Dielectrophoretic cell trapping and parallel one-to-one fusion based on field constriction created by a micro-orifice array

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Micro-orifice based cell fusion assures high-yield fusion without compromising the cell viability. This paper examines feasibility of a dielectrophoresis (DEP) assisted cell trapping method for parallel fusion with a micro-orifice array. The goal is to create viable fusants for studying postfusion cell behavior. We fabricated a micro-fluidic chip that contained a chamber and partition. The partition divided the chamber into two compartments and it had a number of embedded micro-orifices. The voltage applied to the electrodes located at each compartment generated an electric field distribution concentrating in micro-orifices. Cells introduced into each compartment moved toward the micro-orifice array by manipulation of hydrostatic pressure. DEP assisted trapping was used to keep the cells in micro-orifice and to establish cell to cell contact through orifice. By applying a pulse, cell fusion was initiated to form a neck between cells. The neck passing through the orifice resulted in immobilization of the fused cell pair at micro-orifice. After washing away the unfused cells, the chip was loaded to a microscope with stage top incubator for time lapse imaging of the selected fusants. The viable fusants were successfully generated by fusion of mouse fibroblast cells (L929). Time lapse observation of the fusants showed that fused cell pairs escaping from micro-orifice became one tetraploid cell. The generated tetraploid cells divided into three daughter cells. The fusants generated with a smaller micro-orifice (diameter $\sim 2 \mu\text{m}$) were kept immobilized at micro-orifice until cell division phase. After observation of two synchronized cell divisions, the fusant divided into four daughter cells. We conclude that the presented method of cell pairing and fusion is suitable for high-yield generation of viable fusants and furthermore, subsequent study of postfusion phenomena.

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I. INTRODUCTION

In vitro cell-cell fusion is a tool for creation of hybridoma,¹ *in vitro* fertilization,² production of cloned offspring,³ and epigenetic reprogramming of somatic cells.⁴ One commonly used method to obtain *in vitro* cell-cell fusion is electrofusion. It is a purely physical method that does not require any chemicals that might disrupt normal cell functions. The conventional electrofusion method involves two steps. First, the cells are brought into contact with dielectrophoresis (DEP). Then, the cells in contact are exposed to a pulsed electric field to induce membrane potential. When the membrane potential exceeds a certain threshold value, pores are formed in the cell membrane. The pores formed in contact zone, eventually, lead to membrane and cytoplasmic continuity. However, the induced cell membrane potential depends on the cell diameter, which leads to significant low fusion yield and high cell damage. Another drawback is about cell pairing. As fusion is performed in bulk, the random pairing between cells results in unwanted fusion products, such as a fusant formed between the same kinds of cells or fusant formed by more than

FIG. 1. (a) Schematic of the microfluidic chip. The chip design is composed of a fusion chamber separated with a partition. (b) Scanning electron microscope view of the partition with a number of embedded micro-orifices and close view of the micro-orifice.

two cells. Finally, fusant selection protocols are needed to select the fusant of interest among the remaining unfused population. These selection protocols are labor intensive and time consuming.

Recently several groups used microfluidic technology to develop on-chip cell fusion platform. Microfluidic control with hydrodynamic trapping was used to create cell pairs for fusion of different cell types.⁵ Passive delivery function was embedded to electrofusion chips to provide simple and cost effective cell and nutrient delivery for culturing of fusant.⁶ Possibility of high throughput cell fusion chip is demonstrated by integration of microfabricated electrodes into microfluidic channels.⁷ These technologies are important steps toward development of microfluidic cell fusion platform. However, they mainly involve miniaturization of the conventional electrofusion techniques and they do not address the fundamental problems, such as low fusion efficiency in fusing cells with different diameters and fusion of selected cell pairs in one-to-one basis.

