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Design and Implementation of a Microelectrode Assembly for Use on Noncontact In Situ Electroporation of Adherent Cells

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Abstract In situ electroporation of adherent cells provides significant advantages with respect to electroporation systems for suspension cells, such as causing minimal stress to cultured cells and simplifying and saving several steps within the process. In this study, a new electrode assembly design is shown and applied to in situ electroporate adherent cell lines growing in standard multiwell plates. We designed an interdigitated array of electrodes patterned on copper with printed circuit board technology and covered with nickel/gold. Small interelectrode distances were used to achieve effective electroporation with low voltages. Epoxy-based microseparators were constructed to avoid direct contact with the cells and to create more uniform electric fields. The device was successful in the electropermeabilization of two different adherent cell lines, C2C12 and HEK 293, as assessed by the intracellular delivery of the fluorescent dextran FD20S. Additionally, as a collateral effect, we observed cell electrofusion in HEK 293 cells, thus making this device also useful for performing cell fusion. In summary, we show the effectiveness of this minimally invasive device for electroporation of adherent cells cultured in standard multiwell plates. The cheap technologies used in the fabrication process of the electrode assembly indicate potential use as a low-cost, disposable device.

Keywords In situ electroporation · Noncontact · Microelectrode · Adherent cell

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Introduction

Nowadays, electroporation, also known as "electropermeabilization," is a useful technique to introduce foreign impermeable material into the cell cytoplasm. A state of high permeability to ions and macromolecules is achieved by exposing cell membranes to short (microsecond–millisecond) high-electric field pulses (Neumann et al. 1982; Weaver and Chizmadzhev 1996; Teissie et al. 2005). This state can be either temporary (reversible electroporation) or permanent (irreversible electroporation) as a function of the electric field parameters (Wolf et al. 1994; Rols and Teissié 1998; Hui 1995; Lebar et al. 2002). Typical reversible applications comprise drug delivery (Dev et al. 2000), gene therapy (Wolf et al. 1994; Zheng and Chang 1991; Stopper et al. 1987) and introduction of fluorescent probes used in research and functional proteomic treatment (Lambert et al. 1990) as the main representative examples.

The technique can be applied to a wide spectrum of biological preparations, ranging from single cells (Wang et al. 2010) up to whole tissues such as liver, lung and muscle (Dev et al. 2000) and both in vitro and in vivo. Traditional in vitro equipment performs electroporation in cuvettes where cells are suspended in order to apply electric field pulses (Raptis and Firth 2008). Particularly, when adherent cells are electroporated a previous trypsinization process needs to be carried out. However, trypsinizing adherent cells causes an additional stress to the cells that

may affect both the electroporation efficiency and the invasiveness of the operation (Zheng and Chang 1991). As explained in Chang et al. (1992), there are several reasons to believe that in situ electroporation is more suitable for high-efficacy transfection in adherent cells maintaining a reasonable viability.

Some microfabricated devices have been designed to apply electric field pulses directly to the adherent cell monolayer, where cells commonly grow onto the microelectrode surface (Raptis and Firth 2008; Olbrich et al. 2008; Wegener et al. 2002). Some approaches make use of interdigitated microelectrodes deposited into planar glass surfaces with microfabrication techniques to apply the pulses (Huang et al. 2011; Lin et al. 2003). These devices allow lowering of the required voltages to reach the high-intensity electric fields (1–10 kV/cm) due to the small interelectrode distance. For example, in Lin et al. (2003) an electroporation microchip successfully transfected adherent cells using <2 V. The reduction in the required amplitude to create membrane poration reduces the complexity and cost of pulse generators used in traditional systems as well as the requirements for electrical safety of the devices. There are also reports of many single-cell devices that introduce silicon fabrication technology (Braeken et al. 2010) or microfluidics (Geng et al. 2010). However, all the devices described above are custom-built setups and are not suitable for direct use in standard multiwell plates. In addition, in most of them, cells are not attached to a standard cell growing surface; on the contrary, they are in contact with the electrodes or other nonstandard surfaces that may interfere with normal development. Some other commercially available devices have been designed to apply electroporation pulses to cells growing in standard culture plates; these approaches make use of big, flat or wire electrodes positioned above the monolayer very similarly to a cell suspension with relatively high electrode distances (Deora et al. 2007; Raptis and Firth 2008).

