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Electroporation and Electrofusion in Field-Tailored Microstructures*

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Abstract— **Bringing foreign substances into cells is a basic process in cell engineering. So called reversible breakdown of the cell membrane by electrical pulses opens up transient pores on the membrane through which molecules can diffuse into, or contacting cells can fuse. Using micro-fabricated structures with the dimension smaller than that of cells, the field pattern can be designed and tailored, that enables the handling of single cells, as well as the control over the location and the magnitude of the membrane voltage to achieve low invasive high-yield poration or fusion. This paper reviews some of our research, including gene transfection, electrofusion and cytoplasmic transplant.**

I. Introduction

Recent advances in regenerative medicine are accompanied by the needs and seeds for novel methods, including manipulation, measurement, regulation, modification and utilization of cellular functions. A typical example is found in regenerative medicine: tissue cells can be initialized by feeding several genes into cell, whose differentiation then induced by conditioning their environment.

The process has conventionally been done "in dish", where the cells are treated as a mass. However, if the cells are to be used for drug discovery or for clinical purposes, the cell quality, i.e. purity and traceability, must be established. As the cells batch-treated (e.g. transfected) in dish are not necessarily uniform, this requires the handling and assay in single-cell level. Microfabrication that creates the structure comparable or smaller than that of cells can be a powerful tool for such manipulation[1]-[3].

Another advantage of microfabrication, to be addressed in this paper, is its ability to tailor the field pattern for controlling the motion of cells and the membrane voltage. In particular, the field constriction created by micro-orifices can be used to define the location and the magnitude of the induced membrane voltage, through which low-invasive high-yield transfection or electrofusion can be made in highly reproducible manner.

This paper reviews the principle of the field constriction design for the reversible breakdown of the membrane, together with its application to gene transfection, cell fusion, and cytoplasmic transpolant.

II. Reversible Breakdown in Constriction Field

In conventional theory for the reversible breakdown, an isolated spherical cell in a uniform electric field is used as the model. Cell membrane in general is an insulator made of phospholipid bilayer, having the capacitance C_m of c.a. 1 μF/cm^2, regardless of cell type. When a stepwise electrostatic field is applied, the membrane voltage V_m is given by

$$V_m = \frac{3}{2} a E_0 \left(1 - \exp[-t/\tau]\right) \cos\theta \tag{1}$$

$$\tau = a C_m (\rho_{in} + \rho_{out}/2) \tag{2}$$

where a is the cell radius, E_0 is the magnitude of the externally applied field, θ is the azimuthal angle with respect to the external field, τ is the time constant of the charging, ρ_{in} and ρ_{out} are the conductivities of the interior and exterior of the membrane.

When V_m exceeds the critical breakdown voltage V_b, which is about 1 V regardless of type of cells, the partial disruption of the membrane occurs, but so far as V_m is moderate, the disruption is temporary, and spontaneously resealed due to lateral fluidity of the cell membrane (reversible breakdown). In conventional methods, a pulse voltage is applied to an ensemble of cells having size distribution, where large cells receive too much voltage and rupture, while the voltage is too small for small cells to induce effective breakdown. In addition, the cell is not necessarily spherical, in which case V_m depends on the shape and the orientation, making the yield even lower.

Our solution for the problem is to make use of the field constriction. The simplest model is a circular aperture (orifice) on an infinite insulating plate. Biological objects are treated in aqueous solution, which is more or less conducting. The electrical time constant of the medium $\tau_m=\varepsilon_m \rho_m$, where ε_m and ρ_m are the permittivity and the conductivity, is short enough so that $\omega\tau_m<<1$, and the field is conductivity governed,

$$\nabla \cdot \left[(j\omega\varepsilon_m + \sigma_m)\mathbf{E} \right] \approx \nabla \cdot \left[\sigma_m \mathbf{E} \right] = 0 \tag{3}$$

where j is the imaginary unit. The boundary condition at an insulating surface hence becomes $\partial\phi/\partial n=0$ where $\partial/\partial n$ denotes the normal derivative. In this case, the potential distribution around the orifice of radius a is given analytically in cylindrical coordinate (ρ, z) as

$$\phi = 1 - \frac{2}{\pi} \operatorname{arccot} \frac{\sqrt{2}\, a}{\sqrt{z^2 + \rho^2 - a^2 + \sqrt{(z^2 + \rho^2 - a^2)^2 + 4a^2 z^2}}} \tag{4}$$

which is plotted in Fig.1. Along the symmetry axis $\rho = 0$, 90% voltage drop occurs within $z < 6.3\ a$ and 80% within $z < 3.1\ a$. Hence, the voltage drop can be localized around the orifice, and by making the orifice smaller than the cell diameter, the field and thus the induced membrane voltage becomes controllable.

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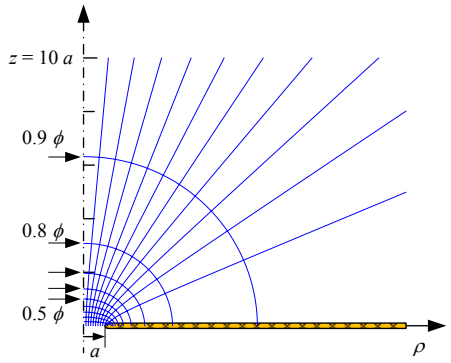

Fig.1 Constriction field produced by an infinite plate with a circular orifice

More detailed analysis including insulating membranes in physiological conditions can be made numerically, approximating the membrane as a capacitor, i.e. assuming the current continuity at the membrane

$$\mu_1 \frac{\partial \phi_1}{\partial n} = \mu_2 \frac{\partial \phi_2}{\partial n} \tag{5}$$

and the membrane charging

$$\phi_1 - \phi_2 = \frac{1}{C_m} \int \mu_1 \frac{\partial \phi_1}{\partial n} dt \tag{6}$$

where μ denotes the conductivity and the subscript 1 and 2 for the inner and the outer of the membrane. An example of the field analysis is shown in Fig.2 for the case of a) one cell on the orifice, and b) a pair of cells sandwiching the orifice. Axial symmetry is assumed, and to simulate inevitable leakage, a small gap is assumed between the cell membrane and the orifice edge.

Without an orifice, for the case of single cell, the voltage utility factor, defined as $\eta = V_{m,max}/V_{app}$, where $V_{m,max}$ is the maximum membrane voltage when steady state is reached, can be deduced from eq.(1) as

$$\eta = \frac{3}{2}\frac{a}{d} \tag{7}$$

where d is the spacing between the electrodes. η, as previously noted, is size-dependent, and is proportional to a/d. On the other hand, as shown in Fig.2, the orifice creates field constriction, where most voltage drop occurs within the area occupied by a cell, thus concentrating the voltage to the membrane bounded by the orifice ($\theta \leq 30°$ in the plot). η = 0.65 in this case due to the gap between the cell and the orifice, and it approaches unity at the limit of small gap and small orifice. The field constriction makes η insensitive to a. The same thing happens in the case of a pair of cells, except that the applied voltage is shared by the two cells.

III. Principle of Field-Constriction-Based Electroporation / Electrofusion

The inherent nature of the field constriction, $\eta \approx 1$, i.e. membrane voltage ≈ applied voltage regardless of the cell size

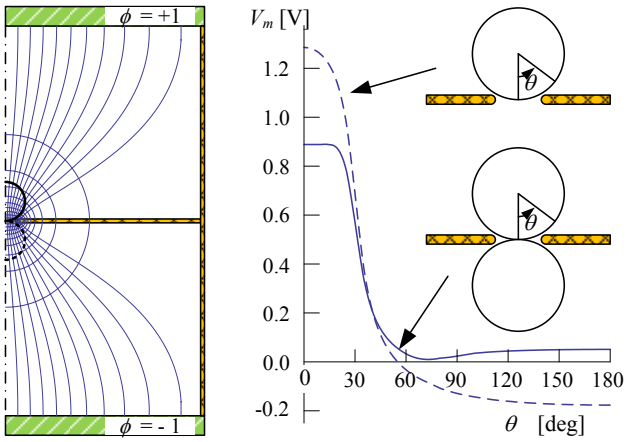

Fig.2 Membrane voltage on a cell and a pair of cells placed at the orifice having a dimaeter a. The electrodes are at $z = \pm 10\ a$, to which ± 1 V is applied. The gap between the cell membrane and the insulator takes minimum value $g = 0.066\ a$ at $\theta = 30°$.

and shape, can conveniently be used for reproducible electroporation and electrofusion, as schematically depicted in Fig.3.

Both adhesive and non-adhesive cells can be the subject of the on-chip electroporation. In the case of non-adhesive cells, they may be positioned onto the orifice by aspiration, while adhesive cells can directly be cultured on the orifice sheet as explained later in more detail.

On the other hand, in the case of electrofusion, the field constriction has dual role, for the positioning and for the breakdown. Two types of cells to be fused are re-suspended in buffers, fed to respective side of the orifice sheet. With the application of a.c. voltage, dielectrophoresis (DEP) drives the cells towards the orifice to form cell pairs there. Then an electrical pulse is applied to impress membrane voltage, which is concentrated at the contacting point in the orifice as

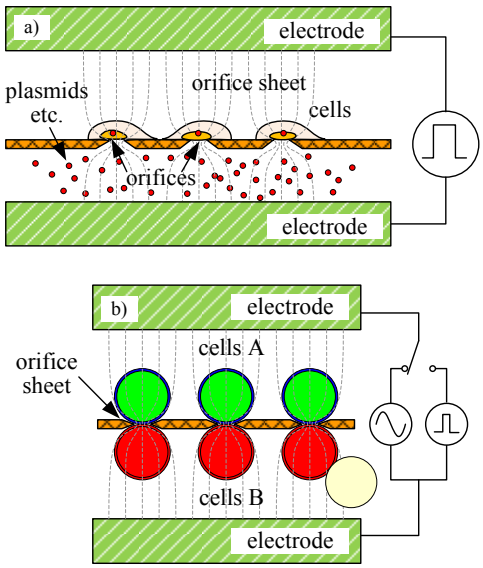

Fig.3 Electroporation (a) and electrofusion (b) based on field constriction at micro orifices

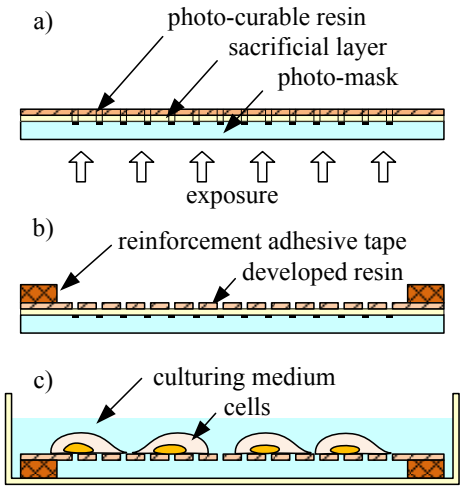

Fig.4 Fabrication of the orifice sheet

shown in Fig.2. Even when third cell may be in contact with the pairing cell (as shown in Fig.3 a), this does not take part in fusion, because no membrane voltage is induced there. In order to perform DEP, low conductivity buffers must be used, but still, the field constriction created by an orifice extends to the distance comparable to the orifice diameter, and are not strong enough to attract cells in the distance, in which case sedimentation can be used for assistance.

For both electroporation and electrofusion, the orifice need not be single, but adaptable to massive parallelism, say in *x-y* array. The orifice sheet can be made by laser ablation of a polymer film, or by a photolithographic process as that depicted in fig.4. Directly on a photo-mask is first coated a water-soluble sacrificial layer, and then a photo-curable resin. Then it is exposed from backside, and the resin is developed. Adhesive tape with mm-sized opening is attached for reinforcement and handling, and the sacrificial layer is dissolved to obtain the orifice sheet. Finally the sheet is surface treated, placed in the culturing medium, on which cells are seeded.

IV. On-Chip Transfection by Electroporation

When the cell membrane on the orifice sheet is permeated by electrical pulses, small molecules (e.g. smaller than protein) can move into the cell by diffusion. To avoid polarization of electrodes, and also the occurrence of breakdowns at locations other than the membrane on the orifice, use of modulated pulse voltage is found to be effective [4]. In fact, by applying 1.5 V, 50kHz, 10 ms burst-wave, intake of fluorescence probe into cytoplasm or nucleus was observed in almost all the cells on the orifice, without perceivable damage to the cell activity, proliferation and division.

The situation is different for plasmid DNA, which is commonly used for gene transfection. The molecule, typically 10kb, is far larger than protein. It must somehow be brought into nucleus for the expression. Our solution to the problem is the use of a dense orifice array together with electrophoretic effect. Fig.5 shows the electron microscope photo of the orifice sheet together with its schematic in comparison to the

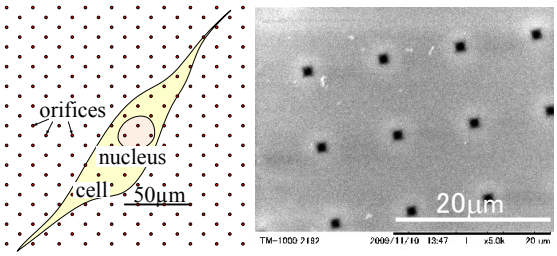

Fig.5 High-density orifice sheet for plasmid transfection

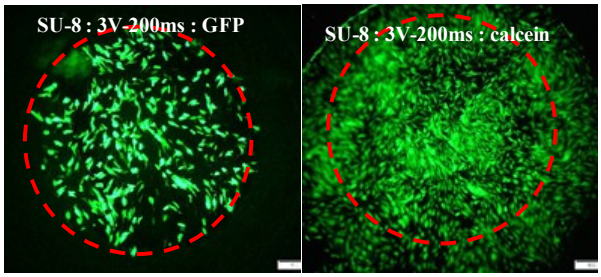

Fig.6 GFP transfection: left: expressed GFP
right: all cells stained by calcein

typical dimension of a cell. Here, the density of the orifice is chosen in such a way that at least one orifice exists below a nucleus of an adhering cell. When relatively long (100-200 ms) square pulse is applied, the reversible breakdown takes place, the electrical fluxes go into the cytoplasm, and penetrates the nucleus through the nucleus membrane pores, so that negatively-charged plasmids can flow along the field-line into the nucleus.

Fig.6 shows an example of GFP (Green Fluorescence Protein) plasmid fed into TIG cell (human diploid cell strain) which is often used for iPS cell studies. The broken-line circle shows the boundary of reinforcement adhesive tape; the electroporation occurs only within the circle, while the cells are seeded anywhere atop (see Fig.4 c) and can proliferate outside. Because the plasmids are electrophoretically transported directly into nucleus, the gene expression starts within a few hours, in contrast to the conventional transfection methods (including electrical) which takes almost one cell cycle for expression. The left photo of Fig.6 is the fluorescence from GFP after 5 hours of 3V-200ms pulsing. The right photo shows all live cells in the same view, stained and visualized by calcein. The comparison of the two shows the gene expression in 20-30% of the cells.

V. On-Chip Electrofusion

Fusion events can be visualized on real-time basis by fluorescence-staining one of the cells. The linear orifice array shown in Fig.7 is fabricated by the self-forming meniscus method[5], different from that of Fig.4. Fig. a) is the superimposed view of the bright field and the fluorescence view before fusion, where there are 5 orifices aligned horizontally. The cytoplasm of the cells on the lower side is stained by calcein, while those on the upper side are not, and hence the former cell looks like an open circle and the latter a solid circle. DEP is used to attract cells to the orifice, to form a pair of an open and a solid circle at every orifice. Then the bright field view is switched off, and a pulse (2.5 V, 200μs) is

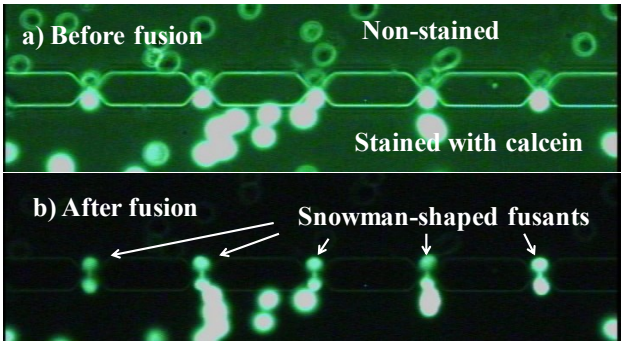

Fig.7 Time-lapse observation of electrofusion

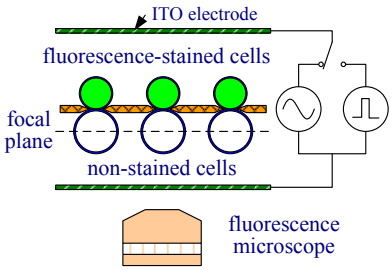

Fig.8 Observation of fusion events in 2-dimensional orifice array

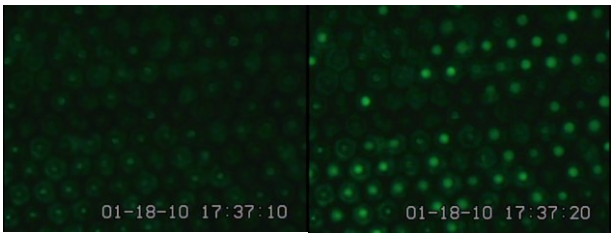

Fig.9 Parallel fusion using 2-D orifice array

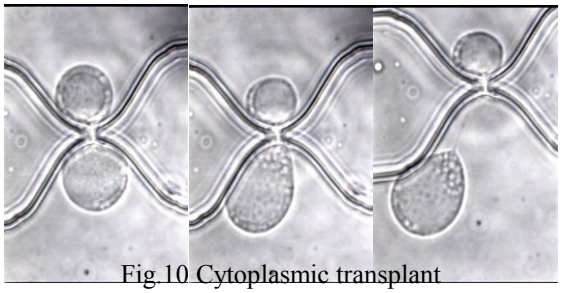

Fig.10 Cytoplasmic transplant

applied. When fusion takes place, the membranes reconnect and cytoplasm mixed, to form snowman-shaped fluorescence, as shown in Fig.b), where the fusion occurred among all cell pairs. Also seen is that, as was shown in fig.3, the third cells do not take part in fusion.

Massive parallelism can be achieved with the use of an two-dimensional orifice array. Fusion can be observed with the same principle as that of Fig.7, except that the observation is made making use of the short focal depth of a high-magnification microscope, as depicted in fig.8. Fig.9 shows the result. The photo on the left is before fusion, with the focal plane on the non-fluorescent cells (lower cells of the cell pair in Fig.8), so that the (upper) fluorescent cells are out of focus and invisible. The photo on the right is after fusion, where the fluorescent-dye influx into the cells clearly visualizes cell contour of the lower cells. The fusion yield here is about 80%.

VI. Cytoplasmic Transplant

Conventional cell fusion in most case is intended for genetic mixing to create a hybrid among two type of cells. However, there are applications where only cytoplasmic factors of foreign cells are to be transferred, and gene mixing should be avoided. Typical examples are in clinical applications, such as the initialization of tissue cells by the fusion with ES cells, or bringing cancer-specific proteins into dendrite cells for immunotherapy. The electrofusion using microfabricated orifice which are small enough compared with nucleus can potentially solve this problem. However, it has been observed that some types of cells are deformable and the fusant can creep through 2μm orifice. In such cases once-fused cell can be separated by an aspiration from one side, as shown in Fig.10. Due to lateral fluidity of the cell membrane, the cytoplasm can be transplanted to one of the cells, leaving the nucleus on the orifice.

VII. Conclusions

Recent progress in regenerative medicine is the driving force for novel methods in cellular and cell-membrane manipulation towards the control of the cellular functions. DEP and the reversible breakdown of the membrane in microfabricated structures can bring about a breakthrough in this field. Conversely, time-lapse observations in single-cell level enabled by the method can bring about new insights into the mechanisms including epigenetic phenomena.

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