



# Fundamental study on a gene transfection methodology for mammalian cells using water-in-oil droplet deformation in a DC electric field



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## ABSTRACT

We have developed a gene transfection method called water-in-oil droplet electroporation (EP) that uses a dielectric oil and a liquid droplet containing live cells and exogenous DNA. When a cell suspension droplet is placed between a pair of electrodes, an intense DC electric field can induce droplet deformation, resulting in an instantaneous short circuit caused by the droplet elongating and contacting the two electrodes simultaneously. Small transient pores are generated in the cell membrane during the short, allowing the introduction of exogenous DNA into the cells. The droplet EP was characterized by varying the following experimental parameters: applied voltage, number of short circuits, type of medium (electric conductivity), concentration of exogenous DNA, and size of the droplet. In addition, the formation of transient pores in the cell membrane during droplet EP and the transfection efficiency were evaluated.

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## 1. Introduction

Electroporation (EP) is the most widely used physical method for delivering cell-impermeable molecules into cells due to its versatility and simplicity. EP generates transient pores in the cell membrane when the transmembrane potential exceeds a critical threshold value, altering the permeability of the membrane by application of an external electric field [1,2]. Many theoretical [3–7] and experimental [6–11] approaches have been investigated in an attempt to explain the mechanisms underlying EP, especially those involved in gene transfer. Advanced EP devices have been developed by combining microfabrication techniques and microfluidics [12–19]. EP offers several important advantages compared to viral, chemical, and liposome-based transfection methods, including low cost, reduced safety concerns, simple operation, and no restriction on the cell type or properties of the exogenous material. However, most commercial EP-based transfection methods require the use of specialized, expensive, pulse generators to produce short electrical pulses at high voltage.

We previously reported a cell electroporation and gene electrotransfer in small volumes using small amounts of cells by water-in-oil droplet actuation in a direct current (DC) electric field (Fig. 1). When an aqueous droplet is suspended in a dielectric liquid such as oil, it can be moved between a pair of electrodes upon application of a DC electric field (droplet bouncing) [20,21]. Our previous study showed that *Escherichia coli* cells were transformed by the bouncing motion of a droplet containing the bacterial cells and plasmid DNA [22]. In addition, we found that a more intense electric field can induce droplet elongation, leading to instantaneous short circuiting caused by the droplet contacting the two electrodes simultaneously. The electric pulses delivered to the cells suspended in the droplet are those linked to these “shorts”. Small pores could be made in the cell membrane during the shorts, allowing the gene electrotransfer. Our further investigation demonstrated efficient transfection of mammalian cells by the droplet actuation in a DC electric field [23]; this methodology is termed “droplet EP” in this paper. However, various parameters affecting the efficiency of droplet EP, such as applied voltage, concentration and exogenous DNA concentration, have not been elucidated for a given electrode geometry. Investigating the effects of these parameters on cell viability and gene expression could

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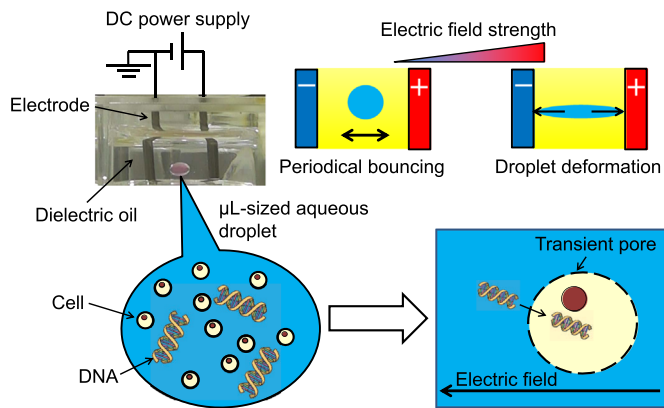


Fig. 1. Schematic illustration of water-in-oil droplet EP.

contribute to not only efficient transfection, but also to exploring the mechanism underlying this novel gene transfer method.

The aim of this study is to characterize the factors affecting the efficiency of droplet EP by systematically varying the applied voltage, number of short circuits, type of EP medium (electric conductivity), concentration of exogenous DNA, and size of the droplet. A luciferase-expressing plasmid DNA was used as the exogenous DNA and high-throughput assays were conducted in 96-well plates. The results of this investigation provided conditions for efficient droplet EP. Furthermore, we also investigated the formation of transient pores on the cell membrane using a cell-impermeable nucleic acid staining dye (YO-PRO-1 uptake assay). Finally, transfection efficiency was evaluated by expression of fluorescent protein (Venus, improved yellow fluorescent protein) and by flow cytometry under the optimized conditions identified from the luciferase-expressing experiments.