In the present report a new electroporation device is described. The electrode assembly proposed was initially designed to monitor the state of cell monolayers with a minimally invasive method by means of electrical bioimpedance spectroscopic measurements. We show the functionality of this electrode system also for in situ electroporation of C2C12 and HEK 293 adherent cells cultured in standard multiwell plates, making use of low voltages.

Materials and Methods

Electrode Assembly

The electrodes were conceived and designed taking into account the principle of in situ use with adherent cell monolayers growing in standard multiwell plates. The main goal was to reduce the invasiveness of the operation in order not to interfere in the regular behavior of cultured cells.

The electrode geometric design was based on an interdigitated structure consisting of six independent lines forming three arrays of electrodes. This design enables bioimpedance measurements in different configurations and, in the case of electroporation, configures three active areas. Each line in the active pairs connected alternately to +V or −V terminals of the pulse generator. Some different designs were tested using different electrode width and spacing (see Fig. 1). In this study the final dimensions were 75 and 150 μm width and spacing, respectively. Electrodes were patterned on copper with printed circuit board technology using as substrate 1-mm-thick FR4 discs with diameter compatible with the dimensions of standard 24-multiwell plates. Due to the toxicity of copper (Cu), once the electrode structure was patterned, a final nickel (Ni)/hold (Au) plating was deposited using the electroless nickel immersion gold technique.

Following the idea of in situ application to cell monolayers, small microseparations were created, to avoid direct contact between the electrodes and the monolayer which could cause mechanical stress or damage to the cells. Noncontact electroporation allows us to minimize the invasiveness of the operation. These separations were constructed using a final photosensible epoxy layer with thickness of 10 μm deposited on the border areas of the surface of the electrodes. Six circular microseparators were patterned on the surface of the electrodes that were equally distributed along the perimeter of the discs. The thickness of cell monolayers is usually 3–8 μm in most cell lines attached to a surface (Durante et al. 1993; Bettega et al. 1998); consequently, 10 μm is enough to avoid direct

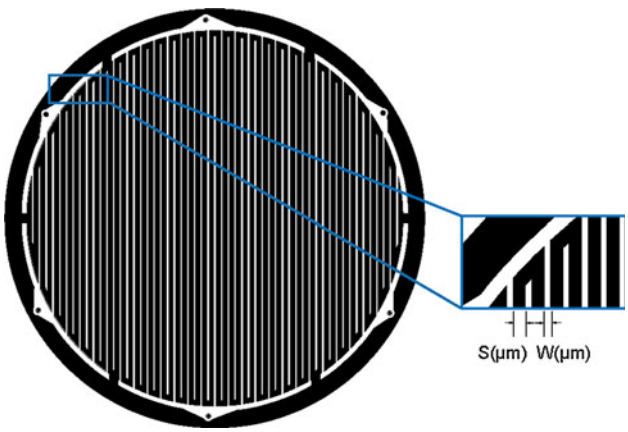

Fig. 1 Interdigitated geometry of the electrodes and detailed view. Six different *lines* are connected to the stimulator terminals. Different separations between electrodes (*S*) and different electrode widths (*W*) were tested

contact with the electrodes. Figure 2a depicts the in situ placement of the electrode assembly above the bottom surface of a culture well, and Fig. 2b is a detailed view of the microseparations described above.

