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# Toward the Precise Control of Cell Differentiation Processes by Using Micro and Soft Lithography

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## 1. Introduction

Regeneration of the central nervous system (CNS), where proliferative potency is limited, is one of the most important research themes in neuroscience and neuroengineering. Pluripotent stem cell lines have attracted broad attentions as an important model system for regenerating damaged brain. The important key for realizing regenerative medicine using pluripotent stem cells is to induce undifferentiated cell into objective cells with high efficiency and reproducibility. Although the methods to induce embryonic stem (ES) cells into specific neuronal subtype with pharmacological treatment have been proposed, however, the handling is difficult and thus the efficiency rate of objective cells is low (Barberi et al., 2003). The main reason for low differentiation rate of pluripotent stem cells is difficulty in precise control of interaction between pharmacological treatment and cell signalling. To overcome these problems, development of alternative methods for precise control of cell differentiation processes is required.

During cell differentiation, both endogenous factors, such as cell-cell signal transmission, and exogenous factors, such as pharmacological application, play important roles. Micro and soft lithography-based surface modification of culture substrate enabled to control stem-cell-aggregation (EB; Embryoid body) sizes and therefore to increase cell differentiation efficiency through promotion of cell-cell signal interactions (Karp et al., 2007; Wang et al., 2009). Particularly, Park et al. reported that small-size EBs of mouse ES cells (100 ~ 200  $\mu\text{m}$  of diameter) tended to differentiate into ectoderm and large-size EB (500  $\mu\text{m}$  of diameter) into mesoderm (Park et al., 2007). These reports suggested that manipulation of EB size and shape could affect endogenous factors of stem cell EBs and thus was an important approach for precise control of differentiation processes. Another important technique for regulating cell differentiation is applying physical stimulation. It was reported that electrical or magnetic stimulation could induce cellular and molecular responses and affected the gene expressions during differentiation (Kimura et al., 1998; Piacentini et al., 2008). Particularly, Yamada et al. reported that applying electrical stimulation induced mouse ES cells efficiently into ectoderm cells (Yamada et al., 2007). These reports suggested

that applying physical stimulation could affect exogenous factors and also differentiation processes of stem cells. Although the experimental methods to regulate the endogenous or exogenous signalling have been proposed as above, increase in differentiation efficiency is inadequate for regeneration therapy, and combination of the two approaches is not reported.

Based on these problems, we have tried to propose a precise control method for cell differentiation by combining EB size treatment and electrical stimulation technique. To do this, we developed microcavity-array device with embedded electrodes (Takayama et al., 2009). Uniform-sized EBs of P19 cells were prepared and aligned in the electrode substrate, and were stimulated uniformly and simultaneously. However, difficulty in handling EBs and limited number of stimulating EBs hindered further analysis. In this chapter, we describe an alternative method to stimulate more number of EBs based on soft-lithography technique and recent attempt to analyse the effects of EB size treatment and electrical stimulation on the gene expression of P19 cells.

## 2. Electrical stimulation of EBs with cell-nonadhesive electrode substrates

In this section, we summarize the methods and the results of ensemble electrical stimulation of P19 EBs with the microfabricated electrode substrates, as we reported before (Takayama et al. 2009).

The experimental procedures for fabricating microcavity-array electrode substrate are described as follows. A glass substrate with a transparent conductive layer (ITO; Indium-tin-oxide, Sanyo Vacuum) was cleaned with acetone (Wako) and isopropyl alcohol (IPA, Wako). Then, SU-8 3050 negative photoresist (Microchem) was spin-coated onto an ITO substrate for 60 s at 1000 rpm with a thickness of 100  $\mu\text{m}$ . The coated substrates were baked on a hotplate for 50 min at 95 °C. The substrates were exposed to UV through a custom-made photomask. A reduced projection exposure system (MM-505, Nanometric Technology) was used for fabricating the photomasks. The photomask featured sixteen microcavities with a diameter of 200 or 500  $\mu\text{m}$  aligned in 4  $\times$  4 matrix patterns. After baking on a hotplate for 10 min at 95 °C, the substrates were developed in SU-8 developer (Microchem) and rinsed with IPA. Because SU-8 resist have good insulating properties, the bottom of each microcavity, where the ITO layer was exposed, acted as the stimulation electrode. After fabrication of the microcavity-array pattern, a glass ring of 1 cm height was mounted onto a substrate with a silicone elastomer (KE-103, Shin-etsu Silicones). A stranded wire of copper was also mounted onto a substrate with an electroconductive paste (Dotite FC-415, Fujikura Kasei). The ITO regions inside a glass ring, except the bottom surfaces of the microcavity, were insulated by coating with a silicone elastomer. The protocols for fabricating the microcavity-array electrode substrate were shown in Fig. 1.

In this research, we used P19 cells as a model system for a stem cell. P19 is a mouse embryonal carcinoma cell line and can differentiate into three germ layers (Bain et al. 1994). For its easy handling and high neuronal differentiation rate, we have used P19 cells as a stem cell model. P19 cells were routinely cultured and passaged every two days in alpha minimum essential medium ( $\alpha$ -MEM, Invitrogen) containing 10 % fetal bovine serum (FBS, HyClone) and 5 - 40 U/ml penicillin-streptomycin (Sigma-Aldrich). To produce uniform size EBs, P19 cells were collected and replated in a 96-well low cell-attachment plate (Sumilon, Sumitomo Bakelite). Due to its spheroid bottom structure, a single EB was formed in each well. The obtained EBs were collected and transferred into

each microcavity of the electrode substrates. To our experience, initial plating of 500 cells resulted in a EB of 200  $\mu\text{m}$  diameter and 4000 cells in a EB of 500  $\mu\text{m}$  diameter at next day of plating. The microcavity-array electrode substrates were previously coated with MPC (2-methacryloyloxyethyl phosphorylcholine) polymer (Lipidure CM5206E, NOF corp.) to prevent nonspecific cell adhesion (Ishihara et al., 1999) and to localize EBs into each microcavity.

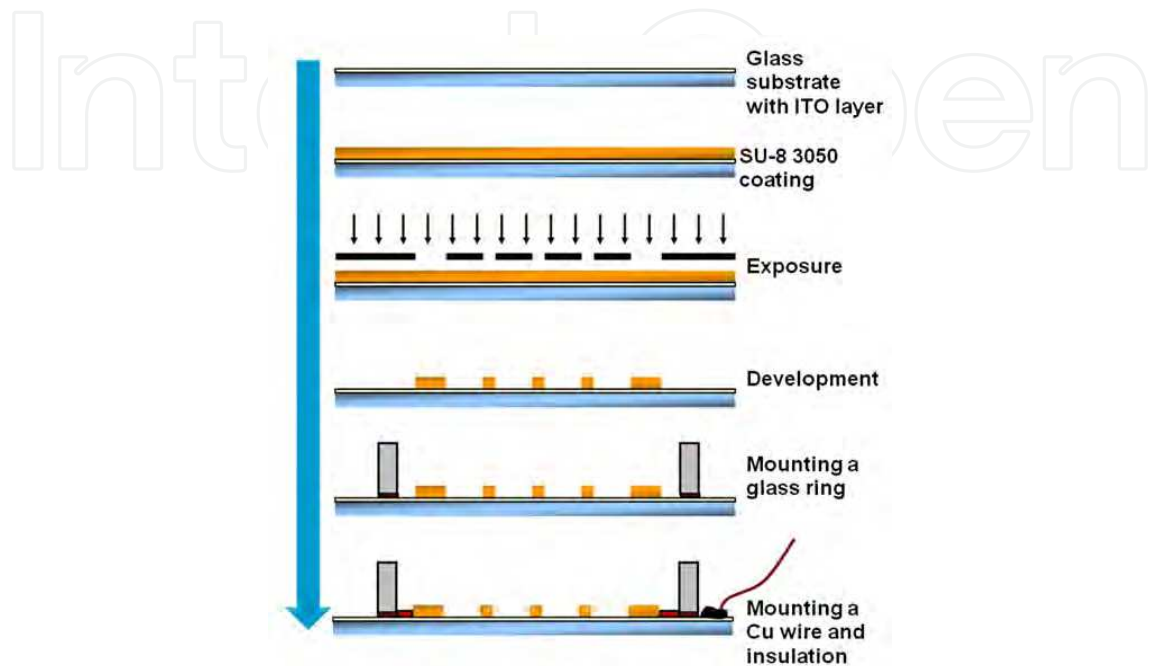


Fig. 1. Schematic of procedures for fabricating the microcavity-array electrode substrate using SU-8 negative photoresist.