## 2. Materials and methods

### 2.1. Cell culture

Human embryonic kidney (HEK) 293 cells (JCRB Cell Bank) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine (Wako Pure Chemicals), 10% fetal bovine serum (FBS, One Shot™ fetal bovine serum, Thermo Fisher Scientific), and penicillin/streptomycin (PS) (Wako Pure Chemicals) at 37 °C, 5% CO<sub>2</sub>. Cells at 50–70% confluence in T25 flasks were treated with 0.25% trypsin-EDTA (Wako Pure Chemicals), harvested by centrifugation, and suspended in DMEM/10% FBS/PS. The cells were then washed with droplet EP medium before final re-suspension in the same medium and the cell concentration was adjusted. Four types of droplet EP medium were used: (1) DMEM with or without 10% FBS, (2) Dulbecco's phosphate-buffered saline without magnesium chloride and calcium chloride (D-PBS (-), Wako Pure Chemicals), (3) a low ionic strength 0.28 M mannitol, and (4) a mixture of D-PBS (-) and 0.28 M mannitol (at a 1:1 ratio).

### 2.2. Plasmid DNA preparation

Gene expression after droplet EP was confirmed using two types of plasmid DNA. For the luminometric assay of luciferase expression, pGL4.51 [luc2/CMV/Neo] vector (Promega), a 6358 bp plasmid DNA encoding firefly luciferase, was amplified in *Escherichia coli* DH5α and purified using a QIAGEN Plasmid Maxi kit (Qiagen) according to the manufacturer's protocol. For the fluorescent protein expression and flow cytometry assays, the Venus yellow fluorescent protein vector provided by Prof. A. Miyawaki

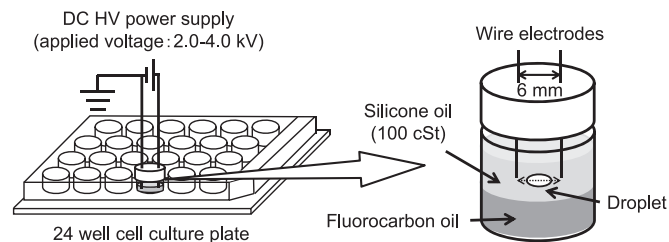


Fig. 2. Experimental setup for droplet EP investigated in this study.

(Brain Science Institute, RIKEN) was used [23,24]. The amplification and purification procedures used in these assays were the same as for the luciferase-expressing vector. Following purification, endotoxin was removed using an endotoxin removal kit (MiraCLEAN®, Mirus). The purified plasmid DNAs were dissolved in sterile water and the concentration of each DNA solution was determined using a UV-vis spectrometer (GeneQuant1300, GE Healthcare).

### 2.3. Water-in-oil droplet electroporation

Fig. 2 shows the experimental setup for droplet EP used in this study. A 24-well cell culture plate (TPP) was used as an oil reservoir. One well of the plate was first filled with 1.5 mL of fluorocarbon oil (Fluorinert™ FC-96, 3M), then 1 mL of silicone oil (KF96-1, 100 cSt kinematic viscosity, 2.74 dielectric constant, 965 kg/m<sup>3</sup> density; Shin-Etsu Chemical Co.) without surfactant was added. A pair of pin electrodes (one ground, one high-voltage (HV)), kindly manufactured by Nepa Gene Co., Ltd., was set in the well. The gap between the electrodes was 6 mm. The appropriately prepared cell suspension (2.0–4.0 μl) was dispensed in the silicone oil and a high voltage was then supplied using a DC HV power supply (HAR-30R10; Matsusada Precision Inc.). During DC HV application, the intense electric field can induce droplet deformation, leading to instantaneous short circuiting caused by the droplet contacting the two electrodes simultaneously. Short circuiting produces a distinctive sound and the number of shorts was counted. Typically, only one short was allowed to occur in one experiment and the DC HV power supply was turned off manually following the designated number of shorts. Following droplet EP, the droplet was recovered and transferred to an appropriate medium for further experiments.

### 2.4. Cell viability and luciferase expression measurement

Cell viability and gene expression measurements were conducted in a single well of a 96-well plate using a ONE-Glo™ + Tox luciferase reporter and cell viability assay kit (Promega). The cell viability assay and luciferase expression assay are based on a fluorometric measurement and a luminometric measurement, respectively. The effects of applied voltage, number of short circuits, EP medium, concentration of DNA, and the volume of the droplet on cell viability and luciferase expression were investigated. An appropriate amount of the luciferase-expressing plasmid DNA was added to the cell suspension prepared as described above, then the suspension was dispensed into the oil and droplet EP was performed. The droplet was then transferred to 100 μl of DMEM/10% FBS/PS in a 96-well opaque microplate (Nunc LumiNunc 96-well plate, Thermo Fisher Scientific). Following 24 h incubation at 37 °C, 5% CO<sub>2</sub>, cell viability and luciferase expression were measured using a multimode microplate reader (Varioskan Flash, Thermo Fisher Scientific) according to the manufacturer's protocol.

