

Recovery of Adherent Cells after In Situ Electroporation Monitored Electrically

BioTechniques 33:348-357 (August 2002)

Joachim Wegener, Charles R. Keese, and Ivar Giaever
Rensselaer Polytechnic
Institute, Troy, NY, USA

ABSTRACT

Here we describe various experiments that address the efficiency of loading extracellular probes into the cytoplasm of adherent mammalian cells (normal rat kidney, Madin-Darby canine kidney, and African green monkey) by means of in situ electroporation. Subsequent cell recovery from the electroporation pulse was monitored electrically in real time for each condition. In this study, small, gold-film electrodes ($5 \times 10^{-4} \text{ cm}^2$) are used as culture substrates and at the same time as an electrode for both the application of the electroporating voltage pulse and the noninvasive electrical monitoring of cell recovery, using a technique referred to as ECISTM. Electroporation has been performed by using ac sinusoidal voltage pulses of varying frequency, amplitude, and duration. Permeabilization and re-closure of the plasma membrane were evaluated by the uptake of the fluorescence probe, Lucifer Yellow, from the extracellular fluid. With the experimental setup described here, efficient electroporation was achieved with voltages less than 5 V. Using ECIS, we followed the morphological response of the cells to the electric field-induced membrane permeabilization. For optimized electroporation conditions, cell recovery was completed in less than 1 h. The introduction of membrane-impermeable substances by electroporation and in situ monitoring of the cellular response may find many applications in cell biology.

INTRODUCTION

The introduction of hydrophilic probes capable of molecular recognition (peptides, antibodies, and nucleic acids) into the cytoplasm of mammalian cells has become a major issue in many fields of cell biology and biomedical research. Typical and important examples are, for instance, the introduction of recombinant genes, gene silencer (e.g., RNAi), and many more within the huge field of functional proteomics. Thus, several techniques to introduce these probes into the cell interior have been developed over recent years, which all have their individual merits and limitations (7). In some cases, organic synthesis allows the generation of membrane-permeable analogs of a given bioactive species that is capable of entering the cytoplasm by simple diffusion. However, synthetic options are rather limited because of the need to preserve biological activity, and they are generally not applicable to biological macromolecules. The most direct but rather difficult approach to get hydrophilic macromolecules into the cells is microinjection. The compounds of interest are directly injected into the cytoplasm or even the nucleus by means of a microsyringe that is controlled by micromanipulators. However, it is a tedious and labor-intensive process to load large numbers of cells by microinjection, and this cannot be done simultaneously. Besides microinjection, fusion of loaded liposomes, or virus shells, another group of alternative techniques supplies the bathing fluid with the probes of interest and

then induces a short-term membrane permeabilization to allow the diffusion of these molecules from the extracellular into the intracellular compartment (7). Transient membrane permeabilization can be achieved chemically (16) by applying acoustical shock waves (sonoporation) (14), high-intensity laser pulses (optoinjection and optoporation) (23), or as most commonly applied, by the exposure of cells to strong electric fields for short time intervals (electroporation) (16,26).

Electroporation is commonly performed with suspended cells that are exposed to the porating electric field in a cuvette-like setup. However, trypsinizing adherent cells before electroporation causes an additional and very severe stress to the cells that may affect both the electroporation efficiency and the invasiveness of the operation (31). Moreover, the cellular properties that are addressed with the probes that have been introduced into the cytoplasm cannot be examined before the cells have been re-plated, have attached, and spread. These limitations can be overcome by the electroporation of adherent cells, and a few experimental approaches have been previously described (6,18,19,24,31). In one of these techniques, the cells are grown on conducting surfaces, and the substrate electrode is used to deliver the permeabilizing voltage pulse (6,18). We have applied this electroporation approach to cells that are adherently grown on small, gold-film electrodes that are commonly used for electric cell-substrate impedance sensing (ECISTM) (Applied Biophysics, Troy, NY, USA) and have thereby combined both techniques.

Research Report

ECIS is a noninvasive electric device that allows one to monitor the viability and morphology of adherent cells with a time resolution that can be reduced to a few seconds (1,9). The ECIS device reads the electrical impedance of the gold-film electrode with weak and non-invasive ac fields (9). When cells attach and spread on the electrode surface, they behave essentially like insulating particles that block the current flow from the electrode and thereby alter the electrode impedance. When a confluent cell layer is established on the ECIS electrodes, most of the current is forced to bypass the cells so that impedance readings as a function of time mirror the dynamics of cell shape and cell morphology, with a sensitivity that is beyond the resolution of an optical microscope (3). In recent years, ECIS has found many applications in cell biology related to following the behavior of mammalian cells in general (11,12,28) or the response of the cells to chemical, mechanical, or electric stimuli in partic-

ular (2,5,6,10,20,25,27,29).

