# SPATIALLY PATTERNED NEURAL AND CARDIAC DIFFERENTIATION OF EMBRYOID BODY (EB) IN A MICROFLUIDIC DEVICE

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## ABSTRACT

Experiments using a micro-device, consisting of two flow channels and 3 pillars standing at the channel crossing, firstly showed that an embryoid body (EB) of iPS cells can be differentiated into neural and cardiac cells in a spatially patterned manner. Theoretical simulation showed that the chemical gradients can be kept at the crossing even with small flowing velocity of 0.1uL/min. It demonstrated that patterned differentiation is induced in one EB when 2 media were perfused simultaneously. The experiments show its advantages in maintaining a low shear while keeping two gradients; it will help to understand the developmental biology.

## KEYWORDS

Patterned differentiation, embryoid body, iPS, neuron, cardiac induction, microfluidics.

## INTRODUCTION

Pluripotent stem cell has been widely applied in both fundamental research and biomedical field; it is expected to be a source of renewable cells for tissue regeneration/replacement therapies because of ability of self-renewal and differentiation into almost all derivatives. However, it is still difficult to control and manipulate over two types of heterogeneous differentiation of pluripotent stem cell. Conventional culturing techniques using only dishes or plates can't generate stable chemical gradients continuously for a long time, and do not allow cultured stem cells to be affected by more than one differentiation media at the same time. Methods using hydrogel or micro-capillary can't stably produce over two kinds of gradients simultaneously for cell culture or differentiation [1]. Therefore, microfluidic device was introduced due to its unique properties such as force effects, surface effect and friction effects. Spatial undiffentiated/differentiated EB model has been reported in a microfluidic device, however, it is not easily controlled because the applied shear stress of 50uL/min to 200 uL/min would wash cells away or exert a reverse effect on cell [2].

In order to generate a stable chemical gradient while keeping proper perfusion flowing condition for spatially dual differentiation, we developed a new microfluidic device, which would support a low level of shear stress, at the same time two chemical gradients can be kept for culturing and cell differentiation; it is expected to control two kinds of differentiation processes by manipulating the culturing conditions (medium kinds, flow rate, factor concentration etc.) in both 'time and space'. Our experimental results of immuno-staining indicated that neural and cardiac lineage cells were successfully differentiated in one EB spatially. It indicated that the chemical gradients play a key role in directing the spatial differentiation and modulating the structure of derivative directly. This suggested that spatially patterned differentiation can be controlled by perfusing two gradient growth factors in a microfluidic device. In the future, multiple chemical gradients more than 2 factors can be expected to control in vitro differentiation, to generate complex cell derivatives for medical test or tissue engineering in the biomedical field. The present experiment is also helpful for understanding embryonic developmental biology.

## MATERIALS AND METHODS

The device was fabricated using a method of photolithography, at first, the device diagram was drawn by Autocad 3.4, based on which a GDS file was prepared, then a shadow mask was fabricated by using a laser exposing system. The mask is developed for UV exposure. Photosensitive epoxy (SU-8 2100) was spun coated onto a cleaned silicon wafer, followed by being baked at 65℃ for 15 min and 95℃ for 90 min, then it was exposed to UV light through the mask. After baking, it was developed in SU-8 developer for 15 min and rinsed with IPA (isopropyl alcohol). The wafer was coated with CHF3, then Poly-dimethylsiloxane (PDMS) was poured on the wafer, after degassing, the PDMS block was heated, then it was trimmed to get PDMS model, after adherence onto a slide glass by oxygen plasma and silicon tubes assembling; the micro-device can be used for culture experiment.

Before culture using the microfluidic device, theoretical simulation using software of Comsol Multiphysics 3.5a was carried out for determining flowing velocity and chemical concentration at the crossing of the flowing channel.

Induced pluripotent stem cell (iPS) (cell line: MEF-Ng-20D-17) was purchased from Kyoto University, cultured on mouse embryo fibroblast (MEF) was maintained in DMEM containing 15% KSR (Knockout Serum Replacement), and LIF with medium changed every day in a humidified incubator (37℃, 5%CO2). iPS cells were dissociated and plated 2000 cells/well onto a non-adherent culture plates to form a spheroid (so called embryoid body, EB). After 2 days of culture, one EB was taken out and seeded onto the crossing of the micro-device. After connecting the microfluidic device to the pumps, medium containing 20%FBS and/or medium containing N2 medium was perfused into the device from the two channels at 0.1ul $min^{-1}$ respectively. In our experiment, cardiac differentiation of EB was induced by a medium containing 20% FBS, neural differentiation was induced by a medium containing N2 supplement. After perfusion culture for 4 days in the microfluidic device, the samples were



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immuno-stained by neural marker of b3-tubulin and cardiac marker of Troponin.

## RESULT AND DISCUSSION

As Figure 1 showed, a microfluidic device is composed of two flow channels, and an EB seeding channel. There is one PDMS pillar and 3 PDMS pillars standing at the flow channels and the crossing respectively for preventing EB from flowing away. The height of PDMS pillar is 430 μm. The diameter of PDMS pillar is 80μm.

