A microfluidic Device for Electrofusion of Two Plant Cells

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Abstract—**In this paper, we have electrofused two cells which are P. japonicum and G. littoralis at the 'Lab on a Chip' level. The microchannel platform was fabricated using poly(dimethylsiloxane)(PDMS) via the conventional softlithographic procedure, and the microelectrode patterns (we e-beam evaporated the titanium (as seed layer) and the gold with the thickness of 200 Å and 3000 Å and patterned by the chemical etching) were created on the surface of glass. Both the PDMS platform and electrode glass were aligned and combined via the oxygen plasma-treatment. The channel has two inlets and two types of cells were slowly introduced by using the syringe pumps and we stopped when the cells were positioned around the electrodes. We have applied 1 – 2 MHz AC field (Amplitude: 8 – 10 p-p) with rectangular wave shape to the microelectrodes to form the pearl-chain between the electrodes. Then, the cell-membranes contact closely each other. For the cell fusion, we again applied short DC pulse (amplitude: 250V/mm, duration: 10 – 100 ms) to the microelectrodes. A little pore was generated and finally two cells were fused to single cell. The fused cells were cultured in Nitsch medium containing growth regulators and some cells were cultured. We will investigate their viability with FDA (fluorescein diacetate). This results may encourage the maximum yield of 1: 1 heterologous fusion under the micro environment.**

Keywords—**Electrofusion, Microfluidic device, *P. japonicum, G. littoralis* , Poly(dimethylsiloxane)(PDMS)**

I. Introduction

Electrical cell fusion is a fundamental step in modern biology and biotechnology, such as the hybridoma for antibody production, the cloning of mammals, genetic make-up of organisms and vaccination against cancer. Compared to the chemically induced cell fusion via polyethylene-glycol (PEG), electrical cell fusion has many advantages such as its independence of the target cell types, the high fusion efficiency and the simplicity.[1-2] The devices with a microscale size have many advantages, such as smaller reagent volumes, shorter reaction times, the possibility of parallel operations, and the promise of integrating an entire laboratory or culture system onto a single chip. However, in plant cell f, such microchip technology has not been employed. In this work, we have electrofused two dune plant cells, which are Peucedanum japonicum and Glehnia littoralis, to get the better species using microchip. The microchannel platform was fabricated using Poly(dimethylsiloxane)(PDMS) via the conventional softlithographic procedure. To make the microelectrode patterns on the glass, we e-beam evaporated the titanium and the gold with the thickness of 200 Å and 3000 Å and patterned by the chemical etching. Both the PDMS platform and electrode glass were aligned and bonded via the oxygen plasma-treatment. The channel has two inlets and two kinds of cells were slowly introduced by using the syringe pumps and we stopped when the cells were positioned around the electrodes. We have applied 1 – 2 MHz AC field (P-P Amplitude: 8 – 10 Volts) with rectangular wave shape to form the pearl-chain between the electrodes. The fused cells were cultured in Nitsch medium containing growth regulators and some cells were cultured. We will investigate their viability with FDA (fluorescein diacetate). This results may encourage the maximum yield of 1: 1 heterologous fusion under the micro environment.

II. Materials and Methods

A. Fabrication of microfluidic devices

The photographs of the total fusion system and the fusion chip are shown in Fig 1(a) and (b) respectively and the mechanism of electrofusion is illustrated schematically in Fig. 1(c). The total fusion system consisted of applied DC-AC voltage, injection of cell and medium and microfluidic device of electrofusion. The microfluidic device consisted of two parts. One is a microchannel and the other is a electrode. The microchannel (height: 50μm, width 200 μm) platform was fabricated using Poly(dimethylsiloxane) (PDMS) via the0020conventional softlithographic procedure. The SU-8 (MicroChem, USA) based master mold was fabricated by the pre-reported procedures [3]. On the 3" silicon wafer, SU-8 which is polymerizable by 365 nm UV light was coated uniformly using the spin coater (P6708, Cookson Electronics Equipment, USA). The coated wafer (thickness: 100 μm) was baked on the hot plate for 10 minutes at the 65 °C and for 30 minutes subsequently at the

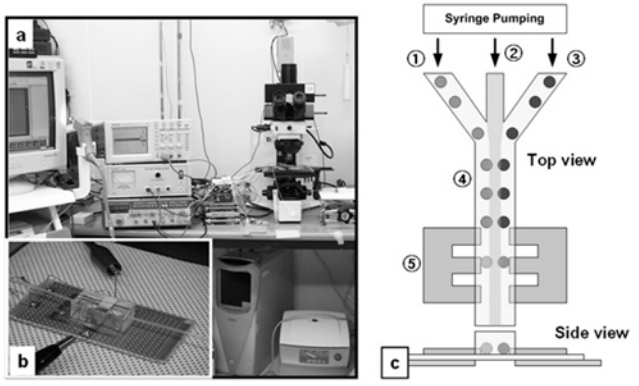

Figure 1 a) Photograph of electrofusion system b) Microfluidic device for electrofusion c) schematics of a micro electrofusion device for cell fusion. Only a couple of electrodes are shown for simplicity. (①, ②, ③ are inlet for cells and CPW respectively.

