31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Ensemble Stimulation of Embryoid Bodies using microfabricated ITO Substrates

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Abstract— **Precise control of differentiation processes of pluripotent stem cell is key component for realization of regenerative medicine. Electrical stimulation is one of the promising techniques, particularly for regulation of neuronal regeneration. In the present study, we developed substrates with embedded electrodes, which allowed ensemble electrical stimulation of embryoid bodies (EBs) of stem cells. Microcavity-array patterns were fabricated on substrates with embedded electrodes using standard photo-lithography. Uniform-size EBs of P19 cells were prepared, inserted one by one in each microcavity, and electrical stimulation was applied through substrate electrodes. Stimulus-induced intracellular calcium transients were successfully monitored by fluorescence imaging. The results suggested that this method would be useful for applying precisely-controlled electrical stimulation to a large number of EBs of stem cells.**

I. Introduction

Generation of neurons from pluripotent stem cells is an important theme for neuronal regeneration because of the inability of normal mature neurons to proliferate. Recently, in particular, the generation of human induced pluripotent stem (iPS) cells [1] accelerates the stem cell- based therapy. However, low differentiation efficiency of pluripotent stem cells is one of the limiting factors in regeneration medicine. Increases in cell-differentiation efficiency, as well as precise control of the differentiation processes are desirable.

During cell-differentiation processes, cell-cell communication plays an important role. Most procedures for inducing cell-differentiation involve the formation of cell aggregates, called embryoid bodies (EBs), generally through suspension culture or hanging drop methods [2]. Microfabrication- based surface modification of substrates was reported to be useful for regulating size of EBs [3,4] and through promotion of cell-cell interactions, an increase in cell-differentiation efficiency was achieved [5]. Another promising technique is physical stimulation. Electrical or magnetic stimulation can induce cellular and molecular responses. It is reported that gene expression is affected by electrical [6,7] and electromagnetic [8,9] stimulation. Combination of cell-aggregate treatment and physical stimulation will be a useful tool to increase cell-differentiation efficiency, as well as precise control of the differentiation processes.

Here in this work, we developed microcavity-arrays with embedded electrodes. Uniform-size EBs of P19 cells were prepared using spheroid-bottom plates and inserted into the microcavities of corresponding dimensions. The EBs trapped in the cavities were electrically stimulated thorough the embedded electrodes and the responses were monitored by intracellular calcium transients.

Manuscript received April 7, 2009

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II. Material & Methods

A. Fabrication of Microcavity-arrays

Microcavity-arrays were fabricated on glass substrates with a transparent conductive layer (indium-tin-oxide: ITO, 40 × 40 mm, Sanyo Vacuum Industries). Microcavity- array patterns were formed by standard photo-lithography. In this work, sixteen cavities aligned in a 4 x 4 matrix were formed. SU-8 3050 (negative photoresist, MicroChem) was spin-coated on the substrate with an approximate thickness of 200 μm. After pre-baking at 95 ^{o}C for two hours, the coated substrate was exposed to UV light through the transparency mask, followed by post-baking at 95 ^{o}C for 30 minutes. The microcavity-array pattern on the substrate was developed in SU-8 developer (MicroChem) for 20 minutes, rinsed with isopropyl alcohol, and dried on hotplate. Then, a glass ring to store recording solution was mounted using epoxy/silicone sealant (KE-103, Shin-etsu Chemical), a stranded Cu wire was connected to the substrate using an electroconductive paste (Dotite FC-415, Fujikura Kasei) and the remaining ITO regions were insulated using same sealant. In this work, two patterns of microcavity-array pattern, each diameter was 200 and 500 μm, were developed. Electrical characteristics of the microfabricated substrates were studied by an LCR meter (3533-50, Hioki) in 1x PBS (Phosphate Buffered Salline) solution, with an Ag/AgCl electrode as the reference.

