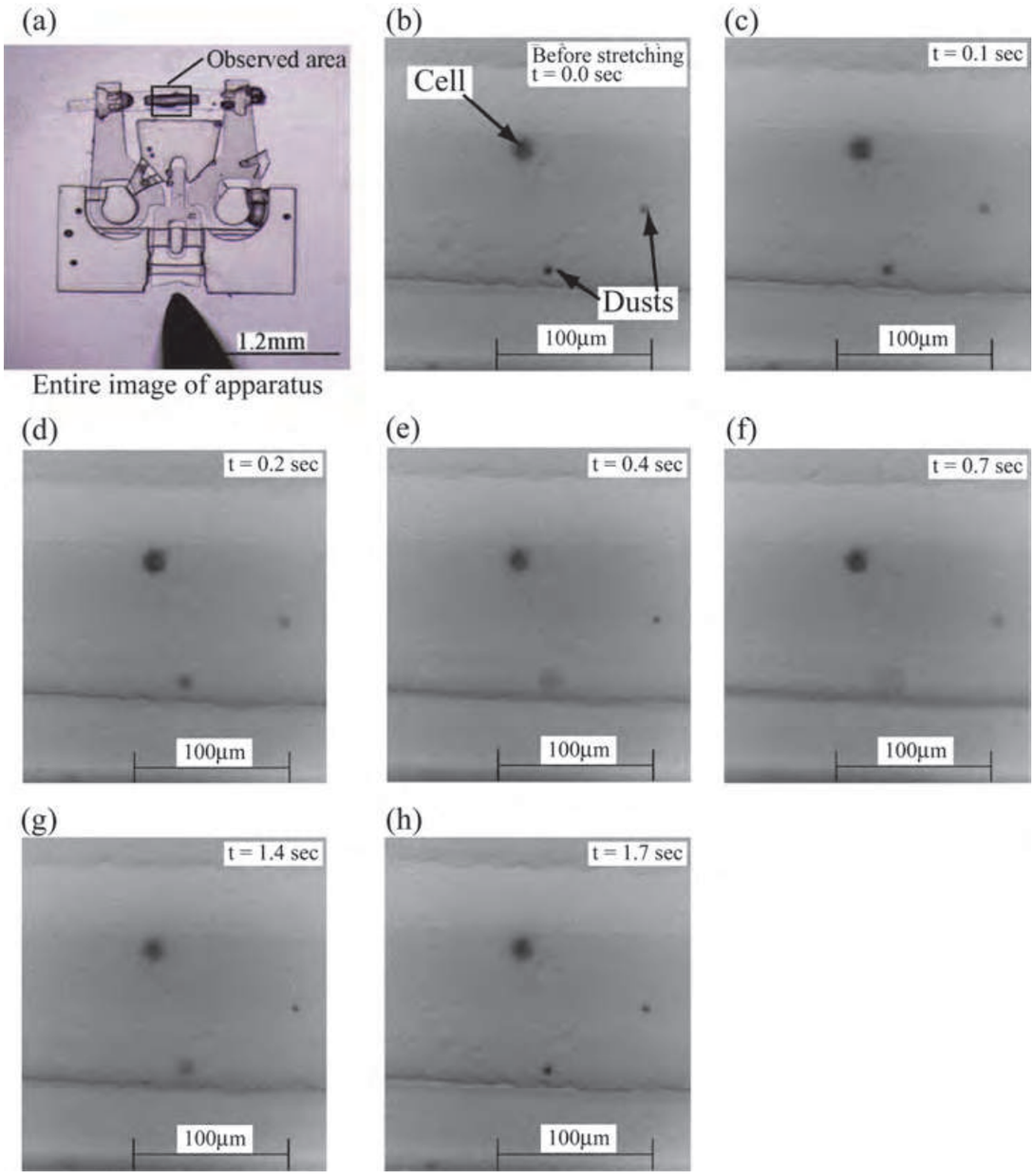


Fig. 16. Sequential time lapse images of a single cell uniaxially stretched using mechanical stimulation micro device. Dark gray indicates higher fluorescent intensity.

particles. Immediately after the maximum stretching, the stretching operation was stopped, and the microchamber returned to the initial state at t = 1.7 s (Fig. 16(h)). As a result, the cell successfully adhered to the stretched substrate and received the stretching stimulation in the microchamber. This result indicates that the fabricated mechanical stimulation device is biocompatible and able to apply dynamic mechanical stimulus to a cell. Also, the observed cell stayed in the field of view, indicating that this device can reduce the rigid displacement of the cell during the stretching operation. Therefore, this device can be used in the observation experiments of reaction to a mechanical stimulus of a cell such as the alteration of cellular morphology and cell differentiation induction.

## 4. Conclusions

This paper demonstrated that two micro devices for dynamic cell differentiation induction. First, we demonstrated that a chemical stimulation micro device consisting of nano-hole array for chemical release and a hydrophobic passive microvalve for its release control is effective in controlling NGF concentration that is required to control stimulation to a cell. Release of chemical solution such as culture medium could be controlled precisely by opening and closing the microvalve because the microvalve was placed very close to the nano-hole array. Furthermore, we succeeded in cell differentiation guidance by controlling NGF release from nano-hole array.

On the other hand, we evaluated the performance of the mechanical stimulation micro device consisting of the culture chamber, the stretching arms, the journal bearings and the slider. Mechanical stimulation to a cell can be controlled by regulating of the chamber contraction with the slider operation. We succeeded in observation of the single cell reaction behavior stimulated by stretching. This result indicated this device can induce the cell differentiation by stimulation control based on cellular reaction.

These devices are able to evaluate the response and morphology transformation of pheochromocytoma based on the chemical/mechanical stimulation. Also, these results suggest that the fabricated two devices can dynamically induce the stem cell differentiation by controlling chemical and mechanical stimulation.

## 5. Acknowledgment

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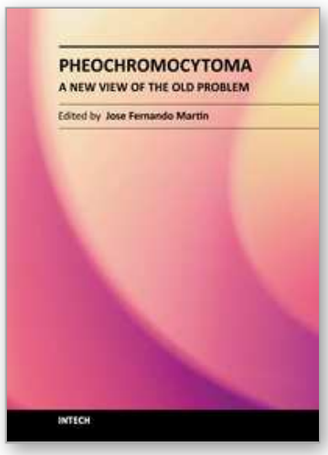


**Pheochromocytoma - A New View of the Old Problem**

Edited by Dr. Jose Fernando Martin

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The book is divided into six sections. The first three sections focus on the pathophysiology of the disease, showing anatomo- and histopathological aspects, experimental models and signaling pathways and programmed cell death related to pheochromocytoma. The fourth discusses some specific aspects of clinical presentation, with emphasis on clinical manifestations of headache and heart. The fifth section focuses on clinical diagnosis, laboratory and imaging, including differential diagnosis. Finally, the last section discusses the treatment of pheochromocytoma showing clinical cases, a case about undiagnosed pheochromocytoma complicated with multiple organ failure and other cases about catecholamine-secreting hereditary tumors. Thus, this book shows the disease "pheochromocytoma" in a different perspective from the traditional approach. Enjoy your reading.

**How to reference**

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