By combining the technical requirements for the electroporation of adherent cells on a conductive substrate with the noninvasive monitoring of the associated changes in cell morphology by using ECIS, we could study the recovery of mammalian cells after in situ electroporation online and in a quantitative fashion. Whereas a lot of work has been done to optimize dc electroporation parameters with respect to the loading efficiency and viability of the cells (13,21,22), little is known about optimized electroporation conditions using ac voltage pulses (4,30). However, to minimize the electrochemical generation of reactive and cytotoxic molecular species at the electrode surface during pulse application, we made use of ac instead of dc voltage pulses. Pulsing parameters (frequency, amplitude, and pulse duration) were optimized for loading efficiency as probed by dye-uptake assays and the invasiveness of the operation.

MATERIALS AND METHODS

Instrumentation and Electrode Arrays

All electrical measurements in this study were based on the well-established ECIS device. The measurement system consists of an 8-well cell culture dish with electrodes deposited on the bottom of each well, a lock-in amplifier with an internal oscillator, relays to switch between the different wells, and a personal computer that controls the measurement and stores the data. The entire system is schematically depicted in Figure 1A. Each well of the 8-well electrode array contains a small working electrode (area = $5 \times 10^{-4} \text{ cm}^2$) and a large counter electrode (area = 0.15 cm^2). Because of the difference in surface area, the total impedance of this two-electrode system is governed by the impedance of the small electrode. The active electrode area of the small electrode is delineated by circular openings ($\varnothing = 250 \mu\text{m}$) in a photoresist overlayer that insulates the rest of the deposited gold film from the bulk electrolyte (magnified scheme in Figure 1A).

To acquire ECIS data, the oscillator applies an ac signal at a defined frequency and 1 V amplitude through a 1-M Ω load resistor to the electrodes that provide a constant current of roughly 1 μA for most frequencies (Figure 1A). The in-phase and out-of-phase voltages across the system are measured by the lock-in amplifier and converted to the complex impedance of the system or its resistance and reactance, respectively. To monitor the morphological response of the cells to the electroporation pulse as a function of time, we followed the impedance of the system at three frequencies, namely 400 Hz, 4 kHz, and 40 kHz (3f-ECIS mode).

To apply electroporation pulses at a given time of the experiment, ECIS data acquisition (3f-ECIS) was paused, and the load resistance was switched from 1 M Ω to 1 k Ω (Figure 1A). Subsequently, ac pulses of defined amplitude, frequency, and duration were applied to the selected electrodes. Figure 1D illustrates the time course of an ac voltage pulse of 40 kHz frequency (25- μs period), 3 V amplitude, and 100 μs duration, as it was used for electropora-