### 2.5. YO-PRO-1 uptake assay with fluorometric detection and microscopic observation

Transient membrane pore formation during droplet EP was confirmed using the YO-PRO-1 uptake assay, conducted as previously described with some modifications [23,25]. YO-PRO-1 was purchased from Life Technologies. A 3.0  $\mu\text{l}$  droplet containing  $1.0 \times 10^4$  HEK293 cells suspended in DMEM with 10% FBS and 1  $\mu\text{M}$  YO-PRO-1 was added to the silicone oil and droplet EP was performed. The droplet was transferred to 100  $\mu\text{l}$  of DMEM with 1  $\mu\text{M}$  YO-PRO-1 in a 96-well opaque microplate (Immuno Standard Modules Black, Thermo Fisher Scientific). After 1 h incubation at 37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , the fluorescence of the cells was measured by the multimode microplate reader using 485/528 nm (excitation/emission) wavelengths, in accordance with the fluorescence properties of YO-PRO-1. Cell proliferation and YO-PRO-1 uptake after droplet EP were confirmed by transferring the droplet to DMEM/10% FBS/PS and 1  $\mu\text{M}$  YO-PRO-1 in an 8-well cover glass chamber (Nunc™ Lab-Tek™ II cover glass chamber, Thermo Fisher Scientific) and incubating for 24 h at 37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . The cells were subsequently microscopically observed using an inverted fluorescence microscope (TE-2000U; Nikon) equipped with a 10 $\times$  objective lens (Plan Fluor; Nikon) and a digital camera (D5000; Nikon).

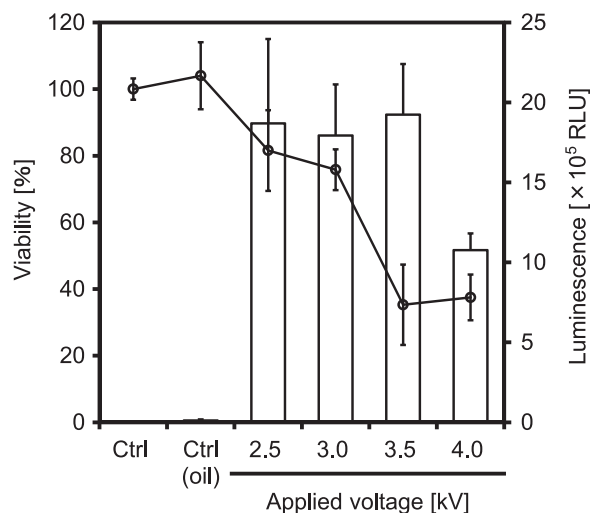
### 2.6. Expression of fluorescent protein and flow cytometry

A 2.0  $\mu\text{l}$  aliquot of DMEM without serum containing  $1.0 \times 10^4$  HEK293 cells and 0.75  $\mu\text{g}$  of Venus-expressing plasmid DNA was added to the silicone oil. The number of shorts was set to one. At least ten droplets were subjected to droplet EP to obtain each experimental data point, and each droplet was transferred to a separate well of a 24-well cell culture plate containing 400  $\mu\text{l}$  of DMEM/10% FBS/PS, then incubated for 48 h at 37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . Microscopic observation was subsequently conducted to confirm Venus expression, as described above. The cell culture was then trypsinized, harvested by centrifugation, and suspended in buffer containing D-PBS (-) and 7-amino-actinomycin D (7-AAD, Beckman Coulter, Inc.) to stain the dead cells. Venus expression and cell viability were measured by flow cytometry with a CytoFLEX flow cytometer (Beckman Coulter, Inc.). At least 10,000 cells were analyzed for each experimental point. Transfection efficiency was calculated by dividing the number of Venus-expressing cells (Venus positive) by the number of live cells (7-AAD negative). Viability was calculated by dividing the number of 7-AAD positive cells by the number of all counted cells.

## 3. Results

### 3.1. Cell viability and luciferase expression

Fig. 3 shows cell viability and luciferase activity 24 h after droplet EP. The results obtained using two negative controls are also shown, where “Ctrl” means that an aliquot of the cell suspension was directly added to the 96-well plate, and “Ctrl (oil)” means that another aliquot of the same suspension was dispersed in silicone oil, kept for 3 min without applying DC HV, then transferred into the 96-well plate. The conditions used were summarized in Table 1. In conventional electroporation, if the applied voltage is too high, the pores formed in the cell membranes will be too large resulting in cell death. If the voltage is too low, the pore sizes will be inadequate for DNA to cross the cell membranes, resulting in unsuccessful gene expression. Thus, electroporation produces a trade-off between viability and transfection efficiency. The results shown in Fig. 3 indicate that cell