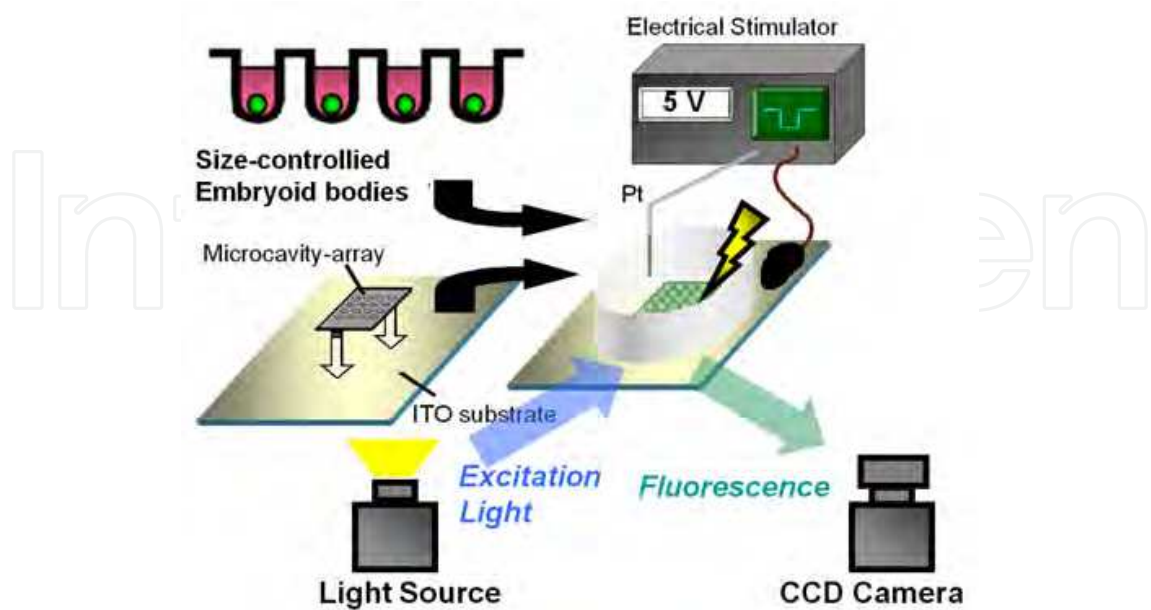


Fig. 2. Schematic diagram of the experimental system. Size-controlled EBs were simultaneously stimulated through the microfabricated ITO substrates.

After insertion of P19 EBs, constant voltage stimulation was applied to the trapped EBs via the bottom surfaces of microcavities with an electrical stimulator (SEN-8203, Nihon Kohden) and an isolator (SS203J, Nihon Kohden). A platinum (Pt) electrode ( $\phi$  1 mm) was used as the counter electrode during electrical stimulation. A single negative-first biphasic pulse with intensity of 5 V and duration of 1 ms was used for stimulation. Stimulation-induced responses of EBs were visualized by calcium imaging technique. The EBs were labelled with a calcium indicator Fluo-4AM (Molecular Probes) and fluorescence signals were detected with a cooled CCD camera (C8800-21C, Hamamatsu Photonics) mounted on an inverted microscope (IX-71, Olympus). The frame rate of 0.5 frame/s was used. The recording solution contained 148 mM NaCl, 2.8 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES and 10 mM glucose. The overview of the experimental system was shown in Fig. 2.

Figure 3 showed photographs of the fabricated substrate and magnified phase-contrast images of the microcavity-array region and the trapped EBs. These microcavity-array patterns were fabricated with fine reproducibility. The EBs of P19 cells prepared by using spheroid-bottom plates also showed high uniformity in size and morphology. Figure 3-c showed the EBs inserted in the microcavities of 500  $\mu\text{m}$  diameter. The EBs were successfully trapped within each microcavity with 100  $\mu\text{m}$  depth. We confirmed that pre-coating with the MPC polymer inhibited EB adhesion to the device surface over several hours. Thus, suspension culture condition was maintained during electrical stimulation experiments. We also confirmed that pre-coating with the MPC polymer did not affect the electrical properties (impedance, phase) of the device by observing with an LCR meter.

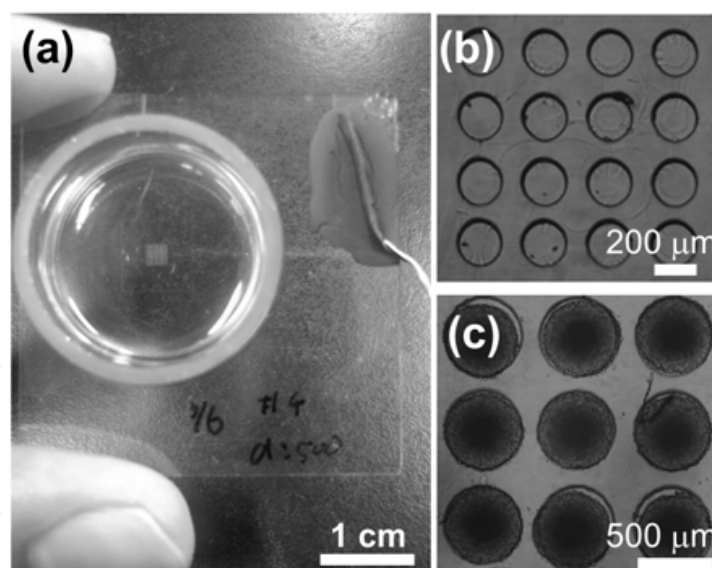


Fig. 3. A microcavity-array dish for ensemble stimulation. (Takayama et al., 2009)

We then applied electrical pulses to the trapped EBs and recorded evoked response by calcium imaging. Figure 4 showed phase-contrast images of EBs in the microcavities and corresponding fluorescence images after applying a single biphasic pulse. The fluorescence images represent the normalized difference ratios in fluorescent intensity between pre- and post-stimulus. Spontaneous calcium transients in cells of P19 EBs were rarely observed before stimulation. By applying electrical stimulation, in contrast, significant elevations of intracellular calcium concentration were observed. For both of microcavity-array patterns,



most EBs in the same observing field showed similar responses. In 200  $\mu\text{m}$  diameter EBs, most of cells in an EB showed calcium transients responded to electrical pulse. In 500  $\mu\text{m}$  diameter EBs, cells in outer region of EB primarily showed calcium transients. In all stimulation experiments, we confirmed that similar results were obtained ( $n=5$  for 200  $\mu\text{m}$  diameter patterns and  $n=3$  for 500  $\mu\text{m}$  diameter patterns). The results indicated that the microcavity-array electrode device could stimulate size-controlled EBs simultaneously and uniformly.