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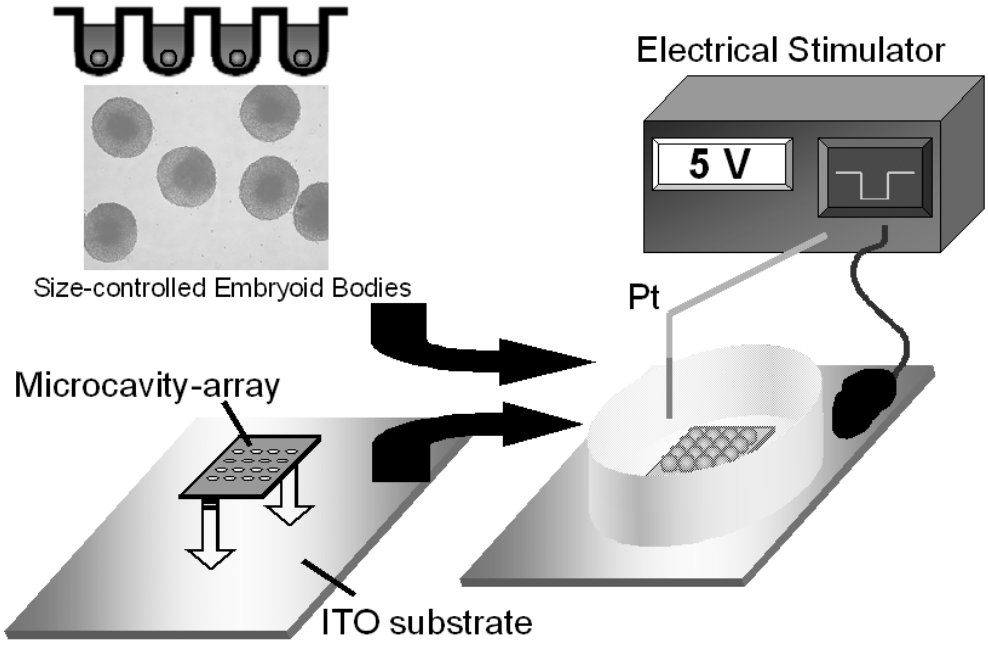

Fig.1. Schematic diagram of experimental system. Size-controlled embryoid bodies of P19 cells were uniformly stimulated through microfabricated ITO electrodes.

B. Preparation of EBs of P19 cells

P19, a stem cell-line established from a mouse embryonal carcinoma, is known to have the potential to differentiate into neurons efficiently [10]. Undifferentiated P19 cells were routinely cultured and proliferated in α-MEM containing 10 % fetal bovine serum and 100 unit/ml Penicillin-Streptomycin (Invitrogen). To produce size-controlled EBs, P19 cells were collected and replated in 96-well low cell-adhesion plates (Sumilon Spheroid Plates, Sumitomo). Due to its spheroid bottom structure, a single EB was formed in each well. Finally, the EBs were transferred to the developed microcavities. The surface of the microcavities was coated with 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer (Lipidure CM5206E, NOF corp.) to prevent cell adhesion to the substrates [11].

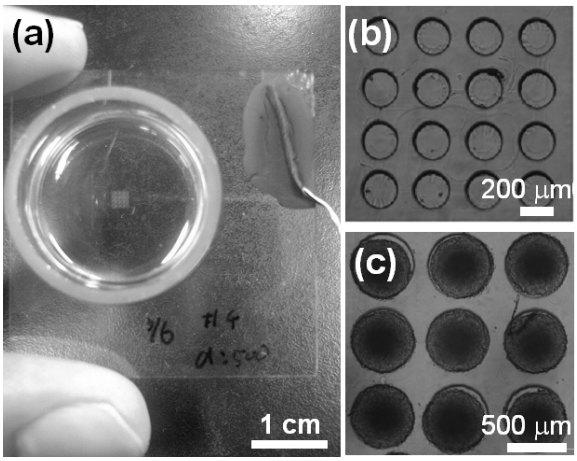

Fig. 2. (a) A microcavity-array dish for ensemble electrical stimulation. (b) Microcavities of 200 μm diameter aligned in a 4x4 matrix. (c) EBs of P19 cells inserted in microcavities of 500 μm diameter.

C. Electrical Stimulation

Constant-voltage stimulation was applied to the trapped EBs through electrodes with an electrical stimulator (SEN-8203, Nihon Koden) and an isolator (SS203J, Nihon Koden). A Pt electrode was used for the counter electrode during electrical stimulation experiments. A single negative-first biphasic pulse with an intensity of 5 V and duration of 1 ms per phase was applied. The EBs were loaded 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 2 s was used. The recording solution contained 148 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose. The overview of the experimental system is shown in Fig. 1.

