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Research Article

Electroporation of micro-droplet encapsulated HeLa cells in oil phase

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Electroporation (EP) is a method widely used to introduce foreign genes, drugs or dyes into cells by permeabilizing the plasma membrane with an external electric field. A variety of microfluidic EP devices have been reported so far. However, further integration of prior and posterior EP processes turns out to be very complicated, mainly due to the difficulty of developing an efficient method for precise manipulation of cells in microfluidics. In this study, by means of a T-junction structure within a delicate microfluidic device, we encapsulated HeLa cells in micro-droplet of poration medium in oil phase before EP, which has two advantages: (i) precise control of cell-encapsulating droplets in oil phase is much easier than the control of cell populations or individuals in aqueous buffers; (ii) this can minimize the electrochemical reactions on the electrodes. Finally, we successfully introduced fluorescent dyes into the micro-droplet encapsulated HeLa cells in oil phase. Our results reflected a novel way to realize the integrated biomicrofluidic system for EP.

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1 Introduction

Electroporation (EP) refers to the phenomenon discovered in 1970s that the cell membrane is temporarily permeabilized in response to the applied electric pulses so that the existing components in the surrounding medium can be introduced into cells. Currently, EP has been widely used in gene and RNA interference transfection, drug delivery and cell fusion in a variety of cell and animal models. The mechanism of EP was intensively investigated. Basically, the applied electric field can trigger a membrane potential. Once this induced potential reaches the critical value (around 1V), the localized rearrangement of the phospholipid bilayer occurs, which results in the formation of nano-pores (as small as 20–120 nm) in the plasma membrane and thus the permeabilization of membrane to foreign molecules. After the expansion and stationary stages, these nano-pores undergo a resealing process within a few seconds to minutes [1]. In gene or RNA interference transfections, EP usually is performed within an electroporator or EP apparatus in a commercial glass or plastic cuvette with two aluminum electrodes. In recently years, several research

groups have reported the successful application of microfluidic devices in EP [2–5].

Microfluidics deals with the behavior, precise control and manipulation of fluids that are geometrically restricted to small, typically sub-millimeter scales. It has attracted ever-increasing interests, particularly in the biological and medical applications, as it requires fewer materials and less time, produces less waste and has the potential for adaptation to highly integrated and computer-controlled reaction processes. In microfluidic devices, droplets that precisely convey a reagent in discrete volumes can be used as miniaturized vessels for chemical and biological reactions [6]. Droplet-based microfluidic systems have been used in many biological applications, such as PCR [7], HPLC [8], proteome analysis [9], clinical diagnosis [10] and protein crystallization [11].

The application of microfluidic devices in EP has several advantages over conventional electroporators and cuvettes: (i) microfluidic device requires much less cell samples and difficult-to-produce reagents; (ii) lower voltage is required for EP due to the smaller channel size; (iii) microfluidic devices made of transparent materials allow for simultaneous microscopic observation; (iv) other applications, including cell culture and detection process, can be integrated for multiple functionality in a single device. Many reports on gene transfection by EP microchips have focused mainly on electrode and chip design for either single-cell or large-cell population transfection by means of a static micro reserve or continuous flow [3], and it

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Abbreviations: EP, electroporation; GFP, green fluorescent protein; PI, propidium iodide; RF, radio frequency

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is now highly anticipated that the process flow including prior and posterior EP processes, such as cell culture, sorting and detection, can be realized in integrative multi-functional chips.

An extraordinary way to control cells in microfluidic devices is micro-droplets encapsulation. By means of particular chip designs, cells can be easily separated into individuals, transported to target location for certain application and reaccumulated for collection. In our previous reports, we have shown that precise control of droplets motions in the microfluidic channel can be achieved by introducing leading droplets of giant electrorheological fluid [12] and hence this is a potential scheme for designing a multi-functional chip for cell processing. T-junctions and flow-focusing structures have been commonly used to encapsulate single cell or particle into a droplet, for the purposes of cell-encapsulated alginate microbeads investigations [13] and worm behavior characterization [14]. Channel size, flow speed and cell or particle size are the key parameters for controlling single-droplet morphology. Because the reported structures for cell encapsulation have been shown to be stable and suitable for various bio-assays, EP of droplet-encapsulated cells could facilitate fully functionalized biomicrofluidics.