In addition, considerably higher and irregular electric fields are presented in the border areas of microelectrodes that could irreversibly damage cell membranes near these areas. More uniform electric fields are created a short distance from the electrode surface (Lin et al. 2003; Lisen et al. 2007). A 2D simplification of the structure was simulated to study the electric field distribution. Simulations were made using commercial software (COMSOL Multiphysics 3.5; COMSOL, Burlington, MA). The conductivity values used for the different parts were $\sigma = 0.004$ S/m for the FR4 substrate, $\sigma = 5.998e^{7}$ S/m for copper electrodes and $\sigma = 0.16$ S/m for poration media. Boundary conditions in the electrodes were set to fixed current ports or ground alternatively. In Fig. 3a the current density distribution is shown. As pointed out above, more uniform distribution is present a short distance from the surface of the electrodes. In Fig. 3b and c cross-sectional plots are shown. Figure 3b depicts the electric field values along a horizontal line separated 15 μm from the electrodes. From this plot it can be stated that cells under the center of the electrodes will not be electroporated because the electric field intensity in these areas will not be high enough. On the other hand, the effect in the areas where the electric field is over the threshold will be very uniform, only 4 % of variation as indicated in the plot. In Fig. 3c the electric field values along a vertical line are plotted. As shown in the figure, there is an electric field reduction in the vertical direction with a maximum decrease of 10 % between the highest and lowest points of the line. From these simulations it can be concluded that the use of microseparations has the advantage of applying more uniform electric fields but that, due to the relative distance between the electrodes and the cell surface, higher voltages need to be applied to obtain suitable electric field intensities for electroporation.

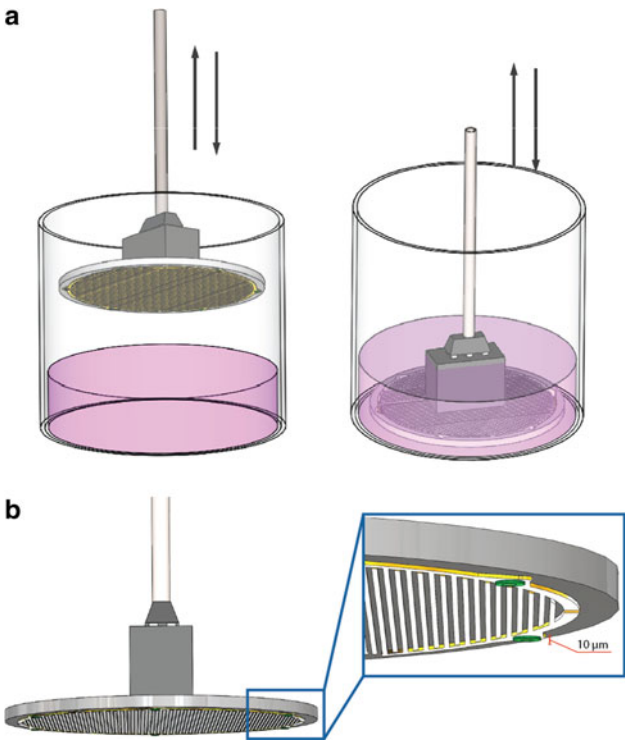

Fig. 2 Illustration of the electrode setup. **a** Representation of the principle of operation, showing how electrodes are placed in the bottom surface of the multiwell plates. **b** Detailed view of the microseparations used to avoid contact between electrodes and cells

Once the microelectrodes were fabricated and tested, the next step was to adapt them for in situ application. A biocompatible acrylic adhesive (LOCTITE 3555™; Henkel, Dusseldorf, Germany) was used to seal the soldering areas between the pads and the connector. Connection wires were introduced in a biomedical silicone tube whose flexibility allowed the discs to be settled uniformly parallel to the bottom surface of the multiwell plates when the assembly was leaned against the surface, and consequently, the electrodes were uniformly parallel to the cell monolayer. The electrode assembly is automatically positioned using a self-constructed positioner which ensures that the same vertical force was applied in all experiments. In addition, using this automatic system, the displacement speed can be controlled, ensuring that with slow enough speed fluid displacement does not harm the monolayer. This electrode assembly is in the patent process.