In this paper, we propose a microfluidic chip based electrofusion method, which employs micro-orifice assisted cell fusion protocol to address problems of conventional electrofusion. By using micro-orifice as a tool to focus electric field, one can induce localized membrane potential, which is not influenced by the cell diameter. Preliminary work⁸ showed that micro-orifice based method allows the fusion of cells with different diameters leading to higher fusion yield and minimum cell damage. A channel network created on the chip enables one-to-one pairing of desired cell types, which prevents creation of undesired fusants. The micro-orifice based method furthermore offers a unique way to immobilize fused cells such that separation of the created fusants from the unfused cells becomes feasible, thus eliminating the requirement for conventional selection protocols and allows studying postfusion behavior of the selected fusants.

The schematic of microfluidic chip is shown in Fig. 1(a). The chip is composed of a fusion chamber with two inlets for introducing different cell types. An insulating partition involving micro-orifice array is located in the middle of the fusion chamber. Figure 1(b) shows scanning electron microscope view of the partition and close-up view of the micro-orifice array. When voltage is applied to electrodes, concentrated electric field lines passing through the micro-orifice generates a high electric field zone. By dielectrophoretic manipulation cells can be moved toward the high electric field zones and cell contact through the micro-orifice is established. Finally, fusion is initiated by applying a pulsed electric field.

II. METHODS

A. Materials and equipment

L929 mouse fibroblasts were used in fusion experiments. Cells were cultured in Dulbecco's modified eagle medium obtained from Gibco Life Tech Corp., Grand Island, NY, supplemented with 10% fetal bovine serum and penicillin-streptomycin. Monolayer culture was harvested when

FIG. 2. (a) Part of the fabricated mold for micro-orifice; (b) the mold after modification with polymer solution; (c) PDMS casting; (d) completed chip.

confluency reached 70%. Low conductivity fusion buffer with 0.1 mM Ca acetate, 0.5 mM Mg acetate, 1 mg/ml bovine serum albumin, and sorbitol sufficient to adjust osmolality to 100 mOsm was used to suspend cells for fusion.⁹

Polydimethylsiloxane (PDMS) (Dow Corning, Sylgard 184, Midland, MI), SU8 photoresist (MicroChem, SU8-25, Newton, MA), barrier coat (Shin-Etsu Chemical, No. 6, Tokyo, Japan), and cytosolic fluorescent dye (Dojindo Molecular Technologies, calcein-acetoxymethylester, Kumamoto, Japan) were used as received in all experiments.

An inverted microscope (IX-71, Olympus, Tokyo, Japan) equipped with a charged-coupled device camera (WAT-221S, Watec, Yamagata, Japan) served as imaging platform. The objective lenses were the UPlanApo 10×/0.4 Ph1 and UPlanApo 40×/0.85 ∞/0.11–0.23. Programmable function generator (WF 1974, NF Electronics, Kanagawa, Japan) was used to supply the required voltage pulse. High speed bipolar amplifier (HSA 4012, NF Electronics, Kanagawa, Japan) was used to add signals to induce cell fusion. Time lapse imaging was done by using stage top incubator (Tokai Hit, INU series, Shizuoka, Japan).

B. Microfluidic chip fabrication

Chip fabrication involved three steps: Mold preparation, modification of the mold, and PDMS casting. The mold was fabricated by spin coating SU8 on silicon wafer to a thickness of 25–45 μm. After the soft bake (65 °C×3 min and 95 °C×7 min), exposure was done with UV dose suggested by the resist manufacturer. The fabrication of the SU8 mold was completed after postexposure bake (65 °C×1 min and 95 °C×3 min) and development in developer supplied by manufacturer (2 min). Figure 2(a) illustrates portion of the fabricated mold with a slit for micro-orifice generation. The modification of the mold was done by spin coating polymer solution on SU-8 mold. The slit had a width of 4 μm. Polymer solution was prepared by diluting barrier coat with toluene (volume ratio of 2%–4%). 1 ml of the polymer solution was deposited on mold and spin coated (1000 rpm×30 s). The coated polymer formed a suspended solid bridge in the slit [Fig. 2(b)]. This bridge was used as a part of the mold as it is (without further baking step). PDMS was prepared by mixing 1 g of curing agent with 10 g of prepolymer. The mixture was degassed for 30 min with a desiccator. Before pouring PDMS on the mold, spacers with thickness of 1 mm were placed to sides of the mold. Then, PDMS was poured on to the mold and a microscope slide glass was used to cover the PDMS layer. The spacers assured the thickness of the