In this study, we report an efficient method for encapsulating cells in droplets, having structural dimensions comparable to those of the cells. Then, droplet-encapsulated cells were electroporated in the silicon-oil phase, and fluorescent dyes were successfully introduced into the encapsulated cells, as observed by laser confocal microscopy. This is an auspicious step toward the ultimate goal, the design and fabrication of integrative cell culture, EP and detection chips for biological research and medical diagnostic applications.

2 Materials and methods

2.1 PDMS chip

The PDMS chip used for the experiments (Fig. 1) was fabricated by soft lithography [15]. The fabrication process flow is shown in Fig. 2. Figure 2A–E shows a negative photoresist SU8-2025 which was first spin-coated on glass wafer at a certain spin rate. After exposure to ultraviolet and development in an SU8 developer, an SU8 pattern was formed. The pattern on the master was then replicated in PDMS, which was produced using an Sylgard 184 silicone elastomer mixture (Dow Corning, Midland, MI, USA) at the weight ratio of Base:Curing agent = 10:1. Then the PDMS slab was released from the SU8 master and bonded with another flat PDMS slab to form the microchannels. To connect the tube parallel to the channel, we make an extra connection using Cu wire with a diameter slightly larger than the PDMS channel and a tubing with an od slightly smaller than the connecting Teflon tubing used to guide the droplets out (Fig. 2F–I).

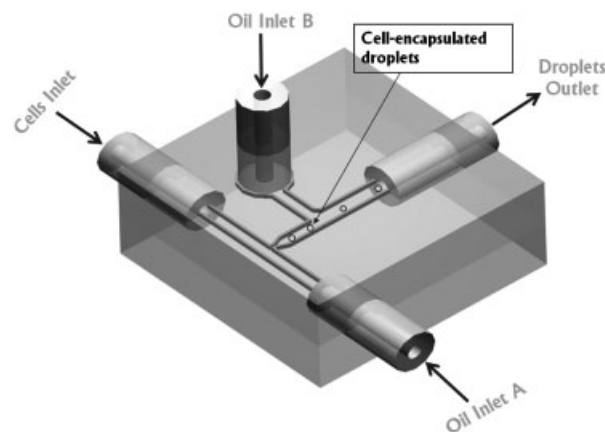


Figure 1. Schematic view of microfluidic chip for cell encapsulation.

2.2 Buffers and chemicals

Modified mannitol poration medium: 260 mM mannitol, 5 mM sodium phosphate, 10 mM potassium phosphate, 1 mM MgCl_2 , 10 mM Hepes, 0.1 mM BAPTA, pH 7.3. Recovery medium: poration medium supplemented with 10% fetal bovine serum and 20 mM MgCl_2 . Propidium iodide (PI) (81845-25 MG) and dextran, rhodamine B isothiocyanate, 70S (R-9379) were purchased from Sigma Aldrich.

2.3 Cell culture

HeLa cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa cells were cultured as a monolayer in minimum essential medium supplemented with 10% fetal bovine serum plus 100 unit/mL penicillin and 100 mg/mL streptomycin. The medium was buffered with NaHCO_3 (2.2 g/1 L minimum essential medium). Cell cultures were maintained at 37°C in a humidified incubator containing 5% CO_2 . The cells were replated every 2–3 days once the monolayer reached confluence. For subculture of the cell monolayer, the old culture medium was removed by aspiration, after which cells were washed by PBS once and then treated with a mixture of trypsin (0.05 mg/mL) and EDTA (2 mg/mL) for about 5 min. Finally, culture medium was added to terminate trypsinization.