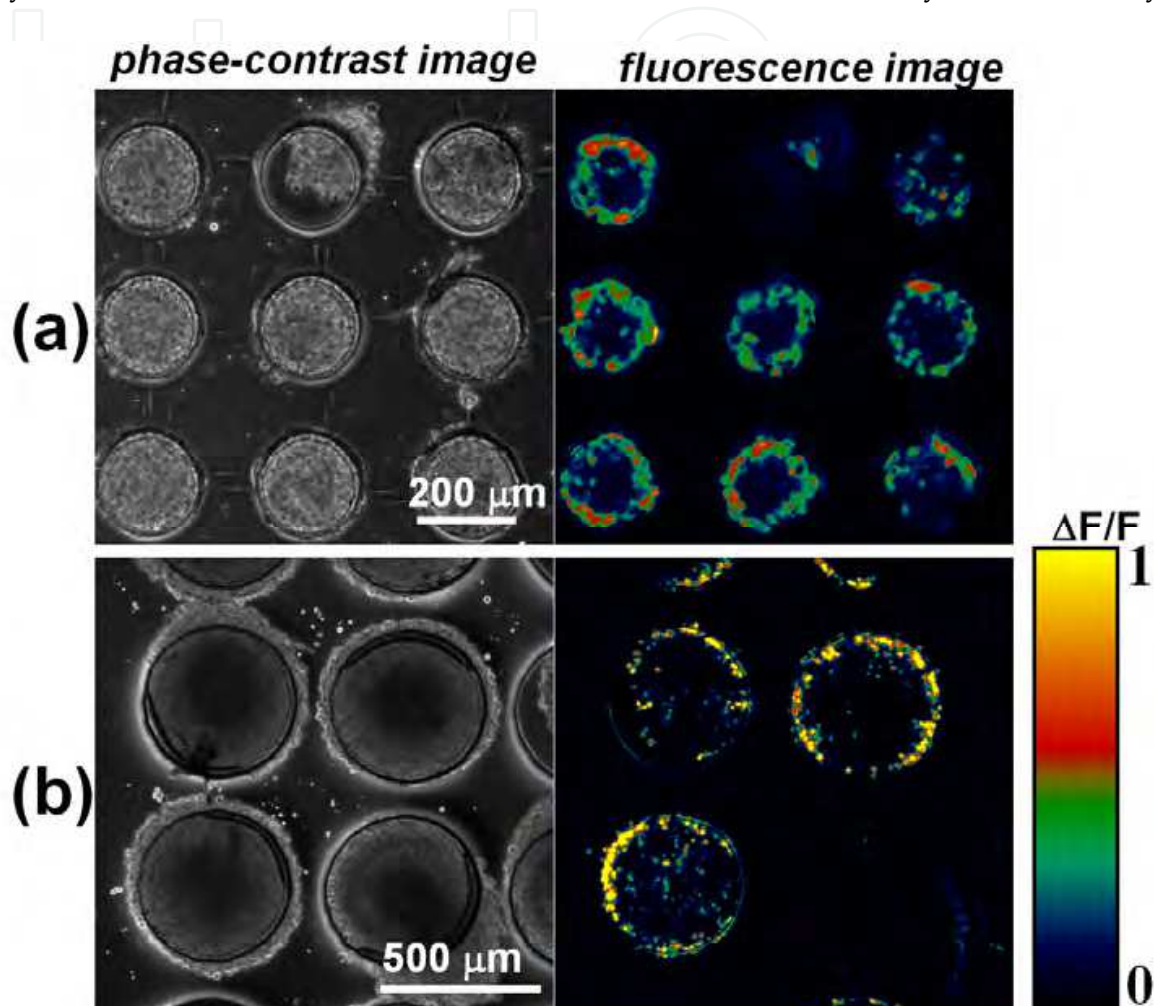


Fig. 4. EBs of P19 cells inserted in the microcavity-array structures (phase-contrast image) and stimulus-evoked intracellular calcium transients (fluorescence image). (Takayama et al., 2009)

In this experiment, by using SU-8 negative thick photoresist, we could fabricate microcavity-array structures onto electrode substrate with enough depth (100  $\mu\text{m}$ ) to trap and localize P19 EBs. By using the electrode substrate, we could stimulate P19 EBs simultaneously. Although several studies have carried out electrical stimulation to EBs, such as a field pacing (Sauer et al, 1999 and 2005; Yamada et al., 2007), there have been no studies that observed cell activity in a large number of EBs simultaneously. This may be due to difficulty in observing randomly floating EBs under suspension culture condition. Thus, localizing EBs in specific regions and detecting cell activity of EBs simultaneously by using microcavity-array structures are important bases for further electrical stimulation experiments and analysis.

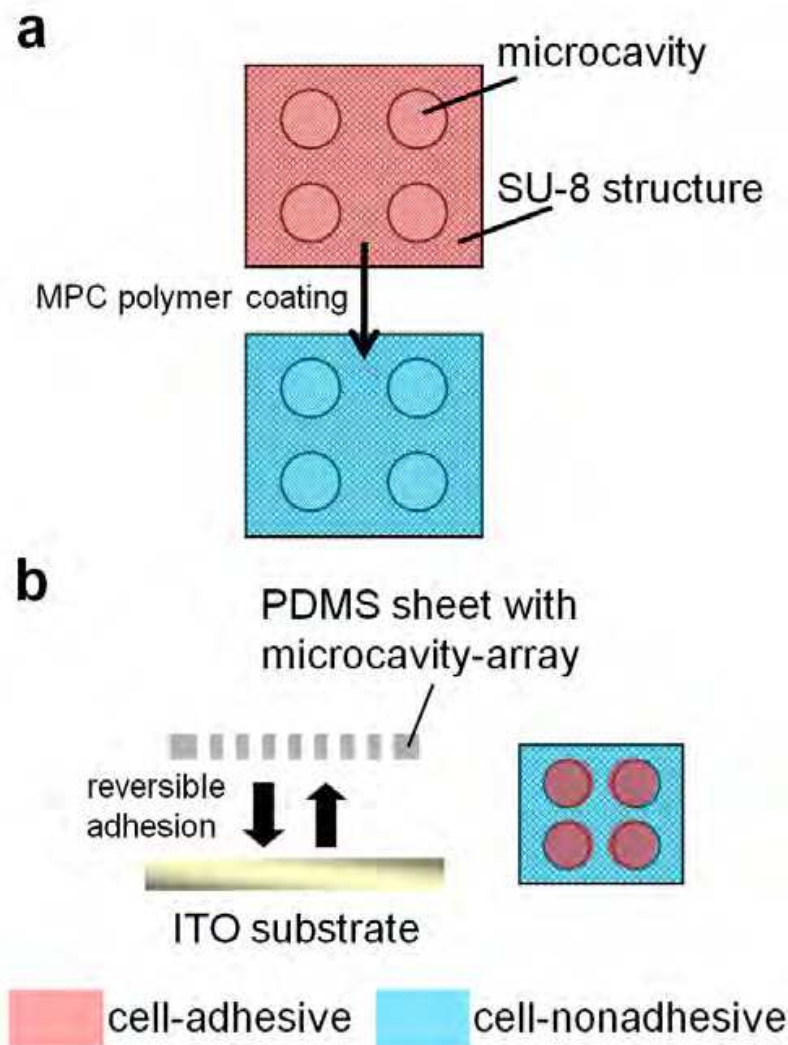
It is widely recognized that intracellular calcium dynamics play important roles in regulation of cell differentiation processes (Dolmetsch et al., 1998; Yamada et al., 2007). Particularly, Spitzer et al. reported that spatio-temporal patterns of intracellular calcium transients regulated neuronal differentiation processes, such as extension of neurite and selection of neurotransmitter (Spitzer et al., 2004). Thus, inducing and controlling intracellular calcium transients in undifferentiated stem cells by applying electrical stimulation, which parameters are flexibly determined, is promising method to affect cell differentiation processes artificially. Optimizing parameters of electrical stimulation to induce desirable spatio-temporal patterns of intracellular calcium transients will be next step for controlling cell differentiation processes.