FIG. 3. [(a) and (b)] Pair formation procedure by tilting the chip. [(c)–(e)] Postfusion handling of fusant.

cured PDMS chip to be 1 mm. Hot plate was used for curing the PDMS ($150\text{ }^{\circ}\text{C} \times 30\text{ min}$). The chip was then released from the mold [Fig. 2(c)]. A microscope slide glass with aluminum electrodes was prepared by physical vapor deposition and standard photolithography. The electrodes had a gap distance of $400\text{ }\mu\text{m}$. The PDMS chip was then placed on the prepared slide glass [Fig. 2(d)].

C. Cell pairing protocol

Cells were introduced into chip by using power-free pumping.¹⁰ First, the chip degassed in a vacuum desiccator (ultimate pressure for pump: 1.3 Pa) for 5 min. As soon as the chip was taken out of the desiccator, a $40\text{ }\mu\text{l}$ of cell suspension (in fusion buffer) was introduced into each inlet. Cell suspension began to fill the fluidic channel network and the cells were transported to the fusion chamber. Then, the chip was tilted, as shown in Fig. 3(a), for 20 s. When chip was tilted, the hydrostatic pressure difference between two inlets created a flow, which carried cells toward micro-orifice. Then ac field was applied to trap the cells at the micro-orifice by dielectrophoretic force. Finally, the chip was tilted the other way, as shown in Fig. 3(b), to move the cells on the other side of the chamber. The ac field was stopped as soon as pair formation was confirmed. After formation of pairs, the number of pairs was confirmed and fusion procedure was initiated.

D. Fusion procedure

Fusion sequence was composed of application of ac field ($10\text{ V}_{\text{p.p.}}$, 1 MHz) for 10 s and then superposing a dc pulse (4 V, $300\text{ }\mu\text{s}$) to ac field and application of ac field for another 10 s. The success of fusion was confirmed by observing the cytoplasmic dye transfer from one cell to the other. Then, the cells are kept undisturbed for 10 min.

E. Separation of unfused cells and postfusion characterization

After fusion procedure the unfused cells in the inlets were removed and fresh fusion buffer was introduced [Fig. 3(c)]. Then, the PDMS chip was gently released from the electrode substrate and placed on a Petri dish with channel surface looking upward [Figs. 3(d) and 3(e)]. During this process, the cell pairs fused in orifice remained in their position while the unfused cells were washed away. Then complete cell growth medium was placed on the chip and the Petri dish was loaded to a stage top incubator for time lapse imaging.

FIG. 4. (a) Image taken in the end of cell formation protocol. (b) Close view of the selected portion.

III. RESULTS

A. Cell pairing

Cell pairing efficiency was characterized by direct observation of paired cells with microscope. Figure 4 shows the phase contrast image of paired cells at the end of the cell pairing protocol. Cell pairs were formed in most of the orifices with a pair formation efficiency of 95%–100%.

B. Fusion

Figure 5 shows still images recorded during fusion experiment. The cells in the lower chamber were labeled with calcein. The cells in upper chamber were not labeled. We used calcein as an indicator of fusion showing cytosolic connectivity and viability of cells after fusion. As soon as fusion was induced a neck between two cells was observed and the fluorescent dye was transferred

FIG. 5. Still images during fusion experiment. The location is the same with Fig. 6(a). (a) Fluorescent image of cells before fusion pulse, (b) Fluorescent image after fusion pulse. (c) Close up view of the selected part after the fusion pulse.