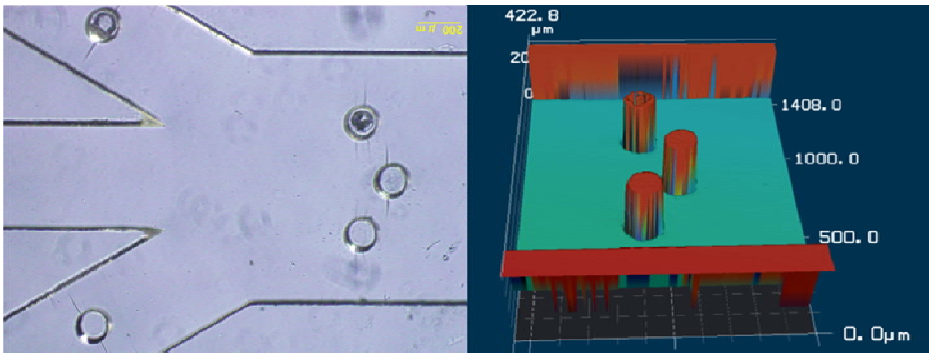


*Figure 1 Two dimensional and three dimensional structures (by laser microscope) microphotographs of the microfluidic device; PDMS pillars (dia.80um, height, 430um) standing at the crossing or channel for preventing from EB washing away;*

Theoretical simulation by using COMSOL Multiphysics indicated that a proper chemical gradient was stably kept at the culture crossing for inducing cell differentiation (Figure 2A), which exists even when the flowing speed was set at 0.1uL/min corresponding to shear of 0.07mPa, which is small enough to avoid its influence on the cells (Figures 2). Calculation by COMSOL Multiphysics also showed that flowing rate at the cross point is nearly 0 (Figure 2B), even after EB is seeded at the center of the crossing (Figure 2B). It indicated that microfluidic device was proper for seeding EBs at the crossing point and culturing for proliferation and differentiation.

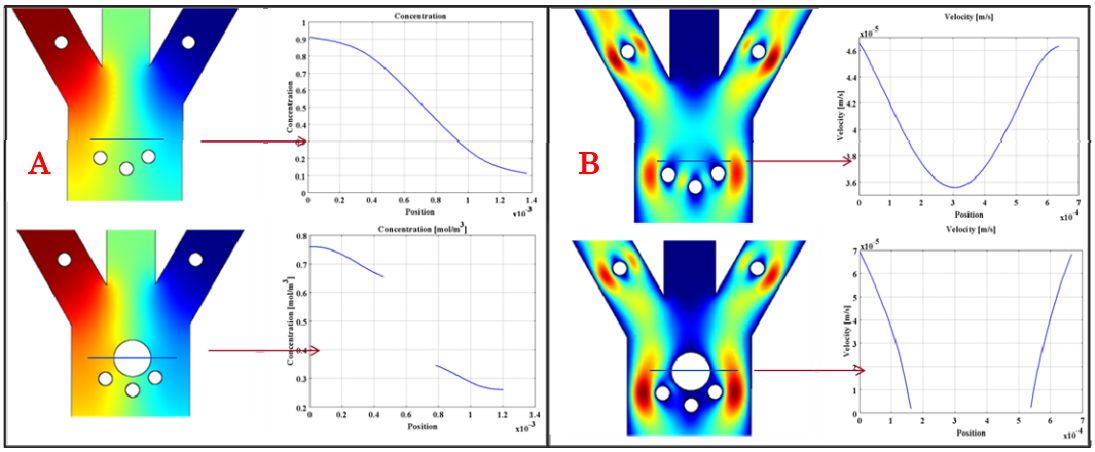


*Figure 2 Simulation of chemical gradients (A) and flowing velocity (B) in the -device with or without EB by Comsol Multiphysics. When two kinds of chemical solutions are perfused into the device, Figures on the right show the chemical gradient (or flowing velocity) along the blue line in the left pictures. It indicated that a proper chemical gradient existed at the crossing (B) and flowing velocity was the least there (B).*

As Figures 3-5 showed, the EB is perfusion cultured in N2, FBS or N2/FBS medium respectively, fluorescence intensity of the EB decreased and disappeared after 2 days of culture in N2, FBS or N2/FBS medium (data not shown). After perfusion culture in N2, FBS or N2/FBS media for 4 days, the EB in the microfluidic device was immuno-stained by using neural marker of b3-Tubulin and cardiac marker of cardiac Troponin together (Figures 3-5). The immuno-staining results demonstrated that 2 days' induction by N2 medium has resulted in the occurrence of neural tissues (data not shown), the neural fiber can be observed very clearly 4 days later (Figures 3). Similar to N2 induction, 2 days' induction by FBS medium has resulted in the occurrence of cardiac tissues (data not shown), the more cardiac tissue can be stained by cardiac marker of Troponin 4 days later (Figures 4). In all, perfusion culture promoted proliferation and differentiation. When EB was perfusion cultured in FBS and N2 medium simultaneously, immunostaining results showed that cardiac marker of troponin and neural marker of b3-tubulin were existed in one EB (Figure 5), which was also testified by real-time PCR (data not shown).