Electric Field Pulse Delivery

Electric field pulses were delivered using a biphasic stimulator developed in our laboratory and first conceived for long-term contraction of cultured muscle cells. The stimulator generates bipolar pulses acting as a fixed current source with intensities (A) ranging from 1 to 800 mA, minimal duration (D) of each part of the bipolar signal of 100 μs and minimal period (T) of 1 ms. There is an additional parameter (T_t) that allows one to set the time separation between the positive and negative parts of the pulse (minimum value 100 μs). The device is fully programmable by an RS-232 connection to a PC. As previously studied by other authors, the use of bipolar pulses enhances transfection efficiency (De Vuyst et al. 2008; Ephrem Tekle and Bonn Chock 1991) and reduces electrolytic gas bubble formation in metal electrodes (Ziv et al. 2009).

Cells and Chemicals

The C2C12 mouse myogenic cell line and the HEK 293 human embryonic kidney cell line were cultured as a

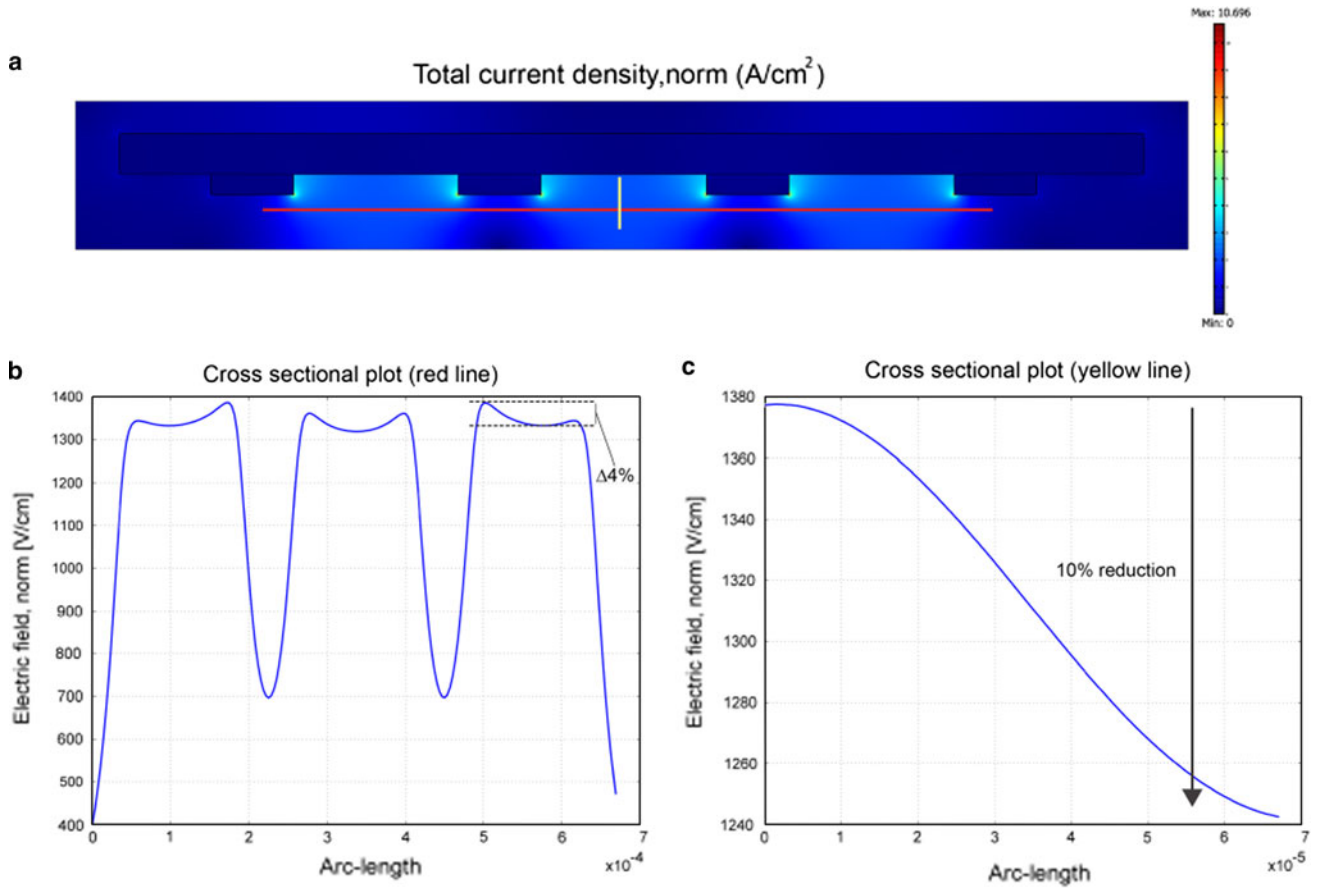

Fig. 3 Electric field simulations run on COMSOL Multiphysics 3.5. **a** The 2D simplification simulated is shown and the total current density is plotted. **b** A cross-sectional *plot* of the electric field distribution along a *horizontal line* 15 μm from the surface of the electrodes (*red line* in **a**). **c** A cross-sectional *plot* of the electric field distribution along a vertical line (*yellow line* in **a**) (Color figure online)

monolayer in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum and supplemented with penicillin, streptomycin and fungizone.