FIG. 6. The image taken after separating the PDMS chip from electrode substrate and placed in a culture dish.

to upper cell. The cells in the upper chamber began to light up, which takes a “snowmanlike” shape [Figs. 5(b) and 5(c)]. We confirmed significant number of successful fusion. The image shows only 14 of them. The fusion efficiency was $>95\%$. After the fusion, the cells were kept undisturbed for 10 min. Then, the PDMS chip was released from electrode substrate. The cell pairs fused in the orifice did not change their positions during this manipulation. Figure 6 shows the image taken after the chip was inverted and placed on a Petri dish with complete cell growth medium. The unfused cells were washed away and the selected fused cells were collected. The necks formed between cells were found to be robust enough for immobilization to separate fused cells from the remaining population.

C. Postfusion behavior

The time lapse observation of the collected fusants was made by loading the PDMS chip to a microscope equipped with on-stage incubator. Figure 7(a) shows five cell pairs fused on the chip at the beginning of the incubation period. The trace diagram for each fusant showing the division of cells and number of daughter cells is illustrated in the same figure. We found that the fusants

FIG. 7. (a) The collected fusants at the beginning of the time lapse imaging and trace diagram of fusants showing cell division. (b) The actual images during cell division for fusant 2 in Figs. 7 and 8. [URL: <http://dx.doi.org/10.1063/1.3422544.1>]

FIG. 8. (a) Synchronized cell division observed on chips with smaller micro-orifice (diameter $\sim 2 \mu\text{m}$). (b) Actual time lapse images of the synchronized cell division. [URL: <http://dx.doi.org/10.1063/1.3422544.2>]

escaping from the orifice divided into three. The created daughter cells divided into either three or two. Figure 7(b) shows the actual images of cell division. A fusant escaped from the orifice divided into three denoted as A, B, and C. One of the daughter cells (cell A) divided into two and the other daughter cell (cell B) divided into three. When the experiments were repeated by using chips having smaller orifice (with diameter $\sim 2 \mu\text{m}$), the fusants did not escape from the orifice until mitotic cell division phase. While fusants immobilized in the orifice, synchronized cell division was observed, as illustrated in Fig. 8(a). Actual images of synchronized cells division can be seen in Fig. 8(b).

IV. CONCLUSION

We fabricated a microfluidic chip to verify feasibility of a DEP based cell pairing method for micro-orifice assisted cell fusion and studying postfusion behavior. Large number of cell pairs were formed and fused with an efficiency $>95\%$. The success of fusion and viability of the fusants were confirmed by fluorescence imaging. The postfusion behavior of the fusants was studied by time lapse imaging. Surprisingly, we found that the fusants, which escaped the orifice, divided into three. Similar ternary division was observed in one of the generated daughter cells. We tentatively attribute this behavior to unequal distribution of chromosomes due to formation of multipolar spindles in tetraploid cells.¹¹ Figure 9 shows a possible situation when a tetraploid cell formed by fusion of two cells. Unequal distribution of the chromosomes generated a tetraploid daughter cell. This suggests the possibility of a repeated ternary division in the next cell cycle. The conventional fusion methods are based on random pairing and one-to-one cell fusion is never guaranteed. We believe this phenomena became observable in cell fusion generated tetraploid cells for the first time by assuring assuring one-to-one cell pairing and selection of fused pairs provided by the proposed technology. Furthermore, when experiments were repeated with micro-orifice having a smaller diameter ($\sim 2 \mu\text{m}$ micro meter), we found that the fusants were kept immobilized at the

FIG. 9. Unequal distribution of chromosomes during ternary division.

micro-orifice until mitotic cell division. The fusant divided into four with two synchronized cell divisions. We conclude that fabricated microfluidic chip based on proposed cell pairing method is suitable for high-yield one-to-one fusion and creation of viable fusants. This technology should be a useful tool to study postfusion phenomena, as it allows imaging of the selected cell pairs during and after the fusion.

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