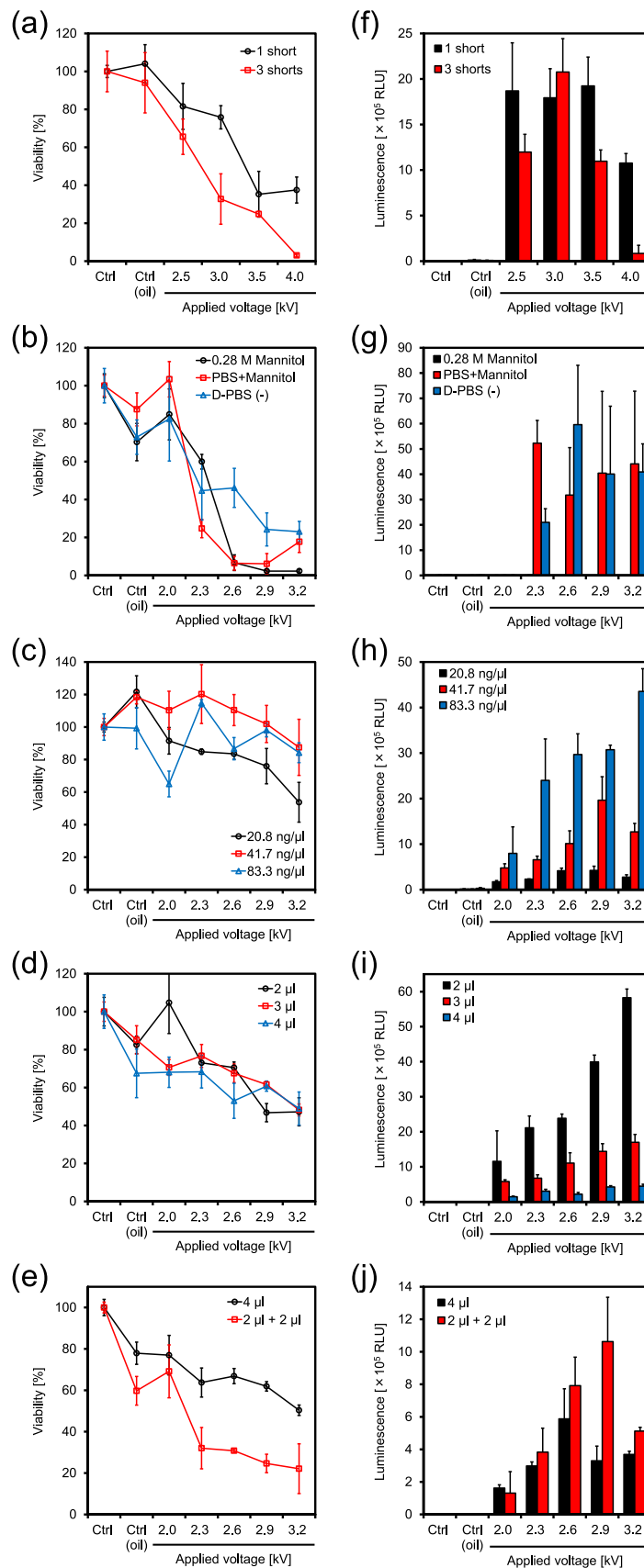
**Fig. 3.** Cell viability (open circle with line) and luciferase activity (bar) 24 h after droplet EP. Data are expressed as the mean  $\pm$  standard deviation (SD) ( $n=3-4$ ). The conditions were: droplet EP medium: DMEM w/o serum, volume of the droplet: 3  $\mu\text{l}$ , amount of plasmid DNA: 250 ng, cell number: 10,000, number of shorts: 1 short.

**Table 1**

Experimental parameters for cell viability and luciferase expression.

Figure #	# of shorts	EP buffer	Amount of DNA [ng] (DNA conc. [ng/ $\mu\text{l}$ ])	Cell # [ $\times 10^4$ ] (cell conc. [ $\times 10^4$ / $\mu\text{l}$ ])	Droplet vol. [ $\mu\text{l}$ ]
3	1	DMEM w/o FBS	250 (83.3)	1.0 (0.33)	3.0
4					
(a), (f)	1, 2, 3	DMEM w/o FBS	250 (83.3)	1.0 (0.33)	3.0
(b), (g)	1	D-PBS(-) 0.28 M mannitol D-PBS+mannitol (1:1)	250 (83.3)	1.0 (0.33)	3.0
(c), (h)	1	DMEM w/o FBS	62.5 (20.8) 125 (41.7) 250 (83.3)	1.0 (0.33)	3.0
(d), (i)	1	DMEM w/o FBS	250 (125) 250 (83.3) 250 (62.5)	1.0 (0.5) 1.0 (0.33) 1.0 (0.25)	2.0 3.0 4.0
(e), (j)	1	DMEM w/o FBS	500, 1000 (250)	0.5, 1.0 (0.25)	2.0, 4.0

viability gradually decreased with increasing applied voltage. On the other hand, luciferase activities markedly increased compared with negative control (without DC HV application). However, significant differences in luciferase activities were not observed in the range of an applied voltage of 2.5–3.5 kV. This could be attributed to well-based fluorometric (viability) and luminometric (luciferase expression) assays. The One-Glo™+Tox luciferase reporter and cell viability kit enables simultaneous measurement of the relative number of viable cells and luciferase activity in a cell culture population. Although this approach is very useful to determine how experimental parameters influence transfection, data obtained from a microplate reader are the sum of the amount of luminescence in the well containing both live and dead cells. The viability at 3.5 kV clearly decreased compared to 3.0 kV of applied voltage, but a significant difference in luciferase activities was not