2.4 EP in conventional cuvette using Bio-Rad apparatus

Cells grown in the mid-log phase were trypsinized and then suspended in the poration medium at a concentration of 4×10^5 cells/mL. In total, 25–100 μL of the cell suspension was mixed with 1 $\mu\text{g/mL}$ of PI and put into a plastic EP cuvette with a 0.1 mm gap between its aluminum plates (Bio-Rad Labs). The cuvette was then placed into the EP

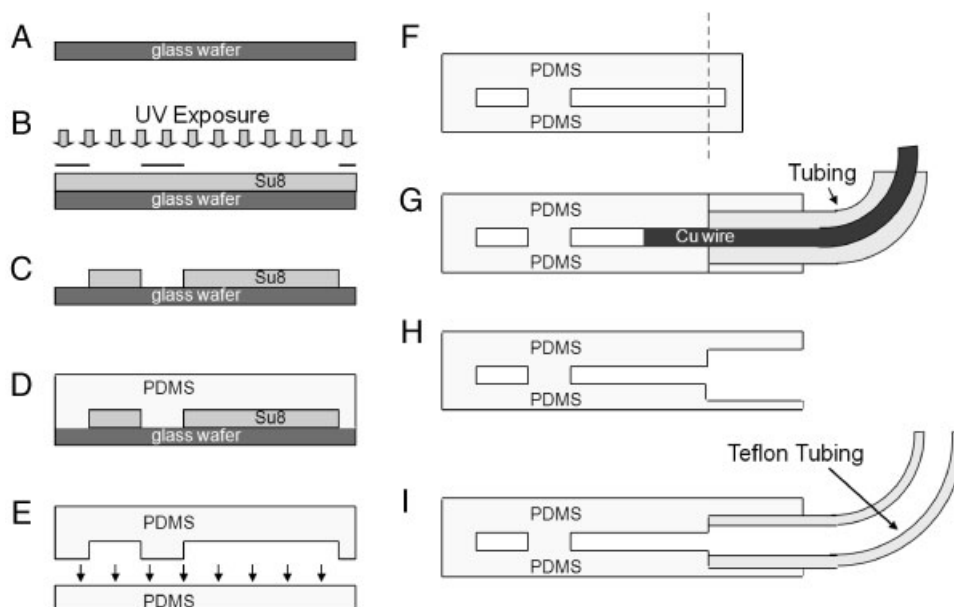


Figure 2. Microfluidic chip fabrication process flow.

chamber connected with the radio frequency (RF)-oscillating electric pulse generator (Bio-Rad Labs). Two trains of electric pulses (5 ms of pulse duration with the voltage of 120 V, the frequency of 30 kHz and a 100 ms interval between pulses) were applied to the cell/dye mixture suspension with an interval of 10 s between each shock. The waveforms of the output electric pulses were monitored using a digital storage oscilloscope (Tektronix 2221A). After EP, cells were incubated in the recovery medium with five times volume of poration medium for 15–20 min. Then cells were spun down, resuspended with culture medium, transferred to a 24-well plate and observed under a fluorescent microscope.

2.5 Data acquisition and analysis

Two imaging systems were employed for observation: (i) an inverted fluorescence microscope (Axiovert 200M, Zeiss) equipped with a cooled CCD camera (Diagnostic Instruments); (ii) a confocal microscope (Leica TCS SP5) equipped with multiple laser sources. The image data acquired from the CCD camera or confocal system were further processed and analyzed by MetaMorph (Universal Imaging) and Leica Confocal System software (Leica), respectively. Then the refined images were achieved by Confocal Assistant v4.0 (Bio-Rad) and Adobe Photoshop (Adobe Systems).