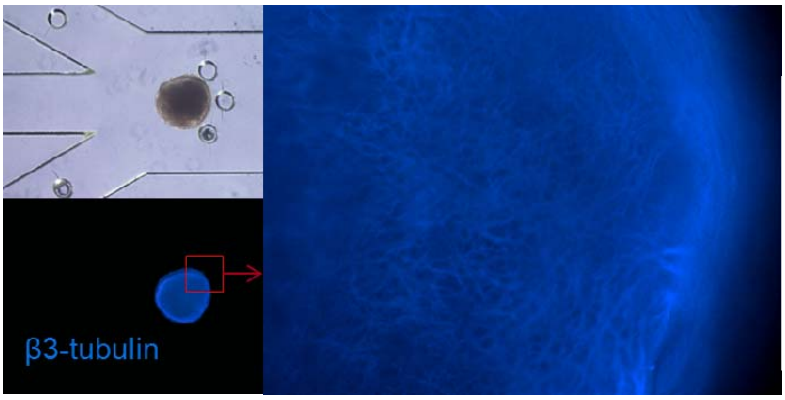


*Figure 3 Neural differentiation of embryoid body of mouse induced pluripotent stem cells in the microfluidic device after 4 days induction in N2 medium. The neural fiber was clearly observed, which is immuno-stained by neural b3-Tubulin.*

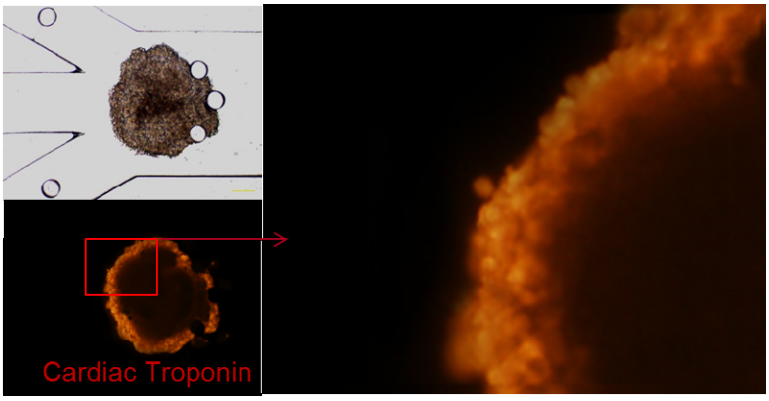


*Figure 4 Cardiac differentiation of embryoid body of mouse induced pluripotent stem cells in the microfluidic device after 4 days induction in FBS medium. The derivatives were immuno-stained by cardiac Troponin*

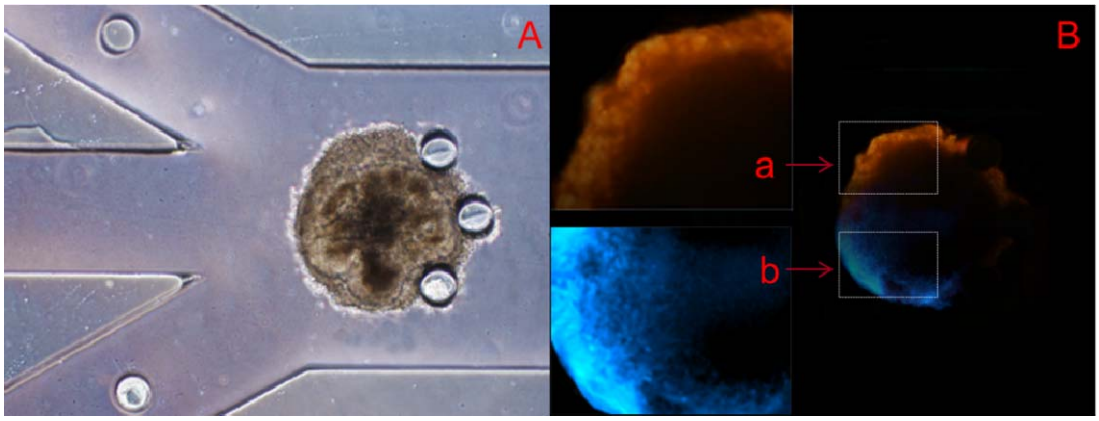


*Figure 5 Patterned differentiation of embryoid body of mouse induced pluripotent stem cell after culture for 4 days perfusion in the microfluidic device, which was immuno-stained by cardiac Troponin and Neural b3-Tubulin.*

## CONCLUSIONS

A simple microfluidic device was developed to mimic in vivo surroundings, which can keep low shear and chemical gradient simultaneously; and a spatially patterned neural and cardiac differentiation was firstly carried out. The results showed that spatially differentiation of neural and cardiac lineage was successfully induced by controlling the flow rate and chemical gradient. The experiments suggested that it is possible to control the differentiation of different cell types of the stem cell artificially by adjusting the flowing velocity or chemical gradients etc. Therefore, it would be a useful tool to induce over two kinds of differentiation simultaneously in vitro in future stem cell researches in the biomedical field such as medical test, tissue engineering, which also would help us to understand in vivo embryonic development well,

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