Chapter 7

Single Cell Electroporation Using Microfluidic Devices

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Abstract

Electroporation is a powerful technique to increase the permeability of cell membranes and subsequently introduce foreign materials into cells. Pores are created in the cell membrane upon application of an electric field (kV/cm). Most applications employ bulk electroporation, at the scale of 1 mL of cells (ca. one million cells). However, recent progresses have shown the interest to miniaturize the technique to a single cell. Single cell electroporation is achieved either using microelectrodes which are placed in close vicinity to one cell, or in a microfluidic format. We focus here on this second approach, where individual cells are trapped in micrometer-size structures within a microchip, exposed in situ to a high electric field and loaded with either a dye (proof-of-principle experiments) or a plasmid. Specifically, we present one device that includes an array of independent electroporation sites for customized and successive poration of nine cells. The different steps of the single cell electroporation protocol are detailed including cell sample preparation, cell trapping, actual cell poration and on-chip detection of pore formation. Electroporation is illustrated here with the transport of dyes through the plasma membrane, the transfection of cells with GFP-encoding plasmids, and the study of the ERK1 signaling pathway using a GFP–ERK1 protein construct expressed by the cells after their transfection with the corresponding plasmid. This last example highlights the power of microfluidics with the implementation of various steps of a process (cell poration, culture, imaging) performed at the single cell level, on a single device.

Key words: Gene transfection, Gene therapy, Drug delivery, Lab on a chip, Electropermeabilization, Single cell analysis

1. Introduction

The introduction of foreign materials in cells is essential for a great variety of applications in medicine and biotechnology such as gene therapy, drug delivery, cell engineering, or microorganism inactivation. Different techniques that rely on various chemical or physical principles exist to cross the impermeable barrier formed by biological membranes and to transiently permeabilize them. One of the most popular techniques is electroporation (1), which is based on the use of an electric field. Upon application of short pulses (exponentially

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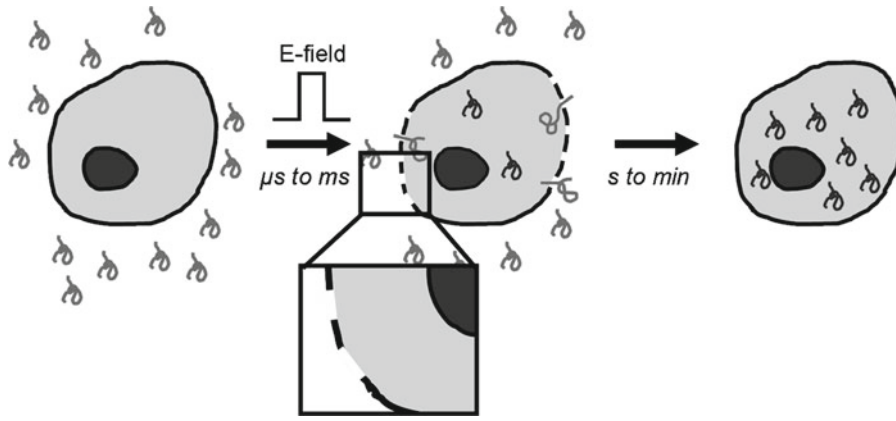

Fig. 1. Principle of cell electroporation. *From left to right* : A cell is incubated in a medium supplemented with foreign substances to be introduced in the cell. The cell is exposed to an electrical treatment consisting of a short pulse (μm to ms) of a high electric field (kV/cm); this leads to the formation of pores in the cell plasma membrane, enabling the entry of foreign substances inside the cell. After a few minutes, the pores seal again, leaving cells loaded with foreign materials (Courtesy of Dr. I. van Uitert).

decaying pulses or square pulses) of a high electric field (kV/cm range) the cell transmembrane potential increases. If the latter reaches the critical threshold of 0.2–1 V (2), a transient molecular rearrangement of the phospholipids forming the membrane occurs, which leads to the creation of aqueous pores in the membrane in a few microseconds, as illustrated in Fig. 1. These pores consist of pathways for molecular exchanges between the intra- and the extracellular media. If the electrical protocol remains mild, pores reseal within seconds to minutes (3), and the cells survive the treatment after having taken up foreign materials added in the solution. However, this viable cell poration is just one of the three scenarios observed when a cell is exposed to an electric field. Alternatively, when the electric field is too weak, the cells do not appear to be affected by the treatment: pores are too small or nonexistent. Conversely, if the electrical protocol is too strong, cells undergo lysis and die. Unfortunately, little is known about the process of pore formation in the cell membrane, and these three possible scenarios are difficult to predict to yield a safe and efficient methodology. What is now acknowledged is that pore formation proceeds in three steps, as demonstrated by molecular dynamic simulations. First, upon application of the electric field, water defects appear in the cell membrane. If these defects are stable enough, they lead to the creation of a water file or hydrophobic pore through the membrane. Finally, the phospholipids in the vicinity this pore rearrange to yield a more stable and hydrophilic pore. However, the precise parameters that influence these three steps of the pore formation process are not identified yet, and therefore, the success yield of the technique remains low (<50%) (4).

Still, the technique of electroporation is widely used for various applications. This popularity is easily explained by the numerous advantages the technique presents compared to other permeabilization approaches. The technique itself is easy to setup, and does not require expensive equipment. Furthermore, electroporation is not toxic to the cells; it is highly reproducible and can be performed at a large scale, in a possibly automated way. Finally, this electrical approach is universal: it has a wide applicability in terms of cell lines that can be treated (including bacteria, plant and mammalian cells); any kind of foreign entities can be delivered into the cells (small molecules, drugs, plasmids, proteins, particles of different sizes…); and the technique can also be employed for extracting material out of the cells for analysis.

The main applications of electroporation are as follows: (1) gene therapy or cell vaccination (5), (2) cell engineering or modification to establish new cell lines for patch-clamp measurements or investigating intracellular signaling pathways, by delivery of either plasmids coding for exogenous proteins to be expressed by cells or siRNA to silence the expression of certain proteins (6, 7), (3) enhanced drug delivery e.g., for cancer treatment (also known as electrochemotherapy) (8), (4) particle delivery (9), (5) bacterial transformation or engineering of plant cells, and (6) protein delivery. For most of these applications, the electroporation treatment is carried out at the level of a whole cell population in bulk. Here, 1 mL of a cell suspension (ca. 10^6 cells) is placed in a cuvette equipped with two electrodes, on which a high voltage (kV range) is applied.

However, the bulk electroporation approach is not appropriate for all applications, and for some particular purposes, the treatment must be miniaturized to the single cell level (3, 10), or even to the subcellular level. Advantages found in this strategy are first the higher control on the electrical treatment to which the cell is exposed and the possible customization of the electrical parameters for each cell; this ultimately results in a higher success yield. Second, as the voltage is created across a single cell, the distance between the electrodes is much shorter (<100 μm), and 1–10 V voltages are typically used, eliminating the risks associated with the use of high voltages (kV range). In addition, this single cell approach is expected to yield basic knowledge on the processes underlying the mechanisms of pore formation. Furthermore, studying the response of single cells to the electroporation signal will generate information on the influence of numerous cell parameters (cell size, shape, membrane composition…) on the outcome of the treatment, and help elucidate the discrepancy found in cell populations. Finally, this single cell electroporation strategy can be seen as a novel noninvasive approach for single cell analysis to study signaling pathways; this relies on either sampling a small amount of the cell content (11, 12) or transfecting cells with a protein of interest

coupled to a fluorescent reporter followed by tracking the protein position after cell exposure to given stimuli (13).

Two main approaches are reported in the literature for single cell electroporation: either using microelectrodes (14) (or micropipettes (15)) or in a microdevice (11–13, 16–26). In the first method, two microelectrodes (or micropipettes) are positioned in close vicinity to a cell with the help of micromanipulators. Subsequently, a voltage is applied between the electrodes to generate a well-defined electric field across the cell. This technique is labor-intensive, time-consuming and requires a skilled operator for precise positioning of the electrodes at a precise distance from the cell membrane. Still, one key advantage is that this approach is suitable for in situ treatment of adherent cells, in a natural environment. Alternatively, the electroporation protocol is implemented in a microfluidic device. The device typically includes micrometer-size structures not only to isolate a single cell from a population and trap it in a given location, but also to locally shape the electric field and create hot spots at the place where the cell is immobilized. This microfluidic-based strategy lends itself well to large-scale (e.g., by using an array of trapping sites) and automated cell poration, even if the protocol is performed at the single cell level (13, 19, 21). On other aspects, on-chip electroporation can also be coupled to other steps such as single cell analysis (e.g., using capillary electrophoresis), and single cell imaging to follow the postelectroporation fate of individual cells (27), which is important when cells have been transfected with genes (13).

In this chapter, we focus on this second approach where microfluidics is exploited for single cell electroporation, and only poration of mammalian cells is discussed, although some bacterial applications are included in Subheading 4.

Specifically, we describe a protocol for single cell electroporation using a glass-silicon microfluidic device that contains a series of independent poration sites (13). These sites consist of 4-μm wide slits where individual cells are trapped and successively exposed to an electric field created using integrated electrodes. The electroporation protocol is first optimized using various cell lines (K562, THP-1, and C2C12 cells) via the uptake or release of fluorescent dyes, and gene transfection is demonstrated using a plasmid coding for EGFP (Enhanced Green Fluorescent Protein). Finally, the single cell electroporation chip and protocol are applied to track the localization of a protein-kinase (ERK1) involved in intracellular signaling. For that purpose, cells are engineered to express an EGFP–ERK1 construct, and imaged on-chip using fluorescence microscopy to follow ERK1 activity upon cell stimulation. This work is an unprecedented example of an on-chip integrated protocol for cell transfection followed by cell imaging to elucidate signaling pathways, with all experiments being performed at the single cell level.

2. Materials

2.1. Cell Culture

1. Cells are cultured in conventional culture medium, and the nature of the medium depends on the cell type, as follows.
2. Human leukemia cells (THP1/K562) are cultured in RPMI-1640 medium supplemented with 10% heat-inactivated and filter-sterilized fetal calf serum, 100 IU/mL penicillin, 100 mg/mL streptomycin, 2 mM l-glutamine, and 250 mg/mL fungizone (RPMI+ medium).
3. C2C12 cells are cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/mL streptomycin, 2 mM l-glutamine, 250 mg/mL fungizone, and 1% sodium pyruvate.
4. Mesenchymal stem cells (MSCs) are cultured in αMEM supplemented with 10% FBS, 1 ng/mL bFGF, 100 mg/mL penicillin, 100 IU/mL streptomycin, and 0.4 mmol/mL ascorbic acid.
5. All media, supplements and antibiotics are purchased from Invitrogen (Breda, The Netherlands), except for ascorbic acid which is purchased from Sigma (Zwijndrecht, The Netherlands).
6. A 0.25% (w/v) trypsin solution in PBS buffer is employed to harvest C2C12 and MSCs cells for culture and cell sample preparation before the electroporation.

2.2. Cell Staining

Cells (10^6/mL) are stained using a 1 μg/mL Calcein AM (Invitrogen, Breda, The Netherlands) solution in PBS buffer prepared by diluting 1,000 times a 1 mg/mL stock solution in DMSO. In this manner, electroporation can be detected via the leakage of the calcein out of the cells.

2.3. Electroporation Buffer

Electroporation experiments are carried out in a dedicated low-conductivity buffer composed of 10 mM HEPES, 140 mM NaCl, 2.68 mM KCl, 1.7 mM $MgCl_2$, and 25 mM glucose. The solution is maintained at a pH of 7.4.

2.4. DNA Solution

For gene transfection experiments, DNA is loaded in the microfluidic chip after trapping of the cells in the device. The working solution introduced in the chip has a concentration of 100 ng/mL; it is prepared by diluting a MilliQ water-based DNA stock solution (450 ng/μL) in electroporation buffer. Two plasmids are employed here: one plasmid coding for the enhanced Green Fluorescent Protein (EGFP) and one plasmid coding for a protein construct composed of ERK1 (signaling protein) and a fluorescent reporter (EGFP).

2.5. Microchip

The essential part of single cell electroporation in a microfluidic format is the microdevice itself. This device must include a number

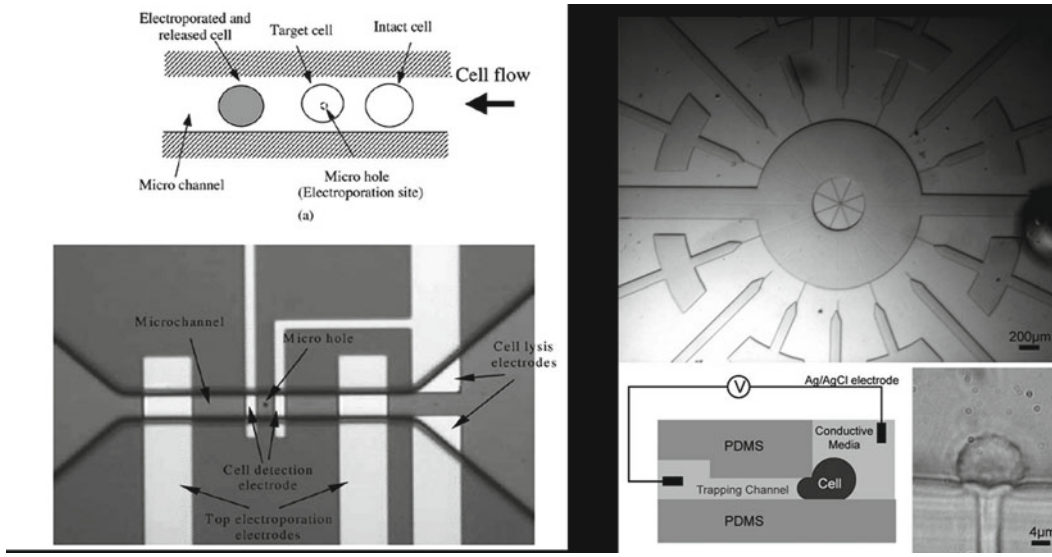

Fig. 2. Examples of miniaturized and microfluidic devices for single cell electroporation. *Left*: the device consists of a main channel where cells flow and at the bottom of which a microhole is added; when a cell passes by, it is trapped in the microhole and exposed to an electrical treatment applied using two integrated electrodes. Subsequently, the cell is released and the same treatment is applied to the next cell flowing in the channel (Reprinted from (Huang, 2003) with permission from Elsevier). Reproduced by permission of The Royal Society of Chemistry. *Right*: the device includes one central chamber connected to two wide channels for loading and removal of the cell solution, as well as an array of channels radiating from the chamber and connected to it via low μm^2-cross-section channels, used for cell trapping (*top*). External electrodes are inserted in the main channel and side channels of the devices. *Bottom*: schematic representation of cell trapping in the constriction channels, and enlarged view of a trapped cell. Reprinted from (Khine, 2005). Reproduced by permission of The Royal Society of Chemistry.

of specific structures, as follows. First, it contains a microfluidic channel where the cell solution is introduced. Second, this channel presents a single trapping site or an array of those, disposed in a planar way (at the bottom of the channel) or in a lateral fashion (along one wall of the channel) in which cells are isolated. For actual trapping of cells, these structures are connected on their backside to another microfluidic structure (e.g., channel or chamber) from which a negative pressure is applied. Finally, electrodes are necessary to create a localized high electric field across the cell(s). They can either be integrated in the device or externally introduced in the reservoirs.

Several single cell electroporation devices are reported in the literature, as illustrated in Fig. 2, with one or several trapping sites, lateral or bottom trapping of cells, and integrated or external electrodes. Here, we focus on one particular device developed in our group.

This single cell electroporation chip consists of two microfluidic channels etched in silicon and connected with each other through an array of nine trapping structures (20 µm width; 4 µm depth),

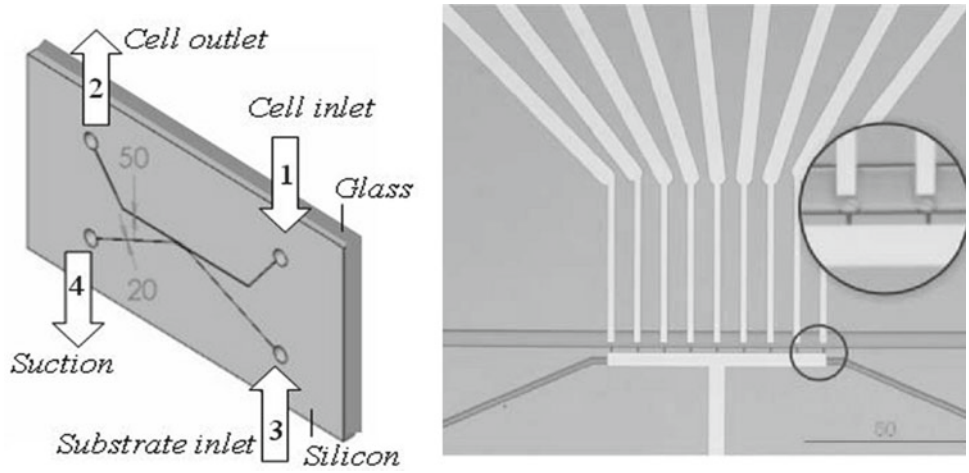

Fig. 3. Single cell electroporation device employed in this chapter including an array of nine independent trapping sites. (**left**) Cartoon of the microfluidic chip showing the two microfluidic channels for cell flow and monitoring cell trapping separated by an array of trapping sites. (**right**) Top view of the microfluidic chip showing the two channels and the electrodes to individually address the nine trapping sites where single cells are immobilized; *inset*: enlarged view of two trapped cells ((Valero, 2008) Reproduced by permission of The Royal Society of Chemistry). Enlarged view on a few trapping sites located between the two microfluidic channels (SEM picture).

shown in Fig. 3. One (wide) channel (50 μm width; 15 μm depth) is used for the introduction of cells and the various solutions in the chip, while the other channel (20 μm width; 15 μm depth) is employed to suck the cells in the traps. This silicon fluidic substrate is bonded to a Pyrex substrate on which Pt electrodes are sputtered: a main and common electrode for all sites as well as separate electrodes for individual addressing of the nine trapping sites. This device is produced by microfabrication techniques in a dedicated clean-room environment; more details on the fabrication process can be found elsewhere (13).

2.6. Experimental Setup

For electroporation experiments, the microfluidic chip is housed in a dedicated chip-holder, which includes fluidic access to the reservoirs of the chip and integrated electrical connections. The chip-holder is connected via an in-house designed interface to a computer equipped with function generator (NI 5041 National Instruments) and acquisition cards (NI PCI-6221, National Instruments) for application of the electroporation signals on the nine independent electrodes and recording of electrical signals. Electroporation experiments are monitored using a LabView interface that controls both NI cards. The chip-holder is placed on the stage of a microscope equipped with an epifluorescence unit and a controlled CCD camera for optical monitoring of the experiments (cell trapping,

cell electroporation, and cell survival). For fluidic handling in the chip, micropipette tips are employed, as well as a pump for applying the negative pressure.

3. Methods

3.1. Microfluidic Device

Before any use, the microfluidic device is sterilized using an autoclave system (20 min at 122°C) (see Note 1). After it has been brought back to room temperature, the chip is filled in with filtered HEPES buffer supplemented with BSA (3–5%) (see Notes 3 and 5), and incubated overnight in this solution. This prevents cells from sticking in the microfluidic channels. Finally, the chip is thoroughly rinsed with filtered HEPES buffer.

3.2. Cell Sample Culture and Preparation

Cells are cultured in the aforementioned media, and medium is refreshed every 3–4 days. C2C12 and MSC cells are first washed with PBS buffer to remove dead cells as well as any protein present in the culture flask. Subsequently, the cells are incubated with a trypsin solution (0.25% w/v) for a few minutes, and complete growth medium is subsequently added to inactivate the trypsin. Following this, the resulting cell solution is centrifuged and cells are resuspended in fresh and prewarmed medium in case of culture, or in electroporation buffer in case of experimentation. For THP-1 and K562 cells, the procedure is the same, but without any trypsin treatment as those cells are already in suspension.

THP-1, K562, and C2C12 cells have been employed to optimize the study of the electroporation process using fluorescence assays (calcein release, PI entry), and C2C12 cells and MSCs for gene transfection experiments.

For some experiments, cell electroporation is detected optically through the release of a dye out of the cells. This dye (typically Calcein AM) is previously loaded in the cells by incubating them in a 1 μg/mL solution for 30–60 min. Thereafter, the cells are washed thoroughly twice with PBS solution at room temperature, and finally resuspended in the electroporation buffer.

3.3. Cell Loading

All reservoirs are filled with electroporation buffer (100 μL), and in reservoir 1, 100 μL of the cell suspension (10× diluted) in electroporation buffer is placed. By removing the buffer (ca. 50 μL) in reservoir 2, a cell flow is created in the main channel. Subsequently, a mild suction (1–2 psi) is applied from reservoir 3 or 4 to attract the cells in the trapping structures. If more than one cell is retained in the trapping structures, a higher flow is created in the main channel to remove the excess of cells, while maintaining the negative pressure in the traps. Once all traps are filled with a single cell, the negative pressure is switched off.

3.4. Preelectroporation Preparation Steps

After trapping of the cells, the buffer in the main channel is refreshed to introduce new buffer supplemented with either plasmids (for gene transfection experiments) or a membrane exclusion dye (for optical detection of pore formation based on the entry of a fluorescent dye in the cells). In the former case, cells are incubated for ca. 10 min with the DNA solution before application of the electroporation signal (see Note 24). This incubation time promotes the formation of DNA–cell adducts, and ultimately enhances the entry of DNA in the cell upon application of the electric field.

3.5. Cell Electroporation

The electroporation signal is applied on the cells trapped in the device. As the electrodes are independently addressable, the cells are treated individually and successively. Typically, an electroporation signal consists of one pulse of 6-ms duration and amplitudes starting from 1 V (see Notes 12–15). If no electroporation is detected (no release of calcein or no uptake of PI, see Subheading 3.7), a second electroporation signal is applied with a longer pulse duration (+1 ms) or a higher amplitude (+0.5 V) until pore formation is observed optically. For C2C12 and MSCs cells, an optimal electroporation protocol consists of one pulse of 2 V and 6 ms, whereas other cell lines require higher field strength before uptake of dye is detected, and this still depends on the type of the cell lines, as shown in Fig. 4.

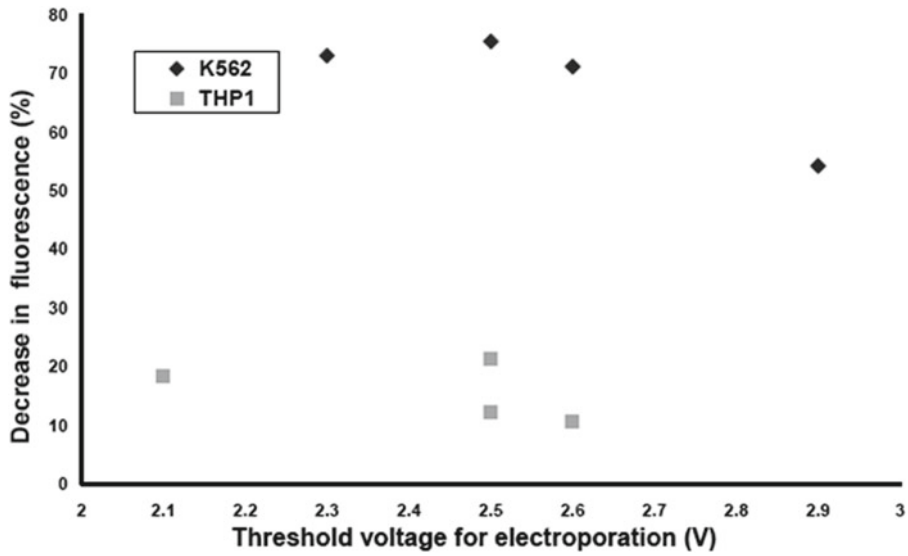

Fig. 4. Discrepancy in the required electrical parameters required to electroporate cells from two different cell lines (K562 and THP1) measured through the release of calcein out of the cells after application of an electrical treatment and the value at which cell poration is detected. K562 cells are more porated than THP-1 cells for a similar electrical treatment (6 pulses of 1 ms, 2–2.9 V amplitude), as observed by the lower decrease in fluorescence intensity measured in the cells (20% for THP1 cells against >70% for K562 cells). This discrepancy can be explained by a difference either in cell size (16 μm diameter for K562 cells against 12 μm for THP 1 cells) or in cell membrane properties (Courtesy Ms. V. Stimberg).

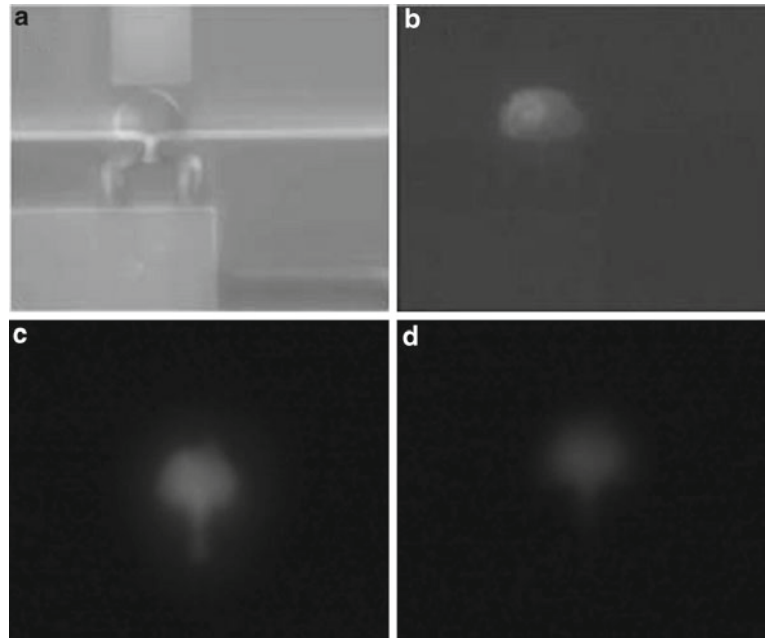

Fig. 5. Examples of fluorescence-based monitoring of cell poration using either the entry of PI into C2C12 cells (*top*), or the release of calcein out of THP-1 cells (*bottom*). C2C12 cells are porated using a single pulse (6 ms, 2 V) ((Valero, 2008) Reproduced by permission of The Royal Society of Chemistry) while THP-1 cells are treated using a series of six pulses (3 ms, 2.4 V) (Courtesy Ms. V. Stimberg). Pictures (**a**) and (**c**) show the cells before the treatment, and pictures (**b**) and (**d**) after application of the electrical pulse(s).

3.6. Postelectroporation Process

For fluorescence assays, cells are imaged in the electroporation buffer (see Subheading 3.7), and once experiments are finished, the chip is cleaned and washed thoroughly to be used again (see Note 2). For DNA transfection experiments, the cells are incubated for an additional 10 min to complete entry of the plasmids in the cells, before the solution is changed to culture medium (see Note 24).

3.7. Detection of Electroporation

Fluorescent membrane integrity markers are mostly used to detect pore formation in the cell membrane (see Note 20). These dyes do not usually cross the membrane unless the latter is damaged. Fluorescence-based detection is done twofold; either by measuring the release of a dye (e.g., calcein AM) previously loaded into the cells (21), or by detecting the entry of another dye (e.g., PI or YOYO-1) (13) added in the electroporation buffer. In the first case, the fluorescent levels in both the cells and the surrounding medium are measured after exposure of the cells to the electric field (see Note 20). In the second case, the dye is a DNA intercalating agent, and upon entry in cells, it moves to the nucleus and binds to DNA to give a bright fluorescent signal. Figure 5 illustrates this principle of fluorescence-based monitoring of cell electroporation, using both approaches of dye loading in the cell (Fig. 5a–b) and dye release out of the cells (Fig. 5c–d). However, all dyes

(calcein, PI, and YOYO-1) are markers for cell death, so a better option would be to use a sequential combination of two dyes (calcein and PI) where the second dye would be added in the medium 30 min after electroporation (when pores are expected to be closed). Thereby, it would be possible to distinguish between viable cell poration (calcein release, no PI uptake) and cell death (calcein release, PI uptake). Alternatively, small ions and FITC-conjugated Dextran particles can be employed to probe the size of the pores created upon application of the electrical signal (see Note 21).

3.8. Gene Transfection

Other fluorescence assays are based on the transfection of a gene coding for a fluorescent protein such as GFP; this enables to demonstrate to only that cells are porated, but also that they are functioning properly as they are able to produce proteins from a gene. For gene transfection experiments, all solutions used (cell solution, electroporation buffer, DNA solution) are cooled on ice before being introduced in the chip (see Note 4). After the poration process and once warm medium has been introduced to replace the electroporation buffer, the chip is removed from its holder, and placed in a Petri dish, covered with warm medium. The Petri dish is kept under controlled conditions (37°C, 5% CO_2) in the incubator for one or several days. Typically, after 24 h, cells are imaged to check for protein expression (positive green fluorescent signal) (Fig. 6), and the fluorescence level in the cell is quantified to determine the amount of plasmids loaded in the cell (13).

3.9. Application: Studying the ERK1 Signaling Pathway

As mentioned in the introduction, one promising application of single cell electroporation in a microfluidic platform is the elucidation of signaling pathways. For that purpose, a plasmid coding for a protein construct is loaded in MSCs cells; here, a construct composed of a kinase protein (ERK1) and EGFP is employed, where EGFP acts as a fluorescent reporter for the localization of the kinase inside the cell (13). As before, one day after cell poration, cells are imaged to check for the expression of the construct protein, and a uniform staining is observed in the whole cell.

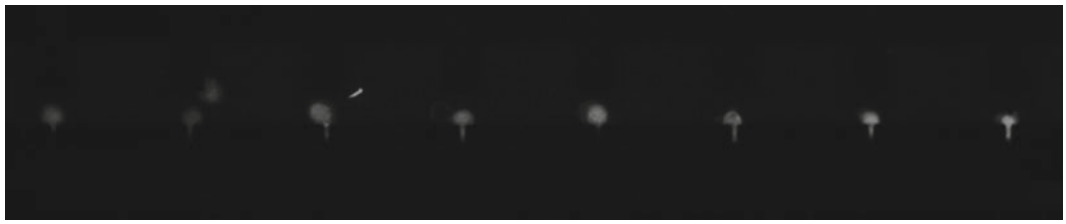

Fig. 6. Gene transfection in C2C12 cells. Pictures showing nine C2C12 cells trapped in the single cell electroporation device, 24 h after on-chip transfection of the gene coding for EGFP using a single pulse (6 ms, 2 V) (Valero, 2008). Reproduced by permission of The Royal Society of Chemistry.

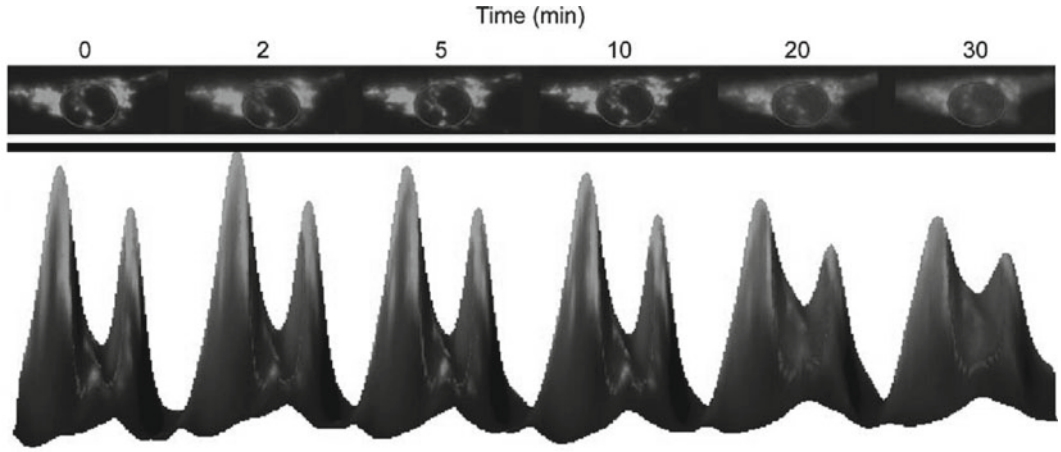

Fig. 7. Imaging the ERK1 signaling pathway using single cell electroporation technology. After having been transfected with a gene coding for the ERK1–EGFP construct (EGFP playing the role of a fluorescent reporter for the protein kinase ERK1), a cell is placed under starvation conditions until ERK1 is solely localized in the cytoplasm (time 0). Subsequently, the cell is stimulated via exposition to a growth factor (FGF-2) for activation of ERK1 and its translocation to the nucleus; this is visualized using real-time imaging by a progressive shift of the fluorescence from the cytoplasm to the nucleus. *Top*: Fluorescent images of a single cell after exposure to FGF-2; *Bottom*: 3D representation of the fluorescent intensity in the cell; as time passes the fluorescent level decreases in the cytoplasm and increases in the nucleus (Valero, 2008). Reproduced by permission of The Royal Society of Chemistry.

Using this protein construct, the ERK1 signaling pathway initiated by the binding of growth factors (FGF-2) to the cell membrane is studied. This binding causes ERK1 activation and its translocation to the nucleus to trigger gene expression (28). In a first step, MSCs cells are kept under "starvation" conditions, in a serum-poor medium (1% instead of 10%) for one day, resulting in the full inactivation of ERK1: after 24 h, it is solely located in the cell cytoplasm, as seen by the green fluorescent pattern limited to the cell cytoplasm. Thereafter, the solution in the chip is changed for a medium containing FGF-2 (10 ng/mL). To follow the activation of ERK1, the cells are imaged continuously for 30 min; Fig. 7 shows the progressive translocation of ERK1 to the nucleus upon FGF-2-based activation, visualized by a shift of the green fluorescent signal from the cytoplasm to the cell nucleus.

4. Notes

General Matters

1. Sterilization of the microchip before any experiment is essential for the outcome of the experimentation. This is done by placing the chip (wrapped in aluminum foil) in an autoclave for 20 min at 122°C. Alternatively, a 70% ethanol solution can be employed. Similarly, the chip-holder must be cleaned thoroughly before and after use with a 70% ethanol solution, followed by rinsing in MilliQ water.

2. Before the chips are reused, they must be thoroughly cleaned to remove any cell debris, by incubating them in a trypsin solution (0.25% w/v) overnight in the incubator. Thereafter, the chip is sterilized anew in the incubator.
3. To avoid clogging issue, all solutions are filtered before being introduced in the chips (0.2-µm nonprotein binding filters, Millipore).
4. A low experimental temperature (0–4°C) is preferred to delay pore resealing and subsequently enhance cell loading efficiency. This is especially important for transfection of genes that penetrate slowly into the cells. This is achieved by placing the chip in a cold environment and by chilling all solutions before use.

Solutions

5. The cell suspension can be supplemented with BSA (3–5%) to prevent cells from aggregating with each other and being trapped as clusters.
6. The pH of the electroporation buffer must be chosen as close as possible to the intracellular pH (e.g., pH 7.2). Similarly, the buffer composition should mimic the composition of the cytoplasm to avoid extensive cell swelling (that would easily lead to cell death).
7. The ionic strength of the buffer should be as low as possible. On the one hand, the ionic strength determines the resistivity of the solution, and subsequently the time constant (RC) of the electroporation process (29). On the other hand, a high ionic content would favor arcing phenomena at the electrodes, which can affect cell viability. The presence of small ions such as Ca^{2+} and Mg^{2+} also promote cell recovery after the electroporation treatment (29).
8. The DNA solution concentration must be adjusted to enhance cell transfection (29).

Electrodes

9. If electrodes are integrated in the chip, the biocompatibility of the materials used to fabricate them must be known. Electrodes are prone to release ions and particles which may be toxic to cells (30).
10. The position of the electrodes must be wisely chosen. When the pulse amplitude is too high, a too small interelectrode distance often leads to bubble formation (through the electrolysis of water), a change in the pH and the production of chemicals (e.g., ROS) which are toxic to cells (3). Subsequently, the addition of a constricted area appears as a more judicious approach to locally create a high electric field.
11. Electrodes suffer from corrosion, especially in case of electrochemical reactions. For repeated use, they can be coated with an insulating layer such as Teflon (31). However, this coating influences the electric field distribution.

Electrical Parameters

12. Mostly, square DC pulses (ns to μs) or exponentially decay pulse (ms) are employed for cell poration. Alternatively, using AC signals, risks for water electrolysis are decreased (32), the signal amplitude is lowered (33) and higher cell transformation yields have been reported (29).
13. The pulse parameters must be optimized depending on the application. Drug delivery requires short and high pulses while DNA transfection is enhanced using lower and shorter pulses, which promote DNA penetration in cells (34). Furthermore, when larger molecules or particles have to be injected in the cells, the pulse amplitude must be increased to create larger pores (35).
14. Similarly, the pulse parameters must be adjusted as a function of the cell type and a number of cell parameters (shape, size, and membrane composition) (36). This is particularly relevant for bacteria and plant cells which possess a different shape and membrane properties than mammalian cells. However, if the cells are truly trapped in a microhole, their size and shape should have little influence on the electroporation outcome.
15. The distribution of the electric field in the device must be carefully studied and modeled to determine the electric field strength across the cells.

Trapping Structures and Trapping Protocol

16. The electrical signal required for cell electroporation correlates with the size of the trapping site; the smaller the site, the higher the electric field across the trap, and so the lower the voltage applied. As already mentioned, one can benefit from this focusing effect when the interelectrode spacing is increased to alleviate unwanted electrochemical reactions.
17. The size of the trapping structures must be adapted for single cell trapping. A too large site leads to multiple cell trapping, and this affects the electroporation treatment. Conversely, cells are not tightly trapped in too small holes, and they can be easily released. Consequently, a single cell electroporation approach is difficult to be implemented for bacteria which have a size of a few microns, as holes in the submicrometer range are required to trap them.
18. Cell squeezing through the trap is observed when the trapping site is too large, or if the suction pressure is too high.
19. The trap size and the suction protocol determine the sealing quality of the cell in the trapping site. In case of a bad sealing, a leakage pathway exists so that a higher poration signal must be applied. Conversely, with a good sealing, the electrical treatment is milder. Furthermore, in this latter case, the cell poration voltage does no longer depend on the cell size and shape; only their membrane composition and their "fragility" influence the poration process.

Monitoring of Pore Formation: Fluorescent or Electrical Approach?

20. Pore formation is mostly monitored using fluorescence microscopy techniques through the release/entry of dyes out of/into the cells. However, in the former case, care should be taken to calibrate the photobleaching behavior of the dyes (negative control) and to also determine the fluorescence level in the solution in the close vicinity of the cells before concluding on cell poration.
21. Fluorescence-based assays can also rely on other probes. For instance, Dextran particles coupled to a fluorophore (FITC) which are available with different sizes (or molecular weights) are employed to probe the size of the pores created in the membrane as a function of the strength of the poration signal (35).
22. In general, fluorescence-based assays are invasive as they imply loading of the cells with fluorescent probes (before or upon cell poration). Alternatively, electrical measurements (patch-clamp recording) are performed to detect pore formation, which results in changes in the cell impedance (37); the latter employs the same electrodes for the electroporation and measurements. This electrical approach is particularly interesting to automate gene transfection processes through real-time monitoring of the electroporation process (20). Furthermore, using this approach, pores which are too small to enable the transport of dye molecules through the cell membrane can be detected.
23. However, for electrical-based detection of pore formation, a good sealing of the cell in the trapping structure ("giga-ohm seal") is mandatory as the presence of a leakage current precludes the detection of pore formation (21).

Gene Transfection Application

24. As stated in the methods, it is essential to incubate on chip the cells in the DNA solution both before and after application of the electroporation signal. In the first case, DNA comes in close vicinity to the cell and has a greater chance to be pulled into the cells upon application of the electrical signal. The role of the second incubation time is to give enough time to the DNA to fully penetrate into the cells.
25. Alternatively, DNA entry in the cells can be promoted with the application of a second lower and longer DC voltage (38), establishing thereby an electroosmotic flow which pulls the negatively charged plasmids into the cells.
26. For some cells, the transfection yield is limited as plasmids injected into the cells by electroporation do not reach the nucleus. In this case, a second series of shorter pulses (ns-range) can be applied to porate the membrane of the nucleus and enhance DNA delivery to the nucleus (39).

Alternative Single Cell Electroporation Approaches

27. The single cell electroporation approach described in this chapter is limited in terms of volume of cells transfected. The current device contains nine trapping sites, and more generally, this approach is limited by the number of electrodes that can be integrated in a single device. Therefore, two other approaches are preferred for treatment of larger amounts of cells, either using a single microhole, where cells are sequentially trapped, porated, and released (19), or using a flow-through device that contains a short constriction through which cells flow as a single-cell line, while a DC voltage is applied in the whole channel (23). However, these two approaches are not compatible with postelectroporation single cell tracking.
28. As mentioned in the introduction, single cell electroporation has also been demonstrated using two microelectrodes positioned close to the cell (14). In this case, one of the electrodes can be replaced by a pipette filled with the solution to be delivered in the cell for localized and subcellular treatment of cells (15).
29. Subcellular treatment has been achieved also using a nanoporous membrane on which cells are grown (40). The electric field is focused through the nanopores, possibly on specific areas in the cell membrane. Furthermore and interestingly, this protocol is applicable to adherent cells directly in their natural environment. Here, cells are grown as a monolayer on the large electrode and a counter-electrode (micropipette, microelectrode) is brought in close contact with the cells from the solution side for localized poration of adherent cells. However, this strategy resembles the microelectrode-based approach and is limited in terms of throughput.

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