**Fig. 4.** Cell viability (a)–(e) and luciferase activity (f)–(j) 24 h after droplet EP using various experimental conditions. Data are expressed as the mean  $\pm$  standard deviation (SD) ( $n=3-4$ ). The experimental variables for each result were: (a) and (f): number of shorts, (b) and (g): droplet EP medium, (c) and (h): concentration of DNA, (d) and (i): size of droplet, keeping the number of cells and amount of DNA in each droplet constant, and (e) and (j) size of droplet, keeping the concentrations of cells and DNA in each droplet constant.

observed. Therefore, higher transfection efficiency at 3.5 kV could be obtained compared with 3.0 kV. The results shown in Fig. 3 demonstrate that the applied voltage significantly affects the outcome of droplet EP and therefore applied voltage was used as an experimental variable in the following experiments.

Fig. 4 shows the effect of applied voltage, number of shorts, droplet EP medium, concentration of plasmid DNA, and volume of the droplet on cell viability and luciferase expression 24 h after droplet EP. Detailed experimental parameters are summarized in Table 1. Experimental data in a pair of viability and luciferase activity were obtained by using a cell suspension prepared with a single cell culture. However, each pair of the data was obtained on different days. Therefore, the cell health under different cell conditions, such as the number of passages, confluency before droplet EP, was not completely the same in different experimental parameters. It could be attributed that some of the same experimental conditions showed different results in Fig. 4. In addition, our previous results based on trypan blue assay demonstrated that the use of silicone oil had little effect on the viability of HEK293 cells in water-in-oil droplets [23]. However, the viability of “Ctrl (oil)” in Fig. 4 varied from 120% to 60%. One possible reason for this could be the sequence of the experiment. In this experiment, the cell suspension without any treatment was initially transferred in a 96-well plate, then droplet EP was performed in order. Finally, the same suspension was dispersed in silicone oil, kept for 3 min without applying DC HV, then transferred into the 96-well plate. Therefore, cells in “Ctrl (oil)” were kept in suspension for a long time, which could have contributed to the cell health in this experiment. The other possible reason is transferring the droplet from the oil to a 96-well plate. This is one of the drawbacks of this transfection method. It is difficult to completely recover the treated cells. Thus this process could affect the reproducibility of the measurement of viability and luciferase activity.

Fig. 4(a) and (f) show the effect of the number of shorts. The distinctive sound caused by shorts meant each short could be counted. The applied voltage was varied from 2.5 to 4.0 kV. Fig. 4(a) indicates that cell viability was affected by increasing both the applied voltage and the number of shorts, with both stronger electric fields and an increased number of shorts inducing more cellular damage. Fig. 4(f) shows an increase in luciferase activity with droplet EP, consistent with the results shown in Fig. 3, whereas the number of shorts did not apparently affect gene expression. As stated previously, decreased luciferase activity is likely attributable to lower cell viability.

Fig. 4(b) and (g) show the effect of the EP medium on viability and luciferase activity using applied voltages in the range 2.0–3.2 kV. The three media tested were: (1)  $\text{D-PBS (-)}$ , (2) a low ionic strength 0.28 M mannitol, and (3) a mixture of  $\text{D-PBS (-)}$  and 0.28 M mannitol (at a ratio of 1:1). The electrical conductivities of the media measured using a conductivity meter (Thermo Scientific) were 15.8 mS/cm, 8.9  $\mu\text{S/cm}$ , and 8.7 mS/cm, respectively. In the other experiments, DMEM without serum was used as the EP medium; the conductivity of this medium is almost the same as that of  $\text{D-PBS (-)}$ , and cell viability and luciferase expression after droplet EP in DMEM without serum showed a similar trend to that observed using  $\text{D-PBS (-)}$  (data not shown).