III. Results and Discussions

Fig. 2 shows photographs of the fabricated device and magnified view of the integrated microcavity region. Fig. 2(b) shows a magnified image of 16 microcavities with diameter 200 μm. The bottom of each cavity was ITO conductive film, which acted as the embedded stimulation electrode. These microcavity structures could be fabricated with fine reproducibility. The EBs of P19 cells prepared by using spheroid-bottom plates showed high uniformity in size and morphology, which was determined by cell density of

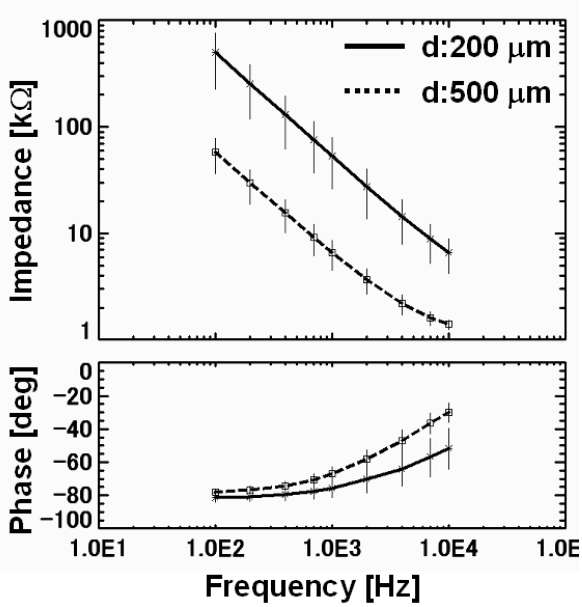

Fig. 3. Impedance spectroscopy of the microfabricated ITO substrates. Means ± SEM are shown. *n*=4 for the 200 μm pattern and *n*=6 for the 500 μm pattern.

suspension. We could establish conditions to obtain 200 and 500 μm EBs. Then, the size-controlled EBs were transferred to the developed substrates. Fig. 2(c) shows the EBs of P19 cells inserted in the microcavities of 500 μm. The EBs were successfully trapped in cavities with 200 μm depth. Pre-coating of the surface with Lipidure could inhibit cell attachment to the surface over several hours. Suspension culture conditions were maintained during electrical stimulation experiments.

Fig. 3 shows the electrical characteristics of the fabricated devices. The frequency dependent magnitudes and phases of the impedance of 200 μm diameter cavity-array substrates and 500 μm diameter substrates were compared. The 200 μm diameter microcavity-array substrate has the higher impedance compared to the 500 μm substrate. The impedance at 1 kHz was 53.3 ± 27.2 (± SEM, *n*=4) and 6.6 ± 2.1 kΩ (± SEM, *n*=6) for 200 μm and 500 μm diameter patterns, respectively. We confirmed that pre-coating with Lipidure did not affect the electrical properties of the device by performing impedance measurements (data not shown).

Electrical stimulation was applied to the trapped EBs and their responses were monitored by intracellular calcium imaging. Phase-contrast images of trapped EBs and the corresponding fluorescence images are shown in Fig. 4. The 200 and 500 μm cavities successfully trapped 200 and 500 μm EBs, respectively. The fluorescence images show difference ratios in fluorescence intensity between pre- and post-stimulus. Before stimulus, no spontaneous calcium transients were observed. In response to single stimulation pulse, clear elevation of intracellular calcium was detected. For both 200 and 500 μm cavities, most of the trapped EBs showed similar responses. This suggested that the developed stimulation device could stimulate the EBs inserted in the microcavities simultaneously. In all the stimulation experiments carried out in this work, we confirmed that similar results could be obtained (*n*=5 for the 200 μm diameter patterns and *n*=4 for the 500 μm patterns).