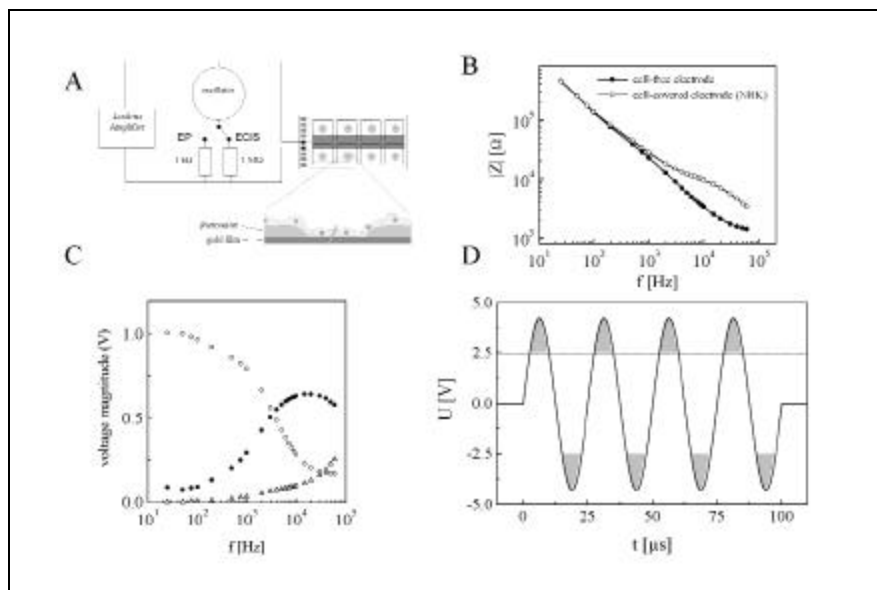


Figure 1. Design and characteristics of the ECIS-electroporation setup. (A) Schematic of the experimental setup to perform noninvasive ECIS measurements with the option to electroporate the cells on the electrode; the series resistance of 1 M Ω (ECIS) is switched to 1 k Ω for electroporation. Details are given in the Materials and Methods section. (B) Frequency-dependent impedance of an ECIS electrode covered with a confluent layer of NRK cells (open circles) compared to the impedance spectrum of the identical but cell-free electrode (filled circles). (C) Frequency-dependent voltage drop across the cell layer (filled circles), the electrode/electrolyte interface (open circles), and the bulk electrolyte (up triangles) when a voltage of 1 V amplitude is applied to the entire system. (D) Time profile of an ac voltage pulse, as typically applied for electroporation. The frequency is set to 40 kHz, the amplitude is set to 3 V, and the pulse duration is set to 100 μs . Electroporation pulses were typically applied for several hundred milliseconds. In this scheme, the duration of the pulse is limited to 100 μs for simplicity. Gray shadings indicate those fractions of the voltage profile in which the voltage magnitude exceeds a certain threshold value that is set to 2.5 V. EP, electroporation.

Research Report

tion in this study (the pulse duration in this graph was limited to 100 μ s for simplicity). Immediately after electroporation, the load resistance was returned to 1 M Ω , and the ECIS data acquisition was continued as before. Switching the load resistance before electroporation became necessary to induce a sufficiently strong electric field across the cell layer, with the output of the oscillator being limited to ac voltages of 5 V amplitude. Due to the small size of the active ECIS electrode, the electric field only extends over a small distance, which allows the generation of electric fields sufficient for electroporation with such low voltages. Because the time of the experiment was kept running even while the ECIS readings were paused, the electroporation pulse was integrated into each experiment in a well-defined manner.

Cell Culture

This study comprises experiments with normal rat kidney (NRK) cells, African green monkey cells (BSC-1), and strains I and II of the epithelial cell line Madin-Darby canine kidney (MDCK). All cells were maintained in a humidified cell-culture incubator [5% (v/v) CO₂] at 37°C and treated identically (unless noted otherwise). We used DMEM with 1 g/L glucose (Sigma, St. Louis, MO, USA) as basal medium, supplemented with 10% FCS (Sigma) and 50 μ g/mL gentamicin. The medium was exchanged every three days. The routine subculturing of confluent cell layers was performed using standard trypsinization techniques [0.25% (w/v) trypsin plus 1 mM EDTA].

Electroporation and Dye-Uptake Studies

To probe electroporation efficiency, we applied defined voltage pulses to confluent cell layers in the presence of the fluorescence probe Lucifer Yellow (4.5 mM). The associated dye uptake into the cytoplasm was evaluated by subsequent fluorescence microscopy. The establishment of a confluent cell layer before an electroporation experiment was routinely monitored by ECIS measurements and verified by phase contrast microscopy. Before electropo-

ration, the confluent cell layers on the ECIS electrodes were incubated with Earle's balanced salt solution (EBSS) (used here without phenol red or calcium, but with 0.9 mM MgCl₂) supplemented with 2 mg/mL Lucifer Yellow. After the cells had been re-equilibrated to incubator conditions, which was followed online by ECIS readings, the ac electroporation pulse of the defined frequency, amplitude, and duration was applied to the cell-covered electrodes. The cells were left in the incubator for an additional 10 min and were then thoroughly washed three times with EBSS. The chamber of the electrode array was then removed, and the cells were inspected and photographed using an epifluorescence microscope (Alphaphot2 with episcopic fluorescence attachment; Nikon, Melville, NY, USA). The color micrographs were digitized and are presented here as grayscale images.

Electroporation and Morphological-Response Studies

To address the morphological response of the cells after exposure to an electroporation pulse, we pretreated the cells as described previously, except that during the experiment, the cells were incubated in EBSS without Lucifer Yellow. After the equilibration to incubator conditions (followed by ECIS), data acquisition was paused, the defined ac electroporation pulse was

applied, and the instrument was then immediately switched back to follow the cell response in the 3f-ECIS mode. It is important to emphasize that all electroporation experiments were performed entirely under sterile conditions at 37°C in a 5% CO₂ atmosphere, using buffered salt solutions with an ionic composition that is routinely used in tissue culture (no ionic substitution was employed to decrease conductivity).

RESULTS AND DISCUSSION

Electroporation and Dye-Uptake Efficiency

The electroporation of adherent cells using a conductive growth substrate to apply the electric field is a rather new approach to introduce membrane-impermeable molecules from the extracellular fluid into the cytoplasm (6,18,19). With the deposited gold-films used here being close to an ideally polarizable electrode whose interface impedance is dominated by its capacitive properties, it seemed reasonable to use ac instead of dc pulses to achieve electroporation without both, major impairments of the electrodes and generation of reactive chemical species. Thus, we first addressed the impact of the various pulsing parameters of the ac pulses, including frequency, amplitude, and duration on electroporation efficiency. The latter was experimentally probed by the cellu-