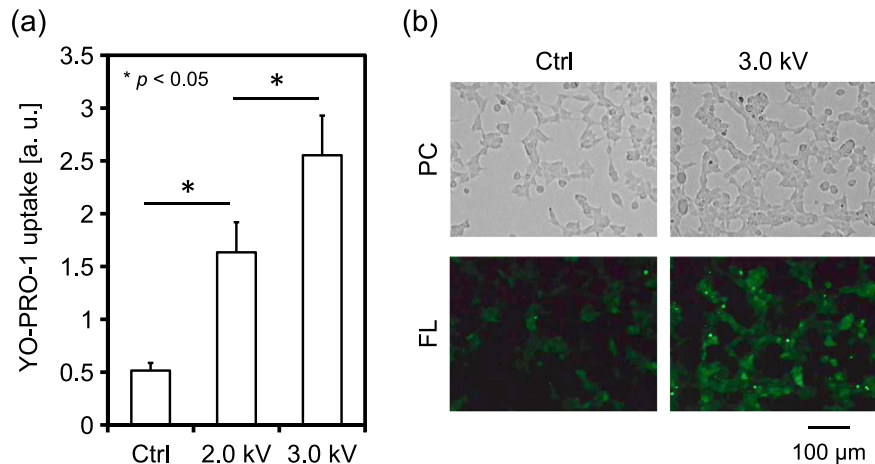
The threshold voltage required for droplet deformation was dependent on the electrical conductivity of the droplet EP medium. No droplet deformation was observed at an applied voltage of 2.0 kV using  $\text{D-PBS (-)}$  and the mixture of  $\text{D-PBS (-)}$  and 0.28 M mannitol. Therefore, droplet bouncing, described in the introduction, was conducted for 3 min at 2.0 kV in both media. Furthermore, no droplet deformation was observed at applied voltages of 2.0 and 2.3 kV using the 0.28 M mannitol and so droplet bouncing was conducted for 3 min at 2.0 and 2.3 kV in this media. Fig. 4(b) shows that cell viability in low electrical conductivity media

decreased drastically with increasing applied voltage compared to when the cells were in high electrical conductivity media, consistent with the threshold voltage required for droplet deformation. Although cell viability in mannitol and  $\text{PBS+mannitol}$  showed similar trend, luciferase activity was not observed in the case of mannitol as shown in Fig. 4(g). This suggests that electric current in the electric pulsation could be required for sufficient gene electrotransfer.

Fig. 4(c) and (h) show the effect of the concentration of exogenous DNA on cell viability and luciferase activity in DMEM w/o serum and keeping all the other parameters the same as described above. Fig. 4(c) shows that the effect of the concentration of DNA on cell viability was smaller than the effect of the number of shorts, whereas luciferase activity was strongly affected by the concentration of exogenous DNA for each applied voltage (Fig. 4(h)), with an increasing concentration of DNA resulting in enhanced gene expression, as generally reported. Viability plotted in Fig. 4(c) expect at 2.0 kV, 83.3 ng/ $\mu\text{l}$  was relatively high compared with the other graphs seen in Fig. 4. In addition, the viability at 2.0 kV, 83.3 ng/ $\mu\text{l}$  was particularly low compared with the other applied voltage. Therefore this low viability could be attributed to some experimental error including transferring the droplet from the oil to a 96 well plate, as mentioned above. Fig. 4(d) and (i) show the effect of size of the droplet as it was varied between 2.0, 3.0, and 4.0  $\mu\text{l}$ . Therefore, a smaller droplet contained both cells and DNA at higher concentration. Fig. 4(d) shows that the viability 24 h after droplet EP was not affected by the volume of the droplets but was affected by the number of shorts, consistent with the effect of the concentration of DNA (Fig. 4(c)). In contrast, Fig. 4(i) shows a marked decrease in luciferase expression with an increase in the volume of the droplets, suggesting that a smaller droplet with a higher concentration of cells and DNA results in more efficient transfection. However, the volume of the droplet should be correlated with the deformation of the droplet. In particular, smaller droplet should be more flattened at the moment of the short, a higher electric field could be created. Therefore, cells inside the droplet would be exposed to different electrical field intensities depending on the volume of the droplet. To examine the effect of the volume of the droplet on viability and luciferase expression while keeping the cell and DNA concentrations the same, a  $2.5 \times 10^3$  cells/ $\mu\text{l}$  cell suspension containing 250 ng/ $\mu\text{l}$  plasmid DNA was prepared in DMEM without FBS. In one experiment, 4.0  $\mu\text{l}$  droplets were subjected to droplet EP, then the droplet was transferred to one well of a 96-well plate. In a second experiment, two 2.0  $\mu\text{l}$  droplets were separately subjected to droplet EP, then both droplets were transferred to the same well of a 96-well plate. This experimental procedure allowed evaluation of the effect of the size of the droplet on droplet EP when maintaining the same cell and DNA concentration and the results are shown in Fig. 4(e) and (j). Fig. 4(e) indicates that the cell viability decreased as the applied voltage increased and that viability decreased as the size of the droplet decreased. This suggests that both stronger electric fields and much smaller droplet size cause more cellular damage. However, luciferase activity was notably increased as the volume of the droplet decreased for each applied voltage (Fig. 4(j)) and again this can be attributed to the electric field strength being stronger in smaller sized droplets.