Low-conductivity electroporation buffer (LCEB) was used in the experiments. LCEB consisted of 10 mM Na_2HPO_4 (pH 7.4), 1 mM $MgCl_2$ and 250 mM sucrose. Conductivity was 1.6 mS/cm. When started, 2.5 mg/ml fluorescein isothiocyanate-dextran, average molecular weight 20,000 Da (FD20S; Sigma-Aldrich, Madrid, Spain) was added to the LCEB as a fluorescence electropermeabilization probe.

Electroporation Procedure

Both C2C12 and HEK 293 cell lines were plated in 24-multiwell plates at cell concentrations of 5×10^4/well and 7×10^4/well, respectively. Plates were cultured at 37 °C in a humidified 5 % CO_2 incubator for approximately 24 h, to reach 50–60 % confluence.

Before application of electric pulses, cells were rinsed with phosphate-buffered saline (PBS); and then 150 μl of LCEB were added to each well. The electrode assembly was sterilized with a 70 % ethanol solution, rinsed with sterile distilled water and finally immersed in LCEB before use. Immediately, the electrode assembly was placed above the cell monolayer and electric field pulses were applied, configuring the biphasic stimulator via RS-232. Eight biphasic pulses, with duration (D) of 100 μs, time separation between the positive and negative parts of the pulse (T_t) of 100 μs and frequency repetition of 1 Hz were applied with different current intensities (A) to create electric fields of 1.2, 1.6, 2 and 2.2 kV/cm in the surface of the electrodes, taking into consideration that the electric field affecting cell membranes is lowered about 10 % because of the relative distance between electrodes and cells. In control cells, no electric pulses were applied, but the electrode was positioned above the cell monolayer for an equivalent period of time. After the electroporation procedure, cells were incubated for an additional 30-min period in the incubator. After this period, the electroporation buffer was removed and cells were rinsed twice with PBS, medium was replaced with fresh culture medium and