95°C (we call this process as soft bake). The repeated coating and soft baking were carried out to get a desired thickness. Onto the baked silicon wafer, we placed the transparent photomask having patterns of culture chamber and radiated the 365 nm UV light (Novacure 2100, EXFO, USA) onto the photomask, and we baked the UV radiated wafer for 1 minutes at the 65 °C and for 13 minutes afterward at the 95 °C (we call this process as hard bake). The desired pattern of culture chamber was designed using CAD (AutoCAD2002, Autodesk Inc., USA) software and was transferred to the transparent photomask using high quality printer. The SU-8 in the region where the UV light was radiated was polymerized. Through the use of SU-8 Developer (MicroChem, USA), the un-polymerized SU-8 was removed and the radiated regions were remained, and the master mold of culture chamber was constructed. Onto this master mold placed inside the Petri dish, the 10:1 mixture of the PDMS prepolymer and curing agent (Sylgard 184 silicone elastomer kit, Dow Corning, USA) was poured with the thickness of 10 mm. The mixture was thermally cured for 2 hours on a hot plate at 80°C, and separated from the mold. Then, the microchannel was generated.

To make the microelectrode patterns on the glass, we e-beam evaporated the titanium and the gold with the thickness of 200 Å and 3000 Å and patterned by the chemical etching. Both the PDMS platform and electrode glass were aligned and bonded via the oxygen plasma-treatment. The channel has two inlets and two kinds of cells were slowly introduced by using the syringe pumps and we stopped when the cells were positioned around the electrodes. We have applied 1 – 2 MHz AC field (P-P Amplitude: 8 – 10 Volts) with rectangular wave shape to form the pearl-chain between the electrodes.

B. Cell preparation

Peucedanum japonicum and Glehnia littoralis are perennial herbal plants living in coastal sand dunes (Figure 2). They have potential values for aromatic compounds and Chinese medicine. For example, P. japonicum was reported that had khellactone esters and coumarine, while G. littoralis had kaempferol and quercetin glycosides. But, these two plants do not inhabit in a same zonation because they have different growth characteristic by light intensity. Hence, we have carried out the protoplast electrofusion to produce hybrid not only with superior growth in broad light intensities and but also with wider secondary metabolites. In contrast to chemical fusion, electrofusion minimally yield comparable results to chemical fusions, at least in systems with robust protoplasts in good chemical fusion protocols, and do better with fragile protoplasts. Practical advantages are the speed of aggregation and fusion manipulations, and the possibility to rapidly determine the fusogenic properties of the protoplasts.

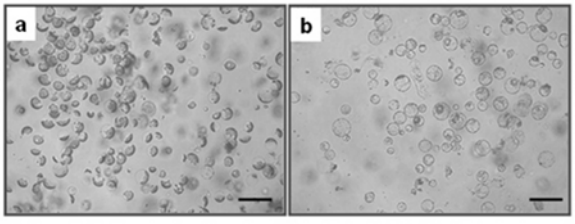

Figure 2. Picture of the protoplasts a) *Peucedanum japonicum* protoplasts (from leaves) b) *Glehnia littoralis* protoplasts (from callus). Scale bars are 50㎛.

Protoplasts were isolated according to a modification of Sun's method [4]. Fully expanded leaves of *Peucedanum japonicum and Glehnia littoralis* were surface-sterilized with 2% NaClO for 15 minutes, and washed with distilled water 4 times. The hypodermal layer was carefully removed with sharp-tipped forceps and cut into small pieces; then, the leaf pieces were carefully placed on the surface of the enzyme solution with the lower surface down. The leaf pieces in the enzyme solution were incubated at 25°C to release the protoplasts. The enzyme solution contained 1.5% (w/v) Cellulase Onozuka R-10, 0.5% (w/v) Macerozyme, 0.1% (w/v) Hemicellulase, 3mM MES, 7mM $CaCl_2 \cdot 2H_2O$, 0.4M mannitol, and pH 5.8. Digested leaf pieces were filtered through a #40 and a #100 steel mesh screen (Sigma, USA). The enzymatic mixture was centrifuged at 700 rpm for 10 minutes. After the upper enzyme mixture was discarded, the protoplasts were re-suspended and, through centrifugation and re-suspension, were washed with a washing solution (0.5M mannitol: 0.2M $CaCl_2$ = 2:1)

three times. The density of the protoplast suspension was adjusted to 2×105/ml. The protoplasts were cultured in the Nitsch medium containing with NLN-vitamin mixture(DUCHEFA BIOCHEMIE BV Haarlem, Netherlands) 0.5g/L, 4.0mg/L NAA, 0.1mg/L BA, 15% (w/v) sucrose, 9% (w/v) mannitol, pH 5.8.

C. Numerical Simulation

Simulations were performed using CFD-ACE (CFD-ACE is a trademark of CFD Research Corporation, AL) run on a personal computer. The finite-element method and 2-D structured grids were employed to calculate species concentrations in the cavity. The simulation was implemented in the transient state. Zero-flux conditions for the mass conservation equation were used for the boundary condition at boundaries of the cavity. The electric field under the applied voltage was solved with the boundary conditions of the fixed voltages at the top of the cavity and the microelectrodes on the glass. Symmetric boundary conditions on the right and left sides of the cavity and the power lawmethod were applied to have higher density grids and obtain better resolutions at the boundaries. The number of calculated nodes and elements were 60720 and 53235 respectively, in the present study.

III. Results and Discussions

Figure 3(a)-(b) show pearl-chain formation of protoplasts in microfluidic devices. We have applied 1 – 2 ㎒ AC field (P-P Amplitude: 8 – 10 Volts) with rectangular wave shape to form the pearl-chain between the electrodes. Figure 3a-c show pearl-chain formation of protoplast in microfluidic devices. The pearl-chain rate (%) of protoplasts depends on the frequency of AC pulse and we discovered that 1 ㎒ pulse shows the best chain formation (Figure 3d). Then, the cell-membranes contact closely each other by the pearl chain formation. For cell fusion, we applied short high voltage DC pulse (amplitude: 250V/mm, duration: 10 – 100 ms) to the microelectrodes. A little pore was generated and finally two cells were fused to single cell. Simulation result of electric field in two type microelectrode is shown in Figure 4. Understanding the electric field distribution between electrodes is important. Figure 4(a) is that simulation result of rectangular electrode. Figure 4(b) is which simulation result of round electrode. Electric field of rectangular electrode is uniformity but electric field of round electrode is not uniformity. Figure 5 shows the process of cells fusion at the microelectrodes Figure 5(a) show a cell alignment, (b) show that applied DC pulse to cell, (c)-(d) are heterokaryon phase and (e)-(f) are Fused cell

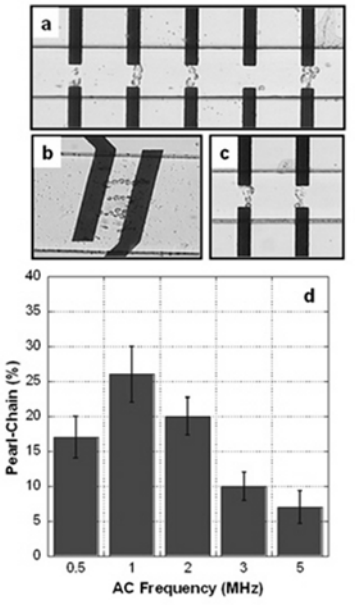

Figure 3. a)-c) Pearl-chain formation of protoplasts to the electrode shape d) Pearl-chain rate(%) of protoplasts according to the change of frequency

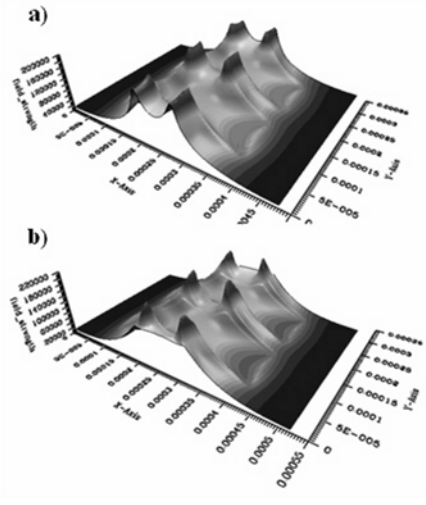

Fig 4 Simulated 3D views of the electric field distributions in the two type electrodes. (*a*) Rectangular type electrode (*b*) Round type electrode.

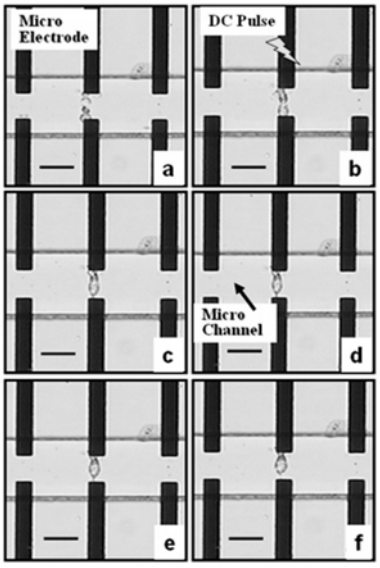

Fig 5 a) Pictures of cell fusion in microchannel and microelectrodes. a) cell alignment b) applied DC pulse c) and d) Heterokaryon phase e) and f) Entire fusion cell. Scale bars are 100 μm

IV. Conclusions

We have designed and fabricated a PDMS-based microfluidic device for electrofusion of two plants cell. We suggest a method satisfying two requirements: effective frequency of pearl-chain and reasonable DC-voltage of cell fusion. For the effective pearl-chain and fusion of cell, the proper frequency of AC voltage and amplitude of DC voltage was determined by the experiments. The fused cells were cultured in Nitsch medium containing growth regulators and some cells were cultured. We will investigate their viability with FDA (fluorescein diacetate). This results may encourage the maximum yield of 1 : 1 heterologous fusion under the micro environment.

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