### 3.2. YO-PRO-1 uptake assay

The results of the YO-PRO-1 uptake assays are shown in Fig. 5. A cell-impermeable nucleic acid staining dye YO-PRO-1 was used to confirm the formation of transient pores on the cell membrane. Fig. 5(a) shows the result of fluorometric measurements 1 h after droplet EP and shows a significant increase in fluorescence intensity compared to in the absence of DC HV application.



**Fig. 5.** YO-PRO-1 uptake assay. (a) Fluorescence intensity 1 h after droplet EP. Data are expressed as the mean  $\pm$  standard deviation (SD) ( $n=4$ ). Statistical analysis was performed using Student's *t*-test. Statistical significance was recognized at  $*p < 0.05$ . (b) Microscopic images after 24 h incubation following droplet EP ((upper) phase contrast (PC) (lower) fluorescence (FL)).

Furthermore, a higher applied voltage resulted in a larger increase in fluorescence intensity. However, not only transient pore formation but also dead cells possibly lead to an increase in fluorescence intensity. To investigate whether the treated cells were viable or not, cell proliferation following droplet EP was confirmed. Fig. 5(b) shows the results of microscopic observation after droplet EP and 24 h incubation. Although the number of cells that appeared in "Ctrl" is less than one in "3.0 kV", comparison of the two phase contrast images shows that the morphology of the treated cells was similar to that of the control. Thus, it can be concluded that there is no notable difference in cell proliferation. In contrast, the fluorescent images are clearly different: cells treated with an applied voltage of 3.0 kV were stained with YO-PRO-1, indicating that YO-PRO-1 molecules entered the cells and bound to intracellular nucleic acids, and that therefore transient pores were formed during droplet EP.

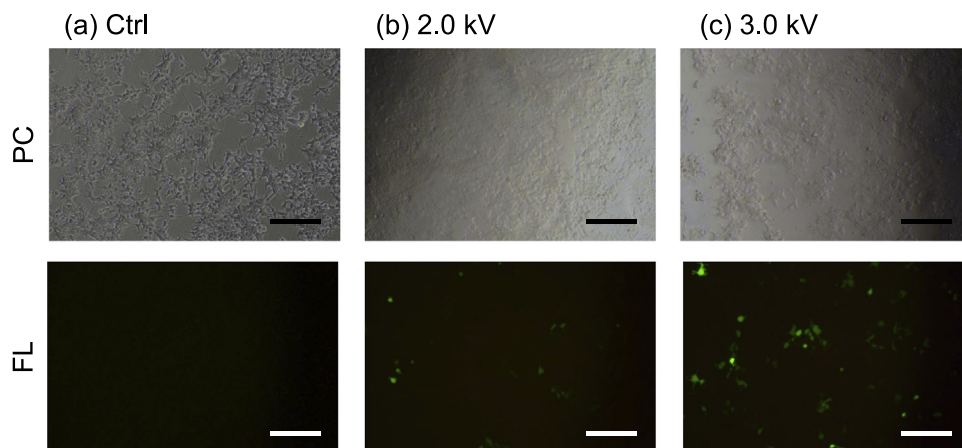
### 3.3. Fluorescent protein expression and flow cytometry

Fig. 6 shows the results of microscopic observation after droplet EP and 48 h incubation. The amount of DNA was increased (750 ng/2.0  $\mu\text{l}$ -droplet) to provide sufficient fluorescence intensity. Fig. 6(a) shows phase contrast and fluorescent images without HV treatment and after 48 h incubation. Consistent with the results in Fig. 5(b), cell proliferation was observed whereas Venus expression was not. Fig. 6(b) and (c) show phase contrast and fluorescent

images after droplet EP with an applied voltage of 2.0 kV or 3.0 kV, respectively, and subsequent 48 h incubation. Comparison of Fig. 6(b) and (c) with Fig. 6(a) shows no notable difference in cell proliferation. In contrast, healthy cells with significant signal intensity are observed in Fig. 6(b) and (c). In addition, comparison of the fluorescent images in Fig. 6(b) and (c) shows a larger number of Venus expressing cells with higher fluorescence intensity in Fig. 6(c). Table 2 summarizes the viability and the transfection efficiency after droplet EP and 48 h incubation. The results show that cell viability gradually decreased with increasing applied voltage, and the expression of fluorescent protein was enhanced with increasing applied voltage. This result agreed with the luciferase-expression experiment.

## 4. Discussion

In this paper, various experimental parameters of the droplet EP method were examined to characterize our method. As shown in Figs. 3 and 4, an increase in the applied voltage resulted in decreased cell viability and enhanced gene expression. The data shown in Fig. 5 indicate that the formation of transient pores during droplet EP and uptake of cell impermeable molecules were enhanced by increasing the applied voltage. The Venus expression experiment showed that cell viability as measured by flow cytometry was consistent with the data shown in Figs. 3 and 4, and the



**Fig. 6.** Microscopic images of fluorescent protein expressing cells after 48 h incubation following droplet EP ((upper) phase contrast (PC) (lower) fluorescence (FL)). (a) Without droplet EP, (b) 2.0 kV applied voltage, and (c) 3.0 kV applied voltage. Scale bars: 100  $\mu\text{m}$ .