3 Results and discussions

3.1 Cell encapsulation

To generate single-cell-encapsulating droplets of smallest size, three frequently used droplet-generation geometries (Fig. 3A–C) were tested. The heights of these channels were

all about 100 μm . Various combinations of cell inlet, carrier flow and droplet outlet were investigated for each geometry, and the results for structure in Fig. 3B is shown in Fig. 3E–G. The smallest droplets diameters for three geometries were 50, 70 and 20 μm respectively. The T-junction geometry shown in Fig. 3A, with outlet A and cell inlet B/C, was found to be the optimal structure. Figure 3D shows that the drops generated by the flow-focusing structure in Fig. 3B were as large as 70 μm both with and without cells.

As derived from the results, the final PDMS chip design for cell encapsulation in droplets is shown in Fig. 1. The T-junction geometry was used to generate droplets. The channel is about 50 μm in depth and 30–300 μm in width. The flow rates of the cells inlet and oil inlet A are 0.005 and 1 mL/h, respectively. Also, the flow rate of oil inlet B could be adjusted over a wide range to further separate the generated droplets by increasing the distance between them. All of the flow rates were controlled by syringe pumps (KDScientific). Figure 3I shows the structure's droplet generation process. It can be seen that the droplet without a HeLa cell is about 20 μm . Once a cell runs through the junction into the droplet, the diameter increases to 40–50 μm . This size difference is an advantage for sorting in downstream so that only droplets with cells could be collected for further manipulation [16]. Moreover, the percentage of generated droplet encapsulating single cell is about 40%.

To avoid the recombination of generated droplets and the accumulation of cells in the connection part of the pipes and PDMS chip, we tried a different means of connection. Traditional connection, represented by oil inlet B in Fig. 1, is by way of holes drilled perpendicularly to channels, through which tubing is inserted and fixed. This type of connection introduces sudden changes of flow direction and

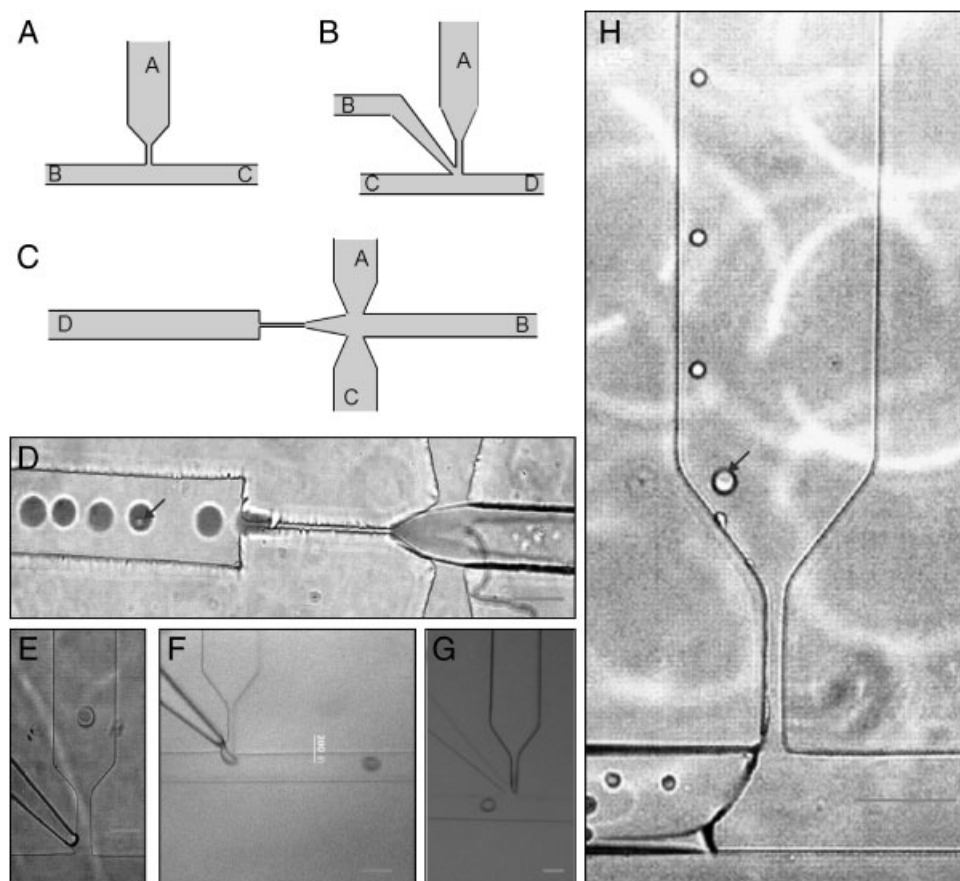


Figure 3. (A–C) Droplet generation designs. In (A) and (B), A–D could be outlet, cell inlet or oil inlet in the investigation of generation geometry, whereas in (C), A and C are oil inlets, B is cell inlet and D is the outlet. Among all the generation geometry, the optimized choice is that: in (A), A acts as outlet, B and C could be inlets, one for cell and the other for oil; (E–G) smallest droplet generated by structure (B) with different water inlet and droplet outlet; (D) and (H) smallest cell-encapsulated droplet generated by geometry (C) and (A). The arrow indicates a cell. All the scale bars are 200 μm .

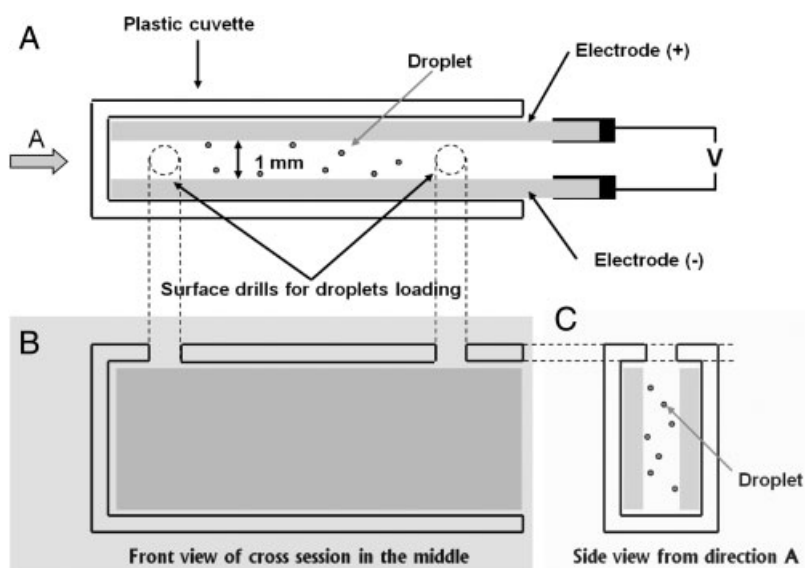


Figure 4. Schematic view of EP chamber. (A) Top view, (B) front view and (C) side view.

channel size, reduces distance between droplets and usually causes merging of adjacent droplets. Meanwhile, many cells will accumulate at the junction in the cell inlet. Thus in our design, we made the cells inlet and droplets outlet parallel to the channels, and connected them to Teflon tubing with

inner diameter about 300 μm . Additionally, the outer diameter of the tubing was made to be about 1 mm, so that the generated droplet could be guided directly to the inlet of EP chamber, which was also 1 mm in diameter (Fig. 4).

3.2 EP of micro-droplets-encapsulated HeLa cells in oil phase

Plasmid DNA encoding green fluorescent protein commonly has been used in EP assessment. However, it requires at least 16–20 h for the transcription and translation of Plasmid DNA into green fluorescent protein in HeLa cells after EP, significantly then, this assessment protocol does not allow for immediate post-EP observation, rendering rapid post-EP cell detection and sorting impossible. We tried another assessment tool, Rhodamine-Dextran, and found that it could indeed be introduced into encapsulated cells. However, its strong background fluorescence in the surrounding medium makes observation problematic (Fig. 5A).

As our third and best choice, we selected PI, a membrane-impermeable fluorescent molecule usually used as a DNA-staining agent in quantitative assessment of DNA content by flow cytometry. Another advantage of PI is its 20- to 30-fold fluorescence enhancement upon being introduced into cells and binding to nucleic acids; resultantly and crucially, very little background fluorescence is detected in the aquatic phase. Therefore, PI could be a good marker for the assessment of EP immediately after application of an external electric field.

We first introduced PI (1 $\mu\text{g}/\text{mL}$) into HeLa cells by means of the conventional EP apparatus (described in Section 2) to determine whether the introduction could be accomplished for the pertinent EP parameters and PI concentration. As shown in Fig. 5B, before application of an RF electric field, very few live cells showed positive fluorescence, whereas after, all of the cells did so. This suggests that the EP parameters and PI concentration were suitable for the EP and follow-up observation.

After successfully encapsulating cells into micro-droplets, we immediately introduced the micro-droplets into a silicon-oil-filled plastic cuvette with removable electrodes inside through the drill (Fig. 4) and acquired images under Leica laser confocal microscope both pre- and post-EP. Figure 5C shows the phase contrast or fluorescence of the representative encapsulated cells with or without the applied electric field. With no electric field, cells in micro-droplets exhibited no PI fluorescence, whereas after application of the electric field, some of the cells showed PI positivity. This suggests that PI had been successfully introduced into micro-droplets-encapsulated cells by means of that externally applied electric field. However, the efficiency ($\sim 5\%$) was not as high as has been achieved with conventional aqueous-phase EP.

There have been several reports on EP of cells in microfluidic droplets [17–20]. For examples, Zhan *et al.* encapsulated China hamster ovary cells into aqueous droplets in oil and then electroporated continuous flow of droplets with a constant voltage applied between the downstream electrodes [19]. However, when we tried a similar method for the purposes of EP in a microfluidic channel, in which the micro-droplets were in contact with the channel walls and electrodes, we found that the droplets

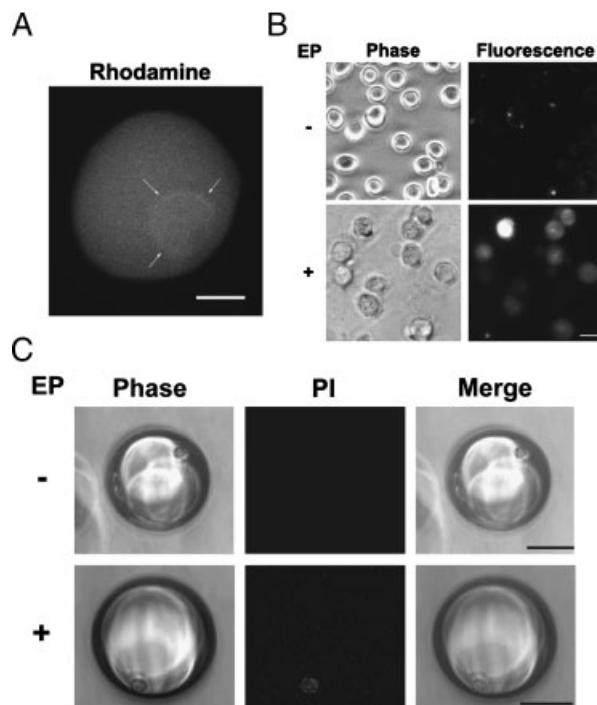


Figure 5. Fluorescent image of encapsulated cells with EP of rhodamine-dextran in oil phase (A) and phase contrast and fluorescent images of HeLa cells with or without EP of PI (B). (A) HeLa cells were encapsulated into rhodamine-dextran contained poration medium composed micro-droplets in silicon oil (as described in Section 3) and then introduced into EP cuvette (Fig. 4). Then, an external RF electric field was applied and the fluorescent image was recorded using a Leica confocal microscope. The circulated fluorescence highlighted region indicated by arrows represents the periphery of a single HeLa cell. Scale bar: 10 μm . (B) HeLa cells were introduced with PI by EP as described in Section 2. The phase contrast and fluorescent images of HeLa cells with or without the external electric field were recorded using a Carl Zeiss Fluorescent Microscope. Scale bar: 15 μm . (C) Phase contrast and fluorescent images of encapsulated HeLa cells without (upper) or with (lower) the application of external electric field. HeLa cells were encapsulated into PI-contained poration medium composed micro-droplets in silicon oil (as described in Section 3) and then introduced into EP cuvette (Fig. 4). Afterward, an external RF electric field was applied. Both phase contrast and fluorescent images before or after the EP were recorded using a Leica confocal microscope. Scale bar: 50 μm .

were easily adhered to the channel walls at the cost of precise control. This serves to be one of the most severe drawbacks of integrating sequential processes in one chip. Therefore, we endeavored to develop a novel EP chip by which an electric field can be applied on continuous oil flow but not to the aqueous droplet directly, which will significantly improve the precision of droplet control inside chips. This thinking may also prevent direct contacts between aqueous droplets and electrodes and therefore minimize the electrolysis at electrodes and avoid the generation of gas bubbles, which can also ruin the precise control.

The idea of EP of cells in oil phase addresses the critical question whether the current generated by external applied

electric field does the matter in the success of EP. Our successful EP process with drop-encapsulated cells in oil phase also gives rise to some basic considerations on the understanding of the EP phenomena. Typically, one can adequately describe the behaviors of an EP system with an LCR representation. The membrane, as well as other external components (including the electrolyte), can be modeled as a parallel arrangement of a capacitor and a resistor, and different components are in series with each other. All the underlying physics can be included when deducing corresponding resistance and capacitance [21]. Different from cells in pure electrolytes, the silicon oil as dispersing media efficiently lowers the global current density even with the electrolyte droplets present [22], as the applied electric field is far below the breakdown threshold of silicon oil. Then, the current in our system would be localized in the droplets and dominated by the charging current of the corresponding capacitors. Thus, it should decay rapidly rather than increase with time, as observed in the previous experiments [21]. What is more, the dielectric constant of silicon oil is around 40 times smaller than that of water, which means that most of the voltage will drop across the silicon oil phase, leaving the transmembrane potential much lower than in conventional EP experiments. This could explain our result that lower efficiency ($30 \pm 9.9\%$) was achieved in the oil-phase EP with the same parameters of electric field as entailed in the conventional EP method. The high efficiency of EP in the oil phase probably could be achieved by employing an electric field with much a higher voltage and this could be easily realized in the micro channel, the width of which is just several tens of micron meters. Besides, the viability determined by cell counting analysis on cultured cells prior to oil-phase EP was around 75%. Although the oil-phase EP is presently suboptimized, our results demonstrate successful EP performance, thereby pointing to the broad application of precisely controlled EP in integrated biomicrofluidic systems.

4 Concluding remarks

We report here a convenient method to encapsulate HeLa cells into peer-sized micro-droplets and realize follow-up EP in oil phase. We successfully encapsulated HeLa cells in micro-droplets with the diameter around 50 μm by PDMS composed microfluidic chip based on the revised T-junction geometry. Using silicon oil as the dispersion media, we electroporated a fluorescence DNA dye into the encapsulated cells in conventional cuvette. This method helps to improve the precise control of individual cells in microfluidic EP chip, thereby making it easier to design and fabricate integrative microfluidic EP chip. Our future study will focus on improving our EP chip, by integrating electrodes and cell culture compartments and optimizing the oil-phase EP conditions.

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The authors have declared no conflict of interest.

5 References

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