We finally attempted to estimate the uniformity of electrical stimulation of EBs using the microcavity-array substrate. Electrical pulses were repeatedly applied with 30 s interval and intracellular calcium transients were monitored. Fig. 5 shows the time courses of the intracellular calcium transients in 200 μm (a) and 500 μm diameter (b) patterns during electrical stimulation. The calcium transients were recorded from cells indicated in the fluorescence image. Nine cells were individually selected from 9 EBs for 200 μm cavities and 8 cells from 4 EBs for 500 μm cavities. In 200 μm cavities, each cell responded to most of the electrical stimulation and showed similar time courses of fluorescence transients. However, in 500 μm cavities, each cell did not constantly response to repeated stimulation and showed inconsistent time courses. This indicated that the microcavity-array could uniformly induced cell responses in the EBs for 200μm diameter pattern and could not for 500 μm pattern. To explore optimal stimulation conditions for uniform induction of cellular responses in EBs of any size will be required.

These results suggested that the developed microcavity-array with embedded electrodes could stimulate large number of size-controlled EBs simultaneously and thus would be useful tool for precisely controlling the differentiation processes of stem cells. Cell-cell interaction could be regulated through the EB-size control, and electrical stimulation had the potential to affect cell-differentiation processes. The next step of our study would be evaluation of the effects of stimulation conditions by analyzing the

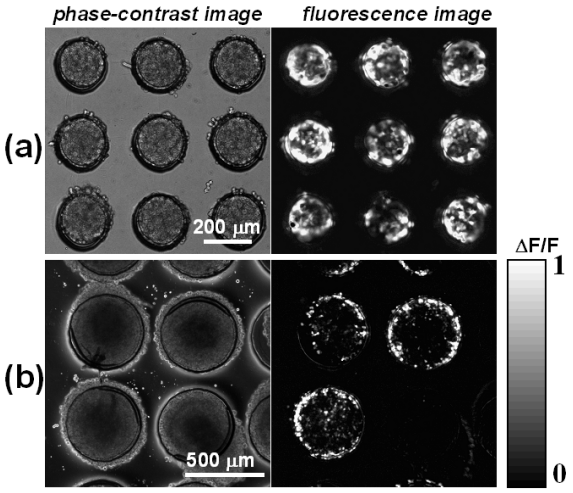

Fig. 4. EBs of P19 cells plated in microcavity arrays (phase-contrast image) and single-stimulus-induced intracellular calcium transients (fluorescence image). Microcavities of 200 μm (a) and 500 μm diameter (b).

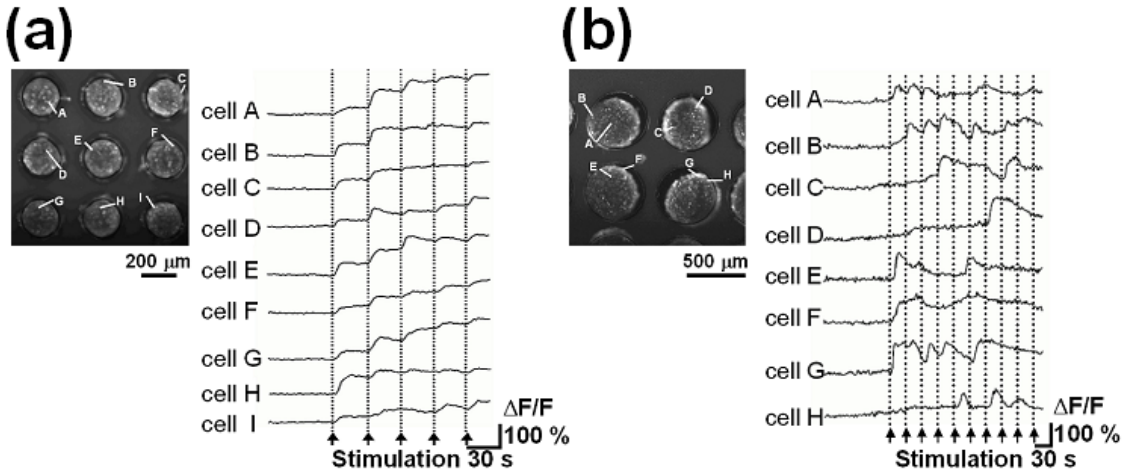

Fig. 5. Cell responses induced by repeated electrical stimulation. Time courses of fluorescence intensity were shown. The positions of the cell analyzed were represented in the fluorescent image. Electrical stimulation was applied 5 and 10 times for 200 (a) and 500 μm diameter (b) patterns, respectively.

expression levels of developmentally-regulated genes in the processes of neuronal differentiation, and the relationship between EB-sizes, stimulation conditions, and differentiation efficiency.