**Table 2**  
Viability and transfection efficiency after 48 h incubation following droplet EP.

Experimental condition	Viability (%)	Transfection efficiency (%)
Ctrl	98	0.1
2.0 kV	96	1.0
3.0 kV	69	13.0

Venus and luciferase expression data showed similar trends. Taken together, the results suggest that the electric field strength is the most critical experimental parameter affecting cell viability, gene expression, and transient pore formation, the latter of which is essential for transfection. This finding suggests that the mechanism underlying droplet EP is similar to that of conventional EP. In addition, Fig. 4(a) indicates that cell viability decreased as the number of shorts increased. The short corresponds to the pulsed electric field in conventional EP and further supports our conclusion that droplet EP is similar to conventional EP. The application of both theoretical and experimental techniques, such as uptake of cell impermeable material and reporter gene expression, showed that higher electric field strength induces a larger number of transient membrane pores in conventional EP [2,25–27]. Since YO-PRO-1 uptake and gene expression observed in this study showed similar trends with conventional EP, it appears that the size and number of transient pores generated by application of a pulsed electric field are the dominant parameters affecting the efficiency of transfection. Our results indicate that approximately 3.0 kV, which is equivalent to an electric field strength of 5.0 kV/cm, is optimal for the experimental configuration used here. However, a more intense electric field > 5.0 kV/cm would be generated near the wire electrodes due to the “edge” effect, as mentioned in the previous report [23]. In addition, the optimum electric field strength will be dependent on the cell line, and therefore further studies are required using different cell lines.

Fig. 4(b) indicates that the electrical conductivity of the droplet EP medium affects cell viability, with viability being drastically decreased in media with lower electrical conductivity as the applied voltage increased. This drastic change in cell viability arises from the threshold voltage for droplet deformation. In addition, luciferase activity was not observed in the case of mannitol as shown in Fig. 4(g). This suggests that electric current in the electric pulsation could be required. However, further electrical conductivity experiments are required to provide more experimental data points. Fig. 4(h) and (i) indicate that an increase in the concentration of DNA and a decrease in the volume of the droplet notably enhance luciferase expression, suggesting that both the concentration of DNA and the cells strongly influence gene expression due to an increase in the frequency of collisions between cells and DNA molecules during droplet EP. One of the advantages of droplet EP is that a smaller volume of cell suspension is required compared with conventional EP, perhaps allowing EP using much higher concentrations of cells and DNA than is possible with conventional EP. Fig. 4(e) and (j) clearly show that the volume of the droplet affects viability and gene expression after droplet EP and may be correlated with deformation of the droplet. As shown in Fig. 1, instantaneous short circuiting caused by the droplet causes an extension of the long axis of the droplet until the droplet spans the distance between the electrodes. The lower viability and higher luciferase activity with a smaller droplet indicate that the cells inside the droplet were exposed to higher electric field. The possible reason is that the thickness of the expanded droplet could affect the electric field forming at the tips of the expanded droplet. Although the current value has not been measured yet, a smaller droplet might lead to an increase of current density and it could also affect the transfection, as suggested in previous report [28].

The characteristics of the electrical pulse delivered by droplet deformation and the correlation with the transfection will be investigated in future work.

Although the transfection efficiency of EP depends on various experimental conditions such as the cell line, confluency of the cells, and pulse parameters, typical EP using commercially available equipment provides transfection efficiency of 10–20% [27,29]. Therefore, the transfection efficiency of our method is comparable, as summarized in Table 2, as was cell viability, but in contrast with conventional methods, an expensive pulse generator and special reagents are not required. In addition, droplet EP works well with 5000–10,000 cells, a number well suited for typical multi-well plate experiments. This enables high-throughput assays and may find applications in life science, for example, in the functional analysis of specific genes, chemicals, and possibly signaling pathways. However, the reproducibility and operability of the droplet EP method requires improvement for these future applications and this can be addressed by improving the experimental setup.

## 5. Conclusion

This paper describes the characterization of droplet EP by varying various experimental parameters and demonstrates that the electric field strength is the most critical experimental parameter affecting cell viability, gene expression, and transient pore formation, which is essential for transfection. Our findings suggest that droplet EP is similar to conventional EP. The number of shorts and electrical conductivity of the medium also affect cell viability. A higher concentration of cells and DNA in the droplet aids efficient transfection. The volume of the droplet affects viability and gene expression after droplet EP and may be correlated with deformation of the droplet. Droplet deformation under the DC electric field is critical in our electroporation method.

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## Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.08.001>.

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