There are several problems, however, with the cell-nonadhesive electrode substrate using SU-8 thick photoresist. In the experiments, we manually transferred EBs one by one from 96 well culture plates into each microcavity using micropipettes. Furthermore, due to the cell-nonadhesiveness of the electrode substrate by the MPC polymer coating, mild mechanical perturbations by moving the electrode substrate easily remove EBs from the microcavities. These problems make it difficult to stimulate more than 20 EBs simultaneously with the SU-8-based cell non-adhesive electrode substrates and to perform subsequent gene expression analysis and culture test after stimulation. On the basis of these disadvantages, we attempt to fabricate PDMS (poly(dimethylsiloxane))-based cell-adhesive electrode substrate to stimulate more number of EBs. The experimental procedures and present results for the cell-adhesive electrode substrate are summarized in a next section.

### **3. Electrical stimulation and gene expression analysis with cell-adhesive microcavity-array electrode substrate**

As described in section 2, it was difficult to stimulate a large number of EBs, over a hundred numbers, when using the cell-nonadhesive electrode substrate with SU-8 negative thick photoresist. This was because that the SU-8 structures were strictly integrated with ITO substrate and thus all surface of the electrode substrate became cell-nonadhesive after the MPC polymer coating of microcavity-array region (Fig. 5-a). Although the cell-nonadhesive surface was advantageous to handle EBs, maintain suspension culture conditions and trap EBs into the microcavities temporally, it was disadvantageous to fix EBs to the bottom surfaces of the microcavities not to remove away by mechanical perturbations. Based on these problems, we attempted to propose an alternative method to stimulate a large number of EBs simultaneously. We fabricated cell-adhesive electrode substrate by attaching a PDMS sheet, featuring microcavity-array patterns, onto an ITO substrate (Fig. 5-b). PDMS is well used elastomeric material in cell engineering research because of its capability of easy processing, biocompatibility and cell-nonadhesiveness (Mata et al, 2005). PDMS is also an insulative material (Nam et al., 2006) and thus useful for insulator as the SU-8 structures used in section 2. By using the cell-adhesive electrode substrate with the PDMS sheet and plating P19 cells onto it, we attempted to obtain a large number of EBs and stimulate them. We also attempted to analyse gene expression changes after ensemble electrical stimulation of EBs by polymerase chain reaction (PCR) and electrophoresis Techniques.

The experimental procedures were described as follows. The microcavity-array pattern was fabricated in PDMS using soft lithography technique. First, column-array structures were fabricated as a master mold onto a standard glass substrate using SU-8 photoresist. SU-8 3050 was spin-coated onto a glass substrate (76 × 52 mm, Matsunami Glass) for 60 s at 1000



(a) Cell-nonadhesive electrode substrate using SU-8 photoresist.

(b) Cell-adhesive electrode substrate using PDMS elastomer.

Fig. 5. Structures of the electrode substrates for stimulating EBs

rpm with a thickness of 100  $\mu\text{m}$ . After exposure and development with the photomasks which had inverted patterns as used in section 2, column-array structures were formed on a glass substrate. In this experiment, 289 ( $17 \times 17$  matrix pattern) and 64 columns ( $8 \times 8$  matrix pattern) were fabricated for 200 and 500  $\mu\text{m}$  diameter microcavities, respectively. Before pouring PDMS, a thin layer of photoresist (OFPR-800, positive photoresist, Tokyo Ohka) with a thickness of 4  $\mu\text{m}$  was formed onto the substrate by spin-coating for 40 s at 2000 rpm and then baking for 30 min at 80  $^{\circ}\text{C}$ . The thin photoresist layer worked as a sacrifice layer to release a cured PDMS sheet from the substrate (Nam et al., 2006). Then, the mixture of PDMS pre-polymer and catalyst (10:1 ratio, Silpot 184, Dow Corning) was spin-coated on the substrate for 80 s at 2500 rpm and 40 s at 2000 rpm, for 200 and 500  $\mu\text{m}$  diameter microcavities, respectively. The PDMS coated substrate was put on a hotplate and then a pre-manufactured thick PDMS annulus (20 mm square and 3 mm thickness) which had a 15 mm diameter hole was immediately attached on the substrate. The PDMS layer and annulus



were baked for an hour at 80 °C. After curing, the PDMS sheet was cut along the thick annulus using forceps and then the substrate was soaked in an acetone to solve the photoresist layer. The released PDMS sheet was rinsed with IPA and distilled water. The microcavity-array pattern electrode substrate was formed by attaching the PDMS sheet onto an ITO substrate (50 × 50 mm). The protocols for fabricating the microcavity-array electrode substrate using PDMS were shown in Fig. 6.