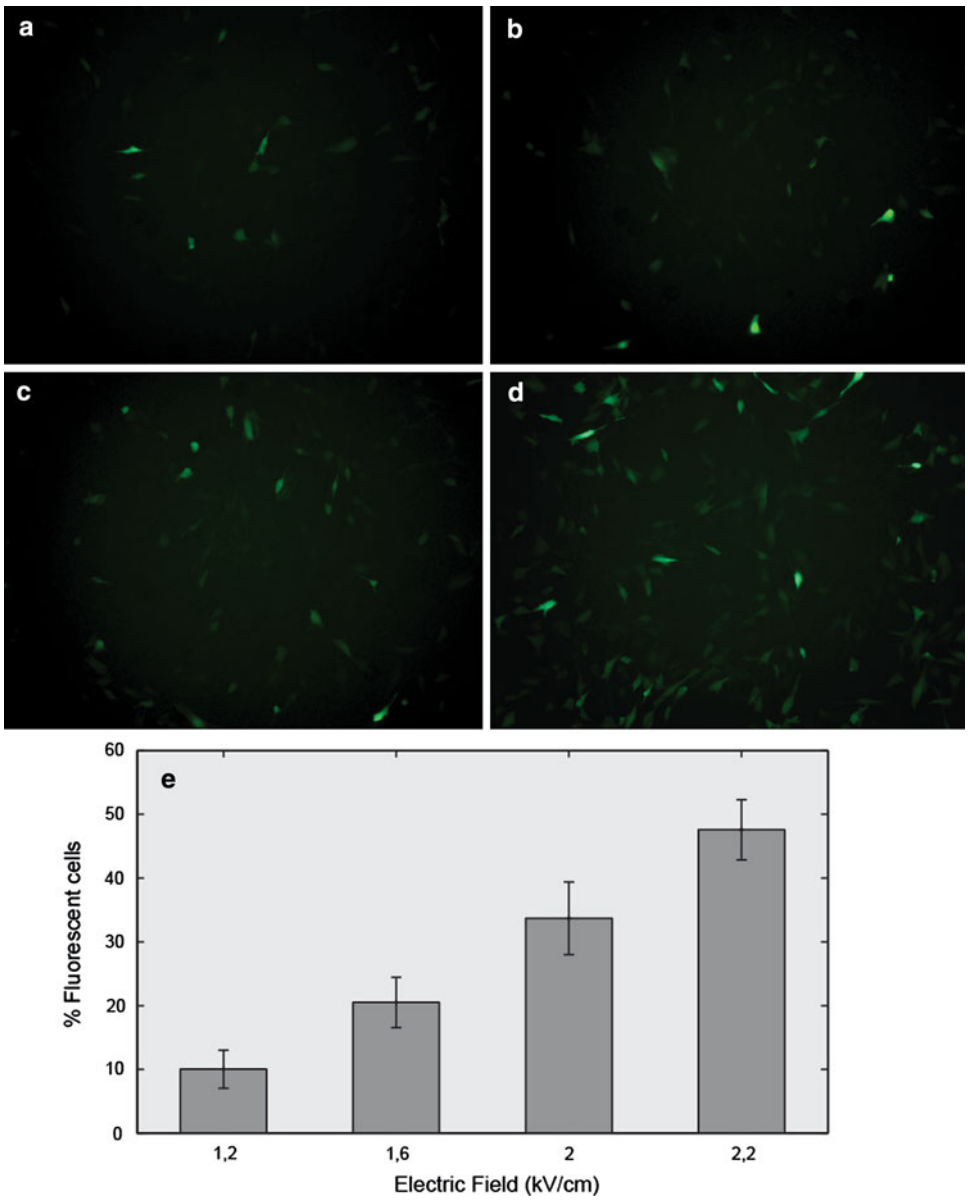

Fig. 4 *Micrograph* of C2C12 cells after electroporation. Cells were electroporated in the presence of FD20S at different electric field intensities: **a** 1.2 kV/cm, **b** 1.6 kV/cm, **c** 2 kV/cm and **d** 2.2 kV/cm. After cell electroporation and recovery, as described in "Materials and Methods" section, images of cell monolayers were taken at ×10 magnification. A representative *image* is shown. **e** Quantitative analysis of the results calculated as the percentage of fluorescent cells. Results are expressed in mean ± SD of at least five measurements

cells were left for 2 h in the incubator for complete resealing. Then, cells were examined under a Leica (Wetzlar, Germany) DMI 4000B inverted microscope for fluorescence, to detect FD20S, which has an excitation wavelength of 485 nm and emission at 510 nm. Images were taken with a digital camera (Leica DFC 300x).