IV. Conclusion

We developed microcavity-array devices with embedded electrodes. Combining size-control techniques of cell aggregates with the microcavity-array, EBs of P19 cells were aligned in a matrix and electrically stimulated The stimulus-induced responses were monitored by intracellular calcium transients. The developed microcavity-array with embedded electrodes could stimulate a large number of size-controlled EBs simultaneously and effectively. This method would be a promising tool to increase cell-differentiation efficiency and to regulate the differentiation processes.

Acknowledgment

This work was partly supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

[1] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka , “Induction of pluripotent stem cells from adult human fibroblasts by defined factors”, *Cell*, Vol. 131, pp. 861-872, 2007.

[2] B. S. Yoon, S. J. Yoo, J. E. Lee, S. You, H. T. Lee and H. S. Yoon, “Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment”, Differentiation, Vol. 74, pp.149-159, 2006.

[3] J. Park, C. H. Cho, N. Parashurama, Y. Li, F. Berthiaume, M. Toner, A. W. Tilles and M. L. Yarmush, “Microfabrication-based modulation of embryonic stem cell differentiation”, *Lab Chip*, Vol. 7, pp. 1018-1028, 2007.

[4] J. M. Karp, J. Yeh, G. Eng, J. Fukuda, J. Blumling, K-Y Suh, J. Cheng, A. Mahdavi, J. Borenstein, R. Langer and A. Khademhosseini, “Controlling size, shape and homogeneity of embryoid bodies using poly(ethylene glycol) microwells”, *Lab Chip*, Vol. 7, pp. 786-794, 2007.

[5] W. Wang, K. Itaka, S. Ohba, N. Nishiyama, U. Chung, Y. Yamasaki and K. Kataoka, “3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells", *Biomaterials*, vol. 30, pp. 2705–2715, 2009.

[6] K. Kimura, Y. Yanagida, T. Haruyama, E. Kobatake and M. Aizawa, ”Gene expression in the electrically stimulated differentiation of PC12 cells”, *J. Biotechnol.*, Vol. 63, pp.55-65, 1998.

[7] M. Yamada, K. Tanemura, S. Okada, A. Iwanami, M. Nakamura, H. Mizuno, M. Ozawa, R. Ohyama-Goto, N. Kitamura, M. Kawano, K. Tan-Takeuchi, C. Ohtsuka, A. Miyawaki, A. Takashima, M. Ogawa, Y. Toyama, H. Okano and T. Kondo, “Electrical stimulation modulates fate determination of differentiating embryonic stem cells”, *Stem Cells*, Vol. 25, pp. 562-570, 2007.

[8] T. Nikolova, J. Czyz, A. Rolletschek, P. Blyszczuk, J. Fuchs, G. Jovtchev, J. Schuderer, N. Kuster and A. M. Wobus, “Electromagnetic fields affect transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells”, *The FASEB Journal*, 10.1096/fj.04-3549fje, 2005.

[9] C. Venture, M. Maioli, Y. Asara, D. Santoni, P. Mesirca, D. Remondini and F. Bersani, “Turning on stem cell cardiogenesis with extremely low frequency magnetic fields”, The FASEB Journal, 10.1096/fj.04-2695fje, 2004.

[10] G. Bain, W. J. Ray, M. Yao, and D. I. Gottlieb, “From embryonal carcinoma cells to neurons: The P19 pathway”, *Bioessays*, Vol. 16, No. 5, pp. 343-348, 1994.

[11] K. Ishihara, E Ishikawa, Y. Iwasaki and N. Nakabayashi, “Inhibition of fibroblast cell adhesion on substrate by coating with 2-methacryloyloxyethyl phosphorylcholine polymers”, J. Biomater. Sci. Polymer. Ed., Vol. 10, pp. 1047-1061, 1999.