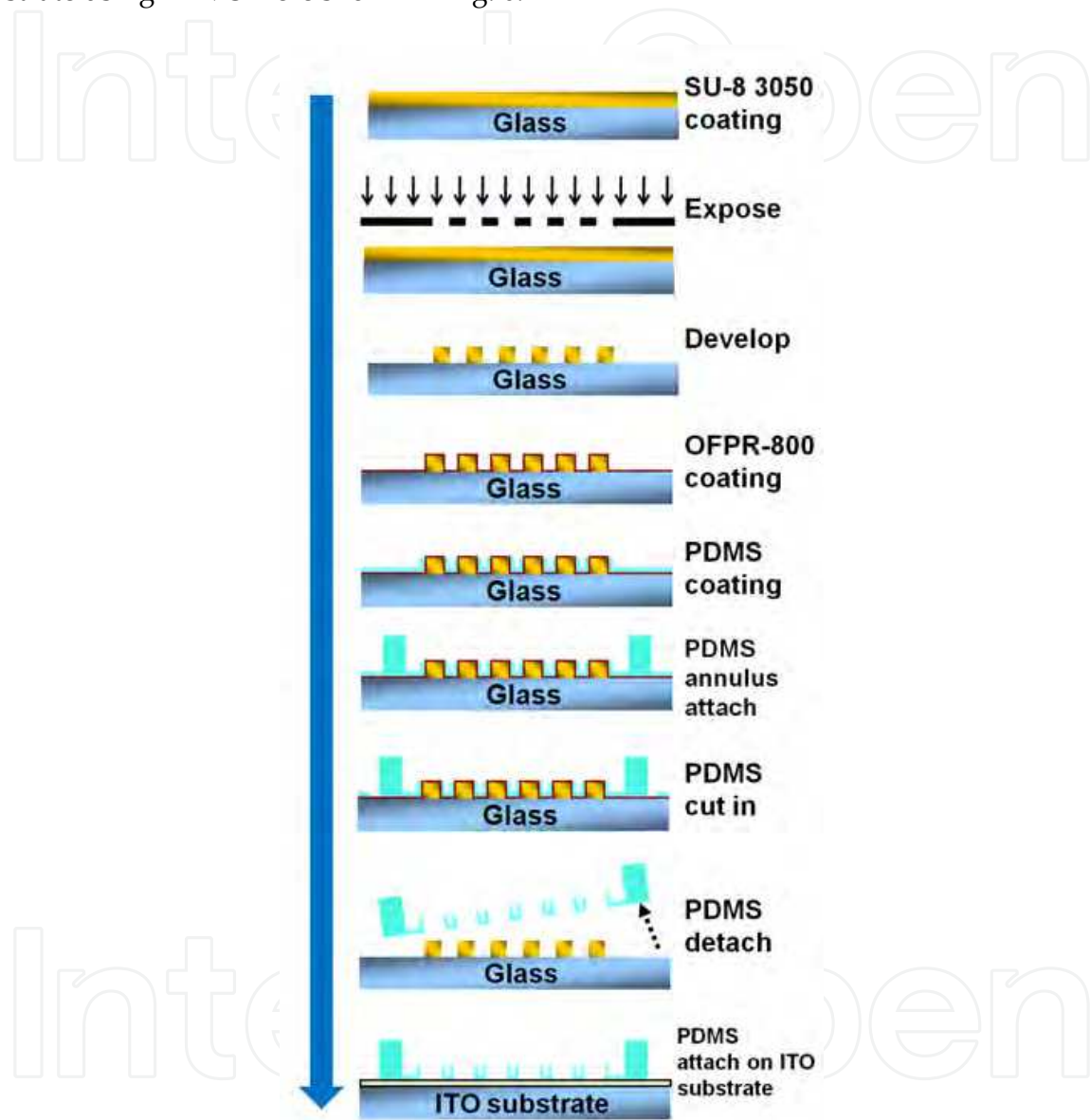


Fig. 6. Schematic of procedures for fabricating the microcavity-array electrode substrate using PDMS elastomer.

Methods for subculture of P19 cells were same as described in section 2. Proliferated P19 cells were collected and then replated onto the microcavity-array electrode substrates. Due to its cell-nonadhesive property of PDMS, P19 cells adhered only to the bottom surfaces of the microcavities. After several hours of plating, non-adhered cells were removed by washing the substrates with culture medium or PBS. P19 cells were proliferated within the microcavities and EBs were self-organizingly formed after 1 day in culture. For electrical

stimulation, a stranded copper wire was attached to the substrate as described in section 2. Electrical pulses were chronically applied to the EBs with culturing them in an incubator after 1 day of cell plating (EB forming). In this experiment, a single biphasic pulse, same conditions as in section 2, was applied at intervals of 30 min for 2 days.

To analyse the effects of chronic electrical stimulation on cell differentiation processes, reverse transcriptase-PCR (RT-PCR) analysis was carried out after electrical stimulation. First, extraction and purification of RNA were carried out by using a commercial RNA extraction kit (NucleoSpin RNA II kit, Nippon Genetics) from the EBs after applying electrical stimulation for 2 days. RNA of the EBs after induction of neuronal differentiation with retinoic acid was also extracted as the control sample. A 250 ng of total RNA was used for synthesis of first strand complementary DNA (cDNA) by reverse transcription for 80 min at 42 °C using a commercial kit (iScript Select cDNA Synthesis kit, Bio-Rad). Then, amplification of cDNA was carried out with 0.5 µl of synthesized cDNA for 30 s at 94 °C and 1 min at 74 °C, for denaturation and primer extension, respectively. The primer information used in this experiment and primer annealing conditions were summarized in Table 1. The PCR products were analysed using an Experion automated electrophoresis system (Bio-Rad). Temperature controls for reverse transcription and PCR reactions were carried out using a C1000 thermal cycler (Bio-Rad).

Gene Name	Forward Primer	Reverse Primer	Annealing temperature (°C)	Cycles	Size (bp; base pair)	Reference
Oct 3/4	cagaagaggatcaccttggg	gtgagtgatctgctgtaggg	60	27	324	Tang et al., 2002
BMP-4	tgccgcagcttctctgagcc	gctctgccgaggagatcacc	64	26	573	Tang et al., 2002
Mash-1	caagttgttcaacctgggtt	gctctgttcctctgggcta	56	30	293	Tang et al., 2002
Wnt-1	acgttgctactggcactgac	ccatttgcaactctgcacag	57	30	244	Gao et al, 2001
GAPDH	accacagtcctatgccatcac	tccaccaccctgttgctgta	63	25	452	Georgiev et al., 2008

Table 1. Primer sequences and reaction parameters for RT-PCR

The Oct 3/4 gene is thought to play important roles in maintaining undifferentiated conditions and thus is widely used as a marker for undifferentiated stem cells (Ronser et al., 1990). The BMP-4 gene is mainly expressed in ectoderm, meanwhile it inhibit the neuronal differentiation. Thus, an expression level of the BMP-4 is transiently increased and then gradually decreased during neuronal differentiation (Wilson and Hemmti-Brivanlou, 1995). The Mash-1 gene is mainly expressed in neuronal progenitor cells and thus is used as a neuronal marker (Lo et al., 1991). The Wnt-1 gene is also used as a neuronal marker, particularly it inhibit glial differentiation and promote neuronal differentiation (Tang et al., 2002). The GAPDH (glyceraldehyde-3-phosphatedehydrogenase) gene is a widely-used housekeeping gene. These genes are also expressed and regulated in neuronal differentiation processes of P19 cells (Bain et al., 1994).

Figure 7 showed a photograph of the cell-adhesive electrode substrate using a PDMS sheet and a phase-contrast image of the microcavity-array region (diameters of 200 µm in this figure). SEM (scanning electron microscope) images of the SU-8 column-array mold and the corresponding PDMS microcavity-array structures were also shown in Fig. 8. The round-shape microcavity-array patterns as in section 2 were fabricated with fine reproducibility. The depth of the microcavities depended on the height of the column mold (100 µm in this experiment) and could be regulated by changing the experimental parameters.

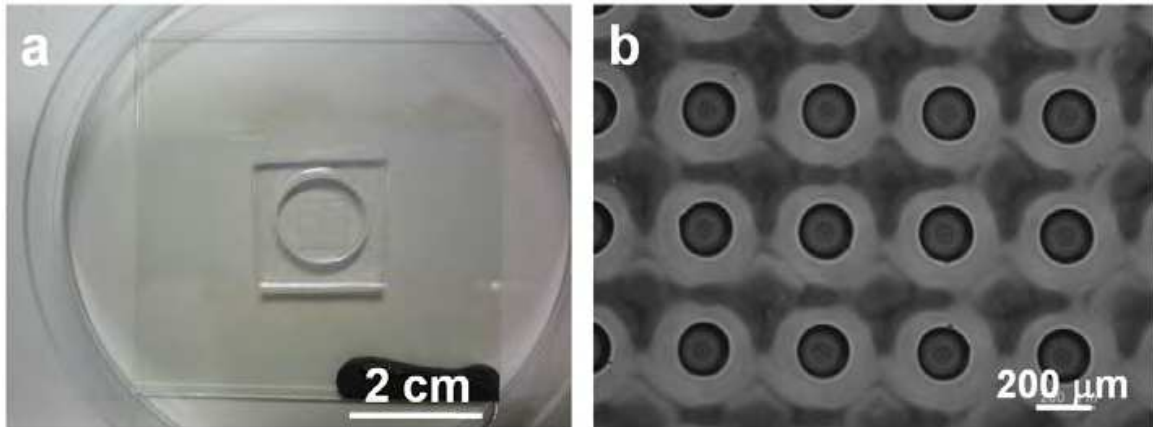


Fig. 7. The cell-adhesive electrode substrate using PDMS (a) the outview of the substrate. (b) the phase-contrast image of the microcavity-array region.

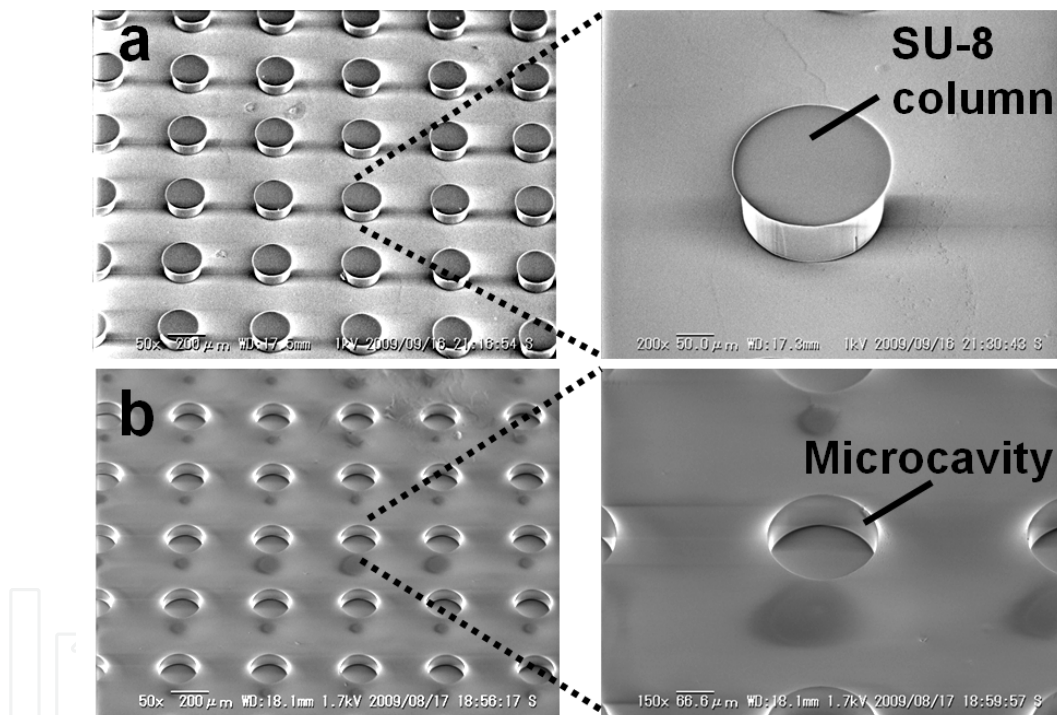


Fig. 8. SEM images of the microcavity-array structures. The diameter of each SU-8 column and microcavity is 200  $\mu\text{m}$ . (a) The SU-8 column-array mold for the microcavities. (b) The corresponding microcavity-array of PDMS.

Then, we plated P19 cells onto the electrode substrate and culture them. The results were shown in Fig. 9. The figure showed the microcavity-array regions of the 200  $\mu\text{m}$  diameter sample (Fig. 9-a) and the 500  $\mu\text{m}$  diameter sample (Fig. 9-b) after 2 days in culture. There were no adhesion and proliferation of P19 cells on the PDMS region. The P19 cells adhered to the bottom surfaces of the microcavities proliferated, aggregated within the microcavities and formed EBs. The characteristic feature of these EBs, different from the EBs in the experiments of section 2, was that they were tightly adhered to the bottom surfaces of the

microcavities. Thus, the EBs were not easily removed away by mechanical perturbations. We confirmed that applying electrical pulses evoked intracellular calcium transients in the EBs as those in the experiments of section 2. We concluded that we could obtain a large number of size-controlled P19 EBs and stimulate them simultaneously by using the cell-adhesive microcavity-array electrode substrate.

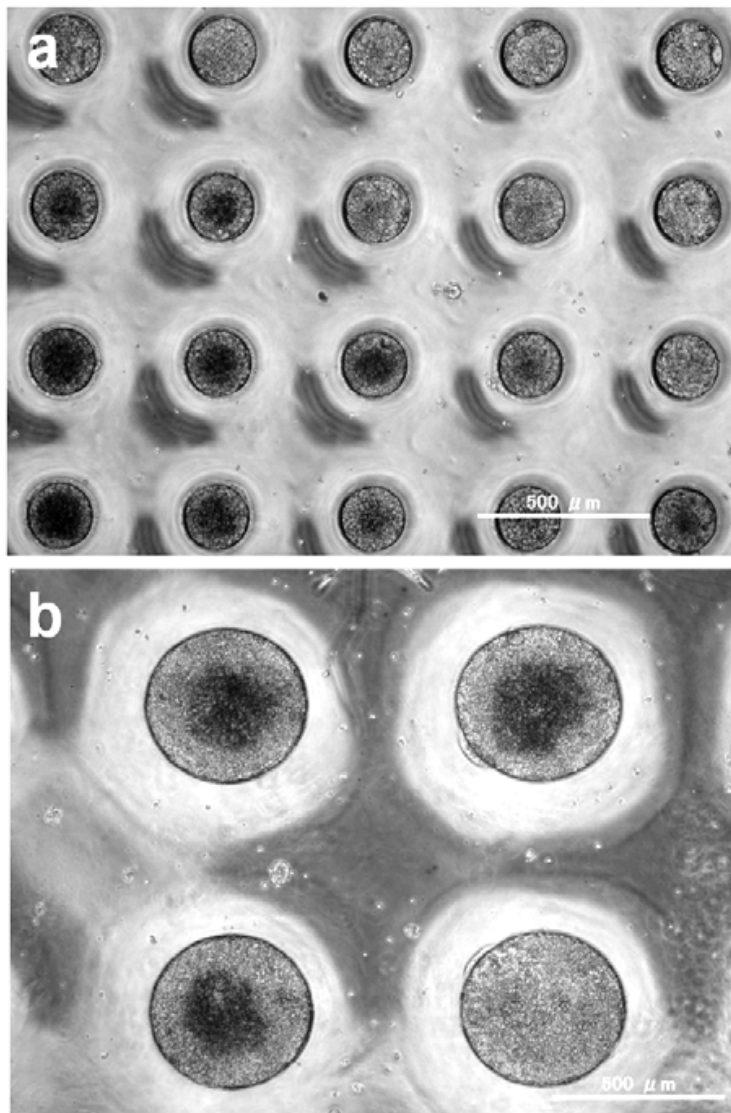


Fig. 9. Self-formed EBs within the microcavity-array substrate using PDMS. (a) the 200  $\mu\text{m}$  diameter pattern sample. (b) the 500  $\mu\text{m}$  diameter pattern sample.

After ensemble electrical stimulation of P19 EBs for 2 days using the cell-adhesive electrode substrate, we attempted to analyse the effects of electrical stimulation on cell differentiation processes of P19 cells by gene expression analysis using a RT-PCR method. Chronic electrical stimulation to P19 EBs was started to be applied after 1 day of cell plating and continued for 2 days with an interval of 30 min. The experimental setup for chronic electrical stimulation of EBs was shown in Fig. 10. A platinum wire was used as a counter electrode as in section 2.



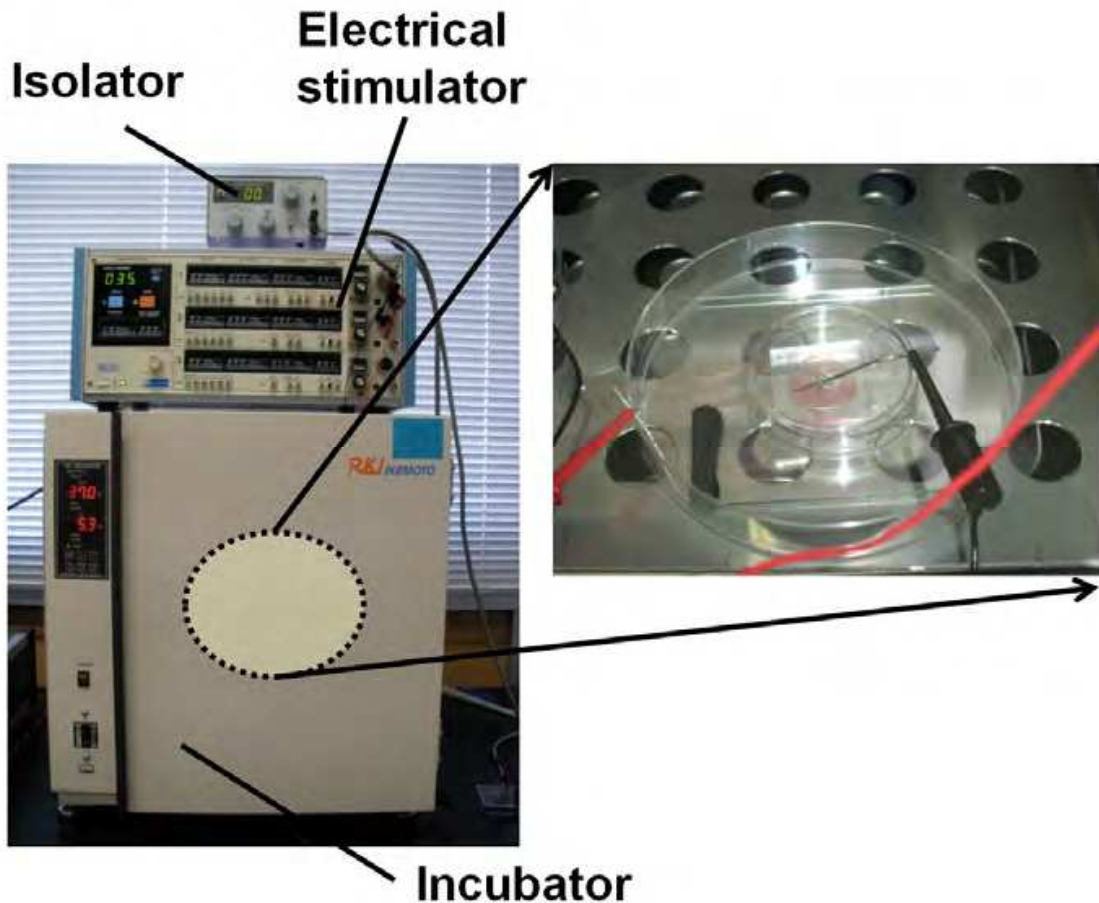


Fig. 10. Chronic electrical stimulation of P19 EBs with the microcavity-array electrode substrate using a PDMS sheet. Electrical pulses were applied for 2 days with an interval of 30 min.

First, we analysed the gene expression of P19 cells induced with retinoic acid under suspension culture conditions for setting a criteria of the gene expression level of neuronal differentiation pathways in P19 cells. To initiate neuronal differentiation, P19 cells were plated into bacteria culture dishes ( $\phi$  100 mm; Fisherbrand) and were allowed to aggregate with  $2 \times 10^6$  cells/dish. During the induction period,  $\alpha$ -MEM containing 5 % FBS and  $1 \times 10^{-6}$  M all-trans-retinoic acid (Sigma-Aldrich) was used as the culture medium. The expression levels of 5 genes described above were shown in Fig. 11. The figure showed the gene expression patterns under five conditions of P19 cells; undifferentiated cells, EBs under suspension culture conditions with retinoic acid (each of 1 ~ 4 days in culture). The presence of white band indicated the expression of the relevant genes.

The expression of the GAPDH gene was confirmed in all conditions, indicating that PCR reactions in the experiment worked well. In undifferentiated P19 cells, distinct expression of the Oct 3/4 was confirmed, while there were no expression of the BMP-4, Mash-1 and Wnt-1 genes. Following the application of retinoic acid under suspension cultures, the expression of the Oct 3/4 rapidly decreased and disappeared. Alternatively, the BMP-4, Mash-1 and Wnt-1 genes showed elevated expression levels in the EBs. We confirmed the inhibition of undifferentiated associated gene and the expression of neuronal differentiation associated

genes during the differentiation processes of P19 cells following the application of retinoic acid.

Then, the results for gene expression analysis of P19 EBs after application of chronic electrical stimulation using the microcavity-array electrode substrate were shown in Fig. 12. The figure showed the results of two samples for each 200 and 500  $\mu\text{m}$  diameter pattern.

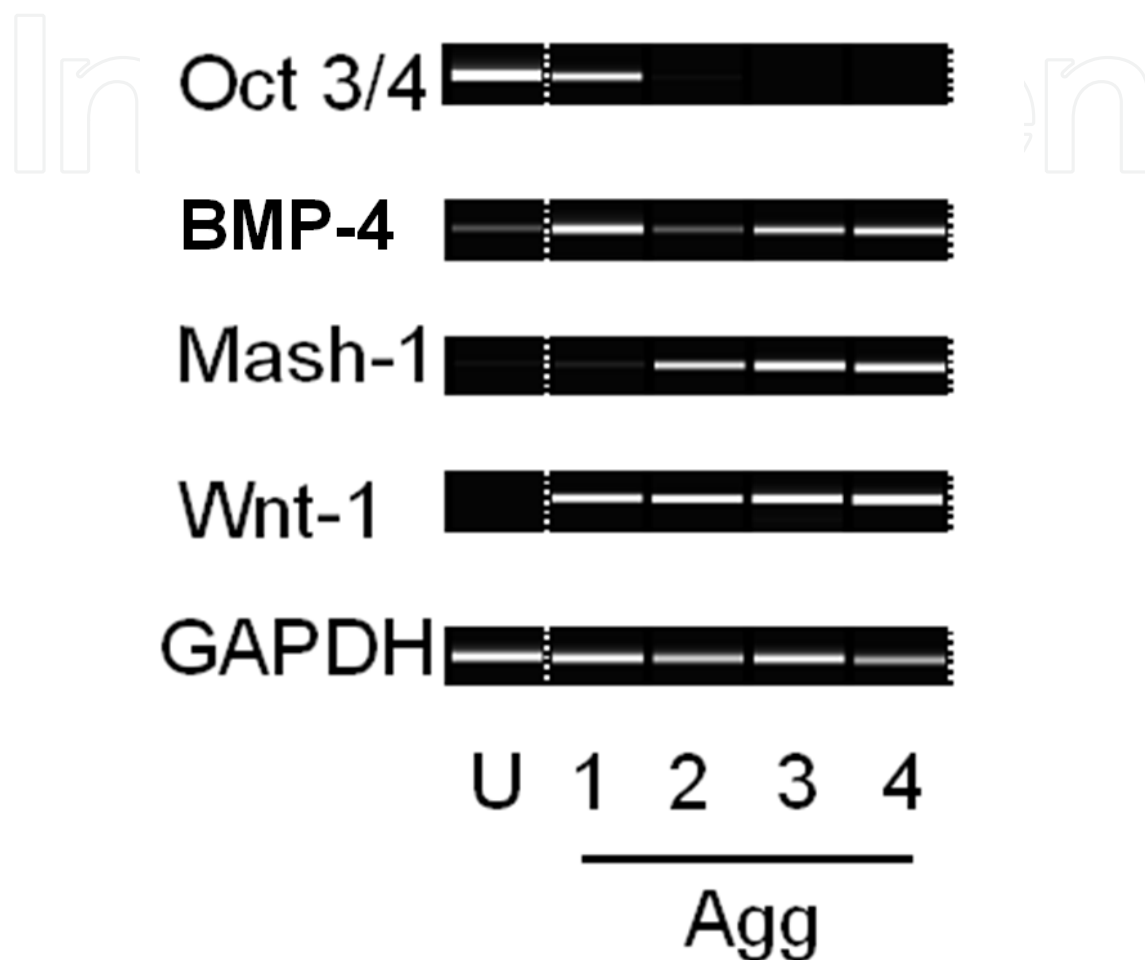


Fig. 11. The gene expression patterns in P19 cells induced by retinoic acid under suspension cultures. U; undifferentiated cells. Agg; EBs in suspension culture. Numbers indicate culture days.

In the four samples used in this experiment, the expression of the Oct 3/4 gene was maintained and there was no distinct expression of the BMP-4, Mash-1 and Wnt-1 genes after electrical stimulation. Thus, in the present situation, we could not induce distinct differentiation processes of P19 cells from its undifferentiated state by electrical stimulation and EBs size treatment.

In this section, we proposed an alternative microcavity-array electrode substrate with PDMS elastomer using micro and soft lithography technique. We could obtain hundreds of size-controlled EBs. The EBs were tightly adhered to the bottom surfaces of the microcavities, thus subsequent electrical stimulation and gene expression analysis could be stably carried out. However, applying electrical stimulation of EBs for 2 days could not induce changes in the gene expression patterns of P19 cells. One of the possible reasons was that an excessive

elevation of intracellular calcium concentration activated protease and thus induced cell death (Berridge et al, 1998). Furthermore, while the cell-adhesive surface was advantageous to fix EBs within the microcavities, adhered culture was not suitable condition for induction of cell differentiation (Bain et al., 1994). Optimization of parameters for electrical stimulation and testing other genes will be required.

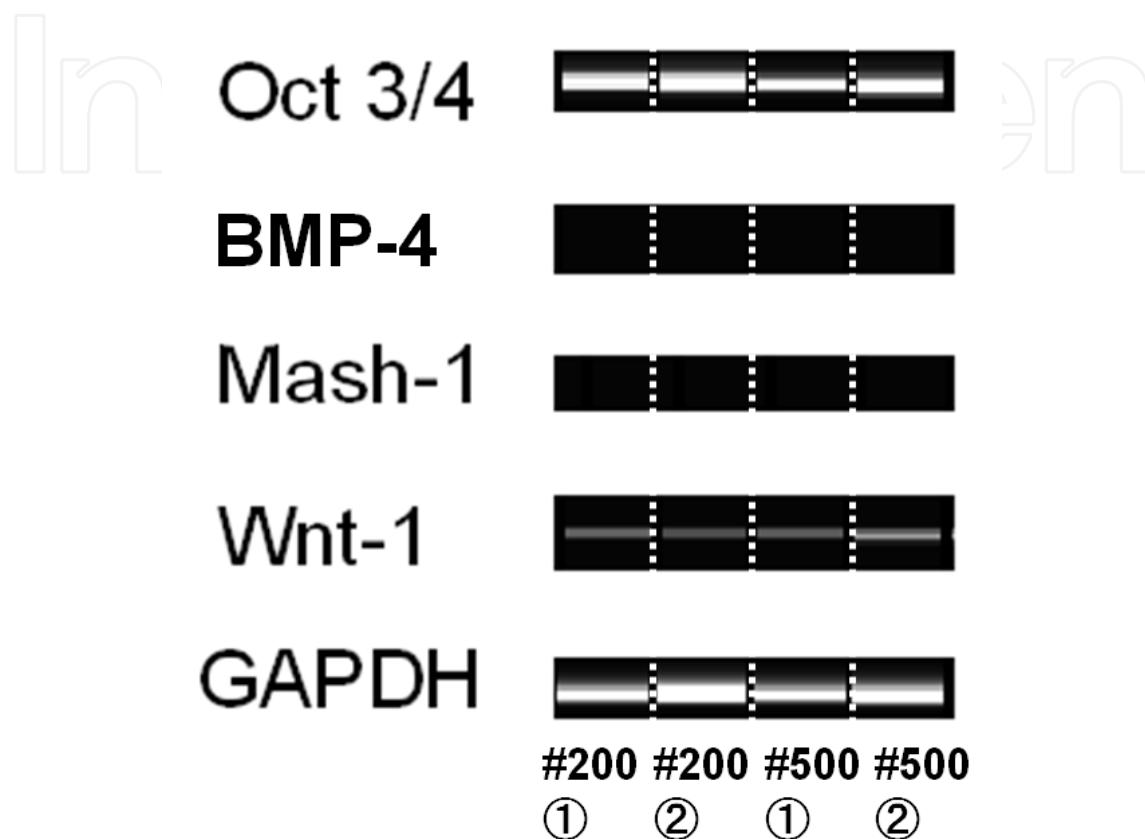


Fig. 12. The gene expression patterns in P19 cells after electrical stimulation for 2 days. #200 indicate the results in 200  $\mu\text{m}$  diameter samples and #500 in 500  $\mu\text{m}$  diameter samples.

#### 4. Conclusion

In this chapter, we proposed the microcavity-array pattern substrate for electrical stimulation of stem cell EBs fabricated by micro and soft lithography technique, aiming at precise control of cell differentiation processes. We could obtain intracellular calcium signals responded to electrical stimulation in EBs simultaneously, indicating that the electrode device would be a promising experimental tool to regulate the cell differentiation processes. We believed that we represented a step on the way to precisely control of cell differentiation processes. Optimization of experimental parameters and improvement of the electrode device structures are current questions.

#### 5. References

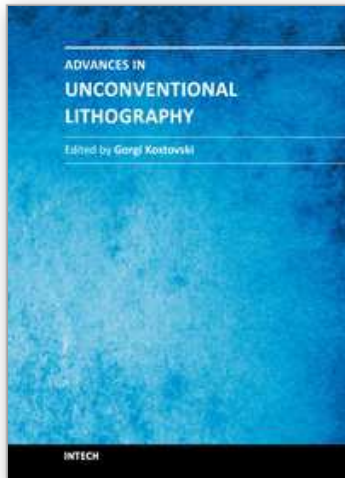
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## **Advances in Unconventional Lithography**

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The term Lithography encompasses a range of contemporary technologies for micro and nano scale fabrication. Originally driven by the evolution of the semiconductor industry, lithography has grown from its optical origins to demonstrate increasingly fine resolution and to permeate fields as diverse as photonics and biology. Today, greater flexibility and affordability are demanded from lithography more than ever before. Diverse needs across many disciplines have produced a multitude of innovative new lithography techniques. This book, which is the final instalment in a series of three, provides a compelling overview of some of the recent advances in lithography, as recounted by the researchers themselves. Topics discussed include nanoimprinting for plasmonic biosensing, soft lithography for neurobiology and stem cell differentiation, colloidal substrates for two-tier self-assembled nanostructures, tuneable diffractive elements using photochromic polymers, and extreme-UV lithography.

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