Results and Discussion

The objective of this study was to test the use of an electrode assembly for in situ electropermeabilization of adherent cells growing in standard multiwell plates with minimal invasiveness of the operation. For that purpose two different cell lines, C2C12 myoblasts and HEK 293 epithelial cells, were subjected to electroporation with this system for intracellular delivery of fluorescent dextran FD20S, which has a molecular radius of 3.2 nm (Ambati et al. 2000). We first performed several tests, in which the duration and amplitude of pulses were varied, to find the optimal electric field parameters that caused effective reversible electroporation. Among the conditions tested for the bipolar pulses, we determined that a fixed, short-duration pulse of 100 μs avoided bubble gas formation caused by electrolysis of water. In addition, we observed that, due to the small distance between cells and electrodes, the cell monolayer detached when bubbles appeared, caused by both pH changes and mechanical stress of the bubbles themselves. In consequence, in the following experiments, we varied the amplitude of pulses at this fixed duration.

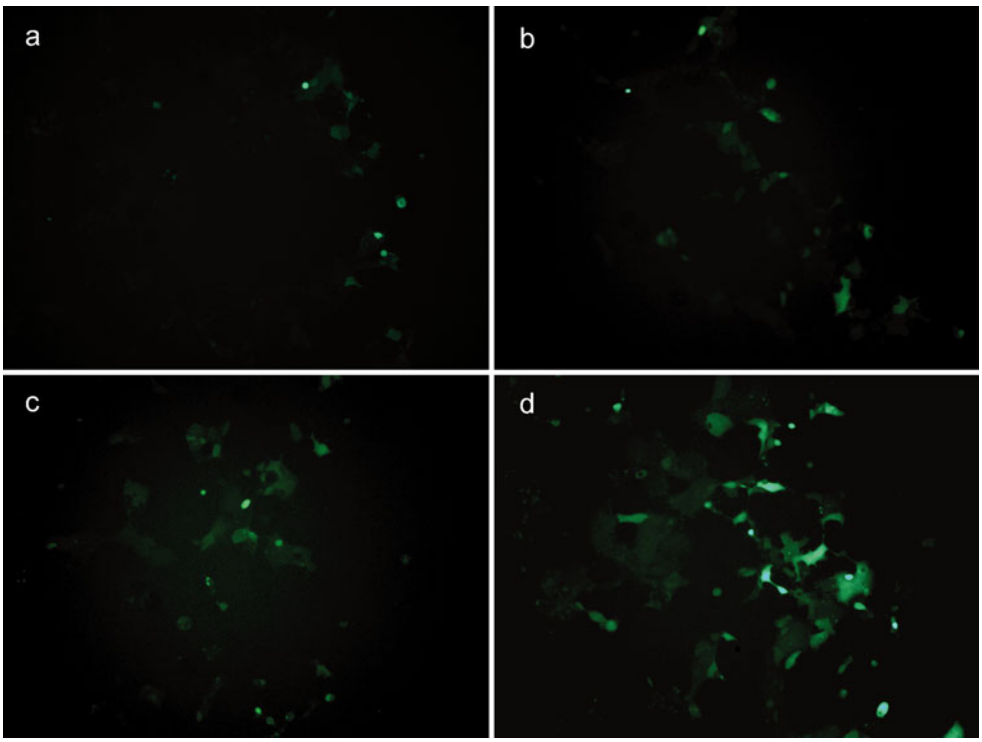

Fig. 5 *Micrograph* of HEK 293 cells after electroporation. Cells were electroporated in the presence of FD20S at different voltages: **a** 1.2 kV/cm, **b** 1.6 kV/cm, **c** 2 kV/cm and **d** 2.2 kV/cm. After cell electroporation and recovery, as described in "Materials and Methods" section, *images* of cell monolayers were taken at ×10 magnification. A representative *image* is shown

In Fig. 4, we show an electric field-dependent fluorescent label of C2C12 myoblasts. Very few fluorescent cells were observed after application of electric fields of 1.2 kV/cm (Fig. 4a). Data also reveal that the permeabilization yield increased with increasing electric field amplitude and reached a maximum when the electric field at the electrode surface was 2.2 kV/cm. Figure 4e shows the quantification of the results as a percentage of fluorescent cells calculated with respect to the total number of cells 2 h after application of the treatment. Thus, these results indicate uptake of FD20S into adhered C2C12 electroporated cells, with up to 50 % efficiency for the best case. C2C12 is a hard-to-transfect cell line with DNA plasmids and usually resistant to chemical methods. These results indicate the potential utility of electroporation as a feasible option when other methods are not suitable.

In Fig. 5 the effect of treatment of cell line HEK 293 with four different electric field amplitudes, 1.2, 1.6, 2 and 2.2 kV/cm, is shown. The micrographs also show, as expected, an increase in the number of fluorescent cells as a function of electric field intensity. On the other hand, in electroporated HEK 293 cells (see Fig. 5c, d) we observed giant cells, which suggests that cell fusion took place with the highest electric fields applied. As also known to those skilled in the field, the application of high electric field pulses can induce fusion of a wide variety of cells under certain conditions. One of the main conditions to achieve cell electrofusion is the establishment of contact between cell membranes. In our case, adherence of cells to the culture plate facilitated cell contact for fusion. Other methods classically forced cell–cell contact by dielectrophoresis, chemicals or centrifugation. The observation of cell fusion in HEK 293 cells but not in C2C12 cells may be due to the fact that the extent of cell electrofusion in vitro and in vivo is cell line-dependent and involves cell type-specific membrane properties and/or secretion of proteases (Salomskaite-Davalgiene et al. 2009). In contrast, in our study conditions for electropermeabilization of both adherent cell lines were very similar. Likewise, relatively little difference in electropermeabilization of plated CHO and B16F1 cells was reported, whereas significant differences were observed between the two cell lines in a suspension (Marjanovič et al. 2010).

In C2C12 or HEK 293 cells, we observed no alteration of the characteristics of the cell monolayer in the course of 24 h after positioning of electrodes in control cells (data not shown), indicating that the device does not cause a major mechanical stress or toxicity that affects cell viability.

Our observations reinforce the principle of minimal invasiveness of the device. An advantage of the system is its in situ application to multiwell plates where cells had

been initially grown, avoiding additional stress to the cell culture by trypsinization or other posttreatment steps.

Conclusions

In this study we tested a new electrode assembly design, based on the principle of in situ electroporation of adherent cell monolayers growing in standard multiwell plates. Using microelectrodes with small distances between adjacent lines, we achieved effective electroporation making use of low voltages. The use of microseparations between the electrodes and the bottom surface of the growing plates provided a significant advantage, avoiding contact with the cell monolayer, thus causing minimal stress to the cell culture. Furthermore, in situ treatment of cells simplified the process and saved several harvesting and processing steps, usually necessary in traditional systems, which may have contributed to improving the yield of the process.

This device was successful in introducing FD20S into two different cell lines with minimal invasiveness of the operation. The success in the electroporation of C2C12 cells, usually resistant to chemical gene transfer methods, suggests it may be valuable to deliver other macromolecules such as drugs, DNA or antibodies. Additionally, as a collateral effect, we observed electrofusion in HEK 293 cells, thus making this device also useful to induce cell fusion.

When compared to the available commercial electroporation equipment, this device has the advantages of simpler cell culturing and preparation processes because of the use of standard culturing plates. Additionally, the cheap technology used in the fabrication of the electrodes and the low voltages needed implies a significant reduction in costs both of the electrodes and of the pulse generator. Once the initial concept has been demonstrated to be feasible, future work will deal with the introduction of active molecules such as DNA plasmids, siRNAs and proteins, and several different cell lines will be tested.

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