Electrostatic bio-manipulation for the modification of cellular functions

Masao Washizu1, 2*
1Department of Bioengineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan
2JST CREST
E-mail: washizu@washizu.t.u-tokyo.ac.jp

Abstract. The use of electrostatic field effects, including field-induced reversible-breakdown of the membrane and dielectrophoresis (DEP), in microfabricated structures are investigated. With the use of field constriction created by a micro-orifice whose diameter is smaller than the cells, controlled magnitude of pulsed voltage can be applied across the cell membrane regardless of the cell size, shape or orientation. As a result, the breakdown occurs reproducibly and with minimal invasiveness. The breakdown is used for two purposes, electroporation by which foreign substances can be fed into cells, and electrofusion which creates genetic and/or cytoplasmic mixture among two cells. When GFP plasmid is fed into MSC cell, the gene expression started within 2 hours, and finally observed in more than 50% of cells. For cell fusion, several ten percent fusion yield is achieved for most cell types, with the colony formation in several percents. Timing-controlled feeding foreign substances or mixing cellular contents, with high-yield and low-invasiveness, is expected to bring about a new technology for both genetic and epigenetic modifications of cellular functions, in such field as regenerative medicine.

1. Introduction
There are emerging needs for artificial control over cellular functions, for instance in reprogramming and/or differentiation control of ES (Embryonic Stem) or iPS (induced Pluripotent Stem) cells. Such processes require technologies for the introduction of foreign genes and substances into cells, followed by the evaluation of the cellular responses. In order to guarantee the purity and the traceability of the product, each step in these processes should be made in single-cell level. In this view, the author and his colleagues are focusing on electrostatic field-effects in micro-fabricated structures to realize such "cell surgery".

The field effects used are a) dielectrophoresis (DEP), an electrokinetic effect to move and position cells, and b) so called "reversible breakdown of the membrane", which uses an electrical impulse to create localized temporary disruption of cytoplasmic membrane. During the breakdown, substances can diffuse into the cytoplasm (electroporation), or the membranes of two cells in contact can reconnect and merge into one (electrofusion). Microfabrication techniques are conveniently utilized here to make structures comparable to a cell to enable the manipulation in single-cell level, as well as to tailor the field pattern around the cell. In particular, with the use of the field constriction created by an orifice

* To whom any correspondence should be addressed.
smaller than a cell, the voltage drop can be concentrated to the cell membrane in contact with the orifice, so that it can be precisely controlled regardless of cell size, shape or orientation. Hence, reproducible and low-invasive breakdown is achieved.

In this paper, the principle of the field constriction is reviewed, and the experimental results of both electroporation and electrofusion are shown.

2. Reversible breakdown of the membrane based on field constriction

The conventional reversible breakdown method uses cell suspension under an uniform pulsed electric field. Modelling a biological cell as a spherical conducting particle with an insulating skin, the induced voltage $V_m$ across the membrane is given by

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

\[ \tau = a C_m (\rho_{in} + \rho_{out} / 2) \quad (2) \]

where $a$ is the cell radius, $\rho_{in}$ and $\rho_{out}$ are the resistivities of the inner and the outer medium, $C_m$ is the membrane capacitance per unit area, $E_0$ is the strength of the uniform applied field, $\theta$ is the azimuthal angle measured from the direction of $E_0$.

The problem here is that $V_m$ is size dependent. For an ensemble of cells with size distribution, large cells receive too much voltage and rupture, while the voltage is too small for small cells to induce effective breakdown. Likewise, for non-spherical cells, the membrane voltage depends on the shape and the orientation, which can make the yield even lower.

To solve the problem, we make use of the constriction field. As schematically illustrated in figure 1, a typical constriction field is produced by an insulating plate with an aperture, which is sandwiched by a pair of electrodes. In the figure is drawn the lines of force, as well as the equi-potential contours. It is seen that the potential drop occurs dominantly near the orifice, in this example case 80% drop occurs between $z = 0$ and 0.26 $d$. Therefore, if a cell, whose diameter is adequately larger than that of the orifice, is placed on the orifice, most potential drop will be impressed on the cell, and the membrane voltage will be virtually independent on the cell size.

Figure 1. A typical constriction field.
An infinitely large insulator plate at $z = 0$, having an orifice with the radius $a_o$, is placed between infinitely large parallel plate electrodes at $z = d$ and $z = -d$, to which potential $\pm$ is applied. The equi-potential lines, as well as the lines of force are drawn for the case of $d = 10 a_o$.

Figure 2 depicts such a field constriction geometry applied for electroporation and electrofusion. An insulator sheet with orifices, may it be single, in a linear array, or $x$-$y$ 2-dimensional array, is used. In the case of electroporation, adhering cells may be cultured on the sheet, while non-adherent cells can be placed onto the orifices by aspiration or DEP. If the cells perfectly seals the orifice, all voltage applied to the electrodes $V_{app}$ is impressed on the membrane extended by the orifice, hence $V_m = V_{app}$. Even if the sealing is imperfect, the field far from the orifice is weak, most voltage drop will take place in the cell, and $V_m \approx V_{app}$. Thus $V_m$ can be controlled regardless of the cell size, shape or
orientation, and reproducible reversibility in the membrane breakdown is guaranteed. Hence the foreign substances in the surrounding medium, say plasmids, can be brought into the cells with a high-yield.

For the case of electrofusion, the cells are positioned onto the orifices by DEP to form cell pairs, as delineated later. Field constriction in this case again plays a role of concentrating the applied voltage to the membrane on the orifice.

![Figure 2](image1.png)

**Figure 2.** Electroporation (a) and electrofusion (b) based on field constriction at micro orifices.

Figure 3 shows a typical result obtained by the numerical analysis of $V_m$ distribution on the cell pair sandwiching the orifice, assuming an axial symmetry, as well as the mirror symmetry. The method itself is delineated in ref. [2]. The calculated result is shown in b) in normalized form, i.e. as the membrane voltage $V_m$ divided by the applied voltage $V_{app}$. It is seen that $V_m / V_{app}$ is about 0.9 in the orifice ($\theta < 20^\circ$ in this case), indicating 90% of $V_{app}$ goes onto the membrane making contact with the other cell, while $V_m$ outside the orifice (say $\theta > 50^\circ$ in this case) is virtually zero. Hence a controlled voltage can be applied to the membrane around the contact point of two cells, and high-yield fusion can be expected. The fusion will be 1:1; even a third cell may be on the side as depicted in figure 2b) right bottom, this cell will not take place in fusion, because there is no $V_m$ here.

![Figure 3](image2.png)

**Figure 3.** Calculated membrane voltage induced on a pair of cell at an orifice.

### 3. On-chip electroporation based on field constriction
Molecules smaller than protein, for example fluorescent dye, glutamate or GFP [3], have diffusion coefficients high enough that they can reproducibly be fed into cells by electroporation with a short ($\approx$ ms) pulse. But in real biological applications, for example in generating iPS cells or its differentiation control, often required is the introduction of plasmid DNA of several ten kilo-base, which is far larger than proteins. In such cases, we make use of the electrophoretic effect induced by a pulse of several hundred ms in length.
To visualize how plasmids can be fed into a cell, we made a real-time observation of the process, fluorescence-labelling plasmids with Quantum dots (Q-dots) [4]. Figure 4a) schematically shows the experimental setup. The device consists of a PDMS (silicone rubber) microfluidic chip having a vertical wall with an orifice at the center, and a pair of electrodes to apply voltage across the orifice. A cell is positioned on one side of the orifice by aspiration, Q-dot labeled plasmids fed on the other side, and an electrical pulse, with the cell side positive, is applied. The plasmids migrate along the field line due to electrophoresis, and move into the cell through the pores formed by electroporation. The nucleus itself has inherent pores which is used for material transport into and out from, so some of the flux penetrates into nucleus, by which the plasmids can be transported directly into nucleus. Figure 4b) is the cell on the right of the orifice, where the nucleus is clearly visible. The orifice itself, and the part further left, is not visible due to halation of many Q-dots. When the pulse is applied, the influx of Q-dots into the cell is observed, some of them into the cytoplasm, and some into the nucleus. Figure 4c) is after the pulse, where two Q-dot labelled plasmids, indicated by arrows, are left in the nucleus.

Figure 4. Fluorescence observation of electrophoretic plasmid transport into nucleus. a) experimental setup; b) before pulse; c) plasmids fed into nucleus (fluorescence view).

The result suggests that plasmids can be directly transported into nucleus, if it is penetrated by the line of force. Therefore, we designed a high-density orifice array sheet, where the orifice pitch is made comparable to the nucleus diameter (when the cell is adhering on the surface and the nucleus is stretched).

Figure 5. Electroporation of GFP plasmid into MSC (Mesenchymal Stem Cell) a) the orifice array dense enough to have more than one orifice beneath nucleus (schematic); b) all alive cells on the orifice sheet stained by calcein; c) fluorescence from expressed GFP (after 5 hours).

Figure 5 shows the result of plasmid transfection. MSC (Mesenchymal Stem Cell), which is adherent, is used as the cell, to which GFP (Green Fluorescent Protein) plasmid is fed. MSC has a typical dimension as drawn in figure a), where the triangular lattice of 15µm satisfies the condition of “at least one orifice beneath nucleus”. Figure 5b) is a fluorescence photo of alive cells as stained by calcein, and c) is the fluorescence from GFP taken after 5 hours of pulsing. The percentage of cells in figure c) divided by that of figure b) gives the transfection yield, which is roughly 50% in this case. In conventional electroporation, it takes about a cell cycle (10-20 hours) for the plasmid to go into
nucleus before it is expressed. In contrast, in the case of this on-chip transfection, the plasmid is transported by electrophoresis into nuclei, and hence the expression starts as quick as 2 hours after pulsing.

4. On-chip electrofusion based on field constriction
Two types of on-chip electrofusion chips are developed, one being one dimensional orifice array to view the fusion process from a side (figure 2b) [5-7], and the other being two-dimensional orifice array for massively parallel fusion [8].

Figure 6. Electrofusion of Jurkat cells.
a) optical view; the orifice is at the center . b) fluorescence view before the pulse; cytoplasm of the cells below the orifice are fluorescence stained, while those above the orifice are not. c) fluorescence view after the pulse; the cytoplasm are mixed and the fusant takes a "snow-man" shape. Orifice diameter~10 µm.

Figure 6 shows the instance of fusion and how cytoplasm is mixed, where Jurkat is used as the cell. The cells whose cytoplasm is stained with calcein are fed into the channel below the orifice, while unstained cells into the channel above. The cells are attracted and positioned onto the orifice by DEP. As optically photographed in a), two cells are making contact at the orifice, and b) shows the same instance viewed by fluorescence, which shows the cells below the orifice are emitting fluorescence, while those above are not. Figure 6c) is just after the pulse, and the fluorescence dye in the lower cell has already been diffused into the upper cell to look like a "snow man". This cytoplasm mixing, at least as observed by the dye, takes only a few seconds. Also to note is that, while there are cells other than making a pair at the orifice, none of them takes part in fusion. This is as it should be, because the membrane voltage builds up only within the orifice. This guarantees that the fusion is exclusively 1:1, in contrast to unpredictable number of cells involved in fusion in conventional methods. The minimum extra gene brought in by 1:1 fusion is desirable in the survival of the fusant.

Time lapse observation of the fusant is made, a typical result is shown in figure 7. By the 1:1 fusion among diploid cells (L929, murine fibroblast), a tetraploid is formed, which in some case divided into two (figure 7a), three (b) or four (c). Such multipolar divisions [9] have been reported in relation to oncogenesis. The fusion method developed here, enabling high-yield timing-controlled 1:1 fusion and its time-lapse observation, is expected to be a novel tool for such biological or biomedical investigations.

Massively-parallel 1:1 fusion can be achieved with the use of a two-dimensional orifice array. A 5×5 mm sheet with 50µm-pitch orifices, having 10^4 orifices altogether, is used in the experiment. Size-independence of $V_{m}$, a property of the field-constriction design, is effectively utilized in the fusion among different-sized cells.
Figure 7. Real-time observation of multipolar division of the 1:1 fusant.

Figure 8. Observation of parallel fusion using x-y orifice array. Influx of fluorescence dye into lower cell is monitored with a microscope viewing the fluorescence from lower cells.

Figure 8 shows the experimental setup. A cell whose cytoplasm is stained with the fluorescence dye calcein is fed above the orifice sheet, while those unstained are fed below the sheet. They are positioned onto the orifices to form pairs by dielectrophoresis with the aid of gravity (flipping the chip over). The observation is made from the bottom by an inverted microscope, with the focal plane set at the lower cell. At this moment, as the fluorescence microscope is focusing on non-fluorescence cells, only out-of-focus view of the cells through the orifice sheet is observed. When the pulse is applied, the cytoplasm mixing, same as that viewed from side in figure 6, takes place and the lower cells become fluorescent, which can now be observed brighter and with a clear contour.

Figure 9 is an example of the experimental result, the fusion among K562 (human leukemia, ~24 µm) and HL60 (human leukemia, ~14 µm). In figure a), the fluorescent cells are out of focus and are hardly visible. When the pulse is applied, each cell pairs fuses, the dye transferred into the non-fluorescent cells on the lower side, and become clearly observable as in figure b). It is found that having a neat alignment is more difficult than the fusion itself. Even so, the fusion yield of ~70% is obtained.

One of the largest application of conventional cell fusion is the creation of antibodies by fusing B cell and myeloma cell. After the fusion operation, the cells are cultured in the selective medium, in which non-fused cells cannot survive, and only fusants can proliferate and form colonies. Whether or not, or how efficiently the fusants obtained by the on-chip electrofusion can form colonies is investigated. The fusion is performed with the same procedure as that in figure 9 using B cell (~7 µm⁴) and myeloma cell (~15 µm⁴), and after fusion, the device is disassembled, the orifice sheet taken out, and placed onto a layer of feeder cells in a dish for culturing.
Figure 9. Electroporation of GFP plasmid into MSC (Mesenchymal Stem Cell). Orifice pitch = 50 µm.

Figure 10. Electroporation of B cell and myeloma cell. Scale bar = 150 µm.

The result is shown in figure 10. The left and the right photo are of the different place in the same culture dish. In the left photo is seen the feeder cells almost uniformly covering the dish surface, and the array of orifices. Encircled are two colonies of cells, which appear no different from that obtained by the conventional fusion method. As the orifice sheet is smaller than the culture dish, and the colonies can float and move during culturing, colonies out of the orifice sheets are observed as well, as encircled in the right photo. About 100 colonies are obtained, using the sheet having 2000 orifices. This is very high yield compared with that of conventional methods, partly due to the yield of fusion event itself is high as seen in figure 9, and partly because the fusion is exclusively 1:1 and the fusant is tetraploid, which can better survive than hyperploids.

5. Conclusions
It has been shown that field tailoring using microfabricated structure can be a novel tool for the investigation, modification and utilization of cellular functions. By precisely controlling the membrane voltage, high reproducibility in the reversible breakdown is guaranteed. Foreign molecules including genetic materials, or the cytoplasmic components of other cells, can be brought into the target cell, and because all operations can be made in a microfluidic chip under a microscope, the cellular response can be evaluated in single-cell level. The technology developed here will find various applications in biological or biomedical field, in particular in regenerative medicine, where the creation, function control, evaluation, and quality control have an essential meaning.
Acknowledgments

The results shown in this paper were done under collaborations and discussions with the following people, Dr. Murat Gel, Dr. Hidehiro Oana, Dr. Osamu Kurosawa, Dr. Yuji Kimura, Prof. Takao Hamakubo, Dr. Hiroko Iwanari, Mr. Yasuhiro Nishigaichi, Mr. Shohei Suzuki of The University of Tokyo, Prof. Hidetoshi Kotera, Dr. Takashi Tada of Kyoto University, Dr. Boonchai Techaumnat of Chulalongkorn University, Thailand. The work was partly supported by JST CREST, NEDO "Dynamic Biology" and "Cell Array" projects, Ministry of Education Kakenhi, ASTEM "Nanomedicine" project and CNBI, U-Tokyo.

References

[1] Zimmermann U, Arnold W M 1988 J. Electrostat. 21, 309
[2] Techaumnat B and Washizu M 2007 J. Phys. D: Appl. Phys. 40 1831
[3] Kurosawa O, Oana H, Matsuoka S, Noma A, Kotera H, and Washizu M 2006 Meas. Sci. Technol. 17 3127
[4] Kurosawa O, Sumita Y, Gel M, Oana H, Kotera H, Kato T, Toguchida J and Washizu M 2010 µ-TAS M49A (Groningen, Netherlands) p 217
[5] Techaumnat B, Tsuda K, Kurosawa O, Gel M, Oana H and Washizu M 2008 IET Nanobiotechnol. 2 93
[6] Gel M, Suzuki S, Kimura Y, Kurosawa O, Techaumnat B, Oana H, and Washizu M, 2009 IEEE Trans. Nanobiosci. 8 300
[7] Gel M, Kimura Y, Kurosawa O, Oana H, Kotera H and Washizu M 2010 Biomicrofluidics 4 022808
[8] Kimura Y, Gel M, Techaumnat B, Oana H, Kotera H, Washizu M 2011 Electrophoresis 32 2496
[9] Vitale, Senovilla L, Jemaa M, Michaud M, Galluzzi L and Kepp O et al. 2010 The EMBO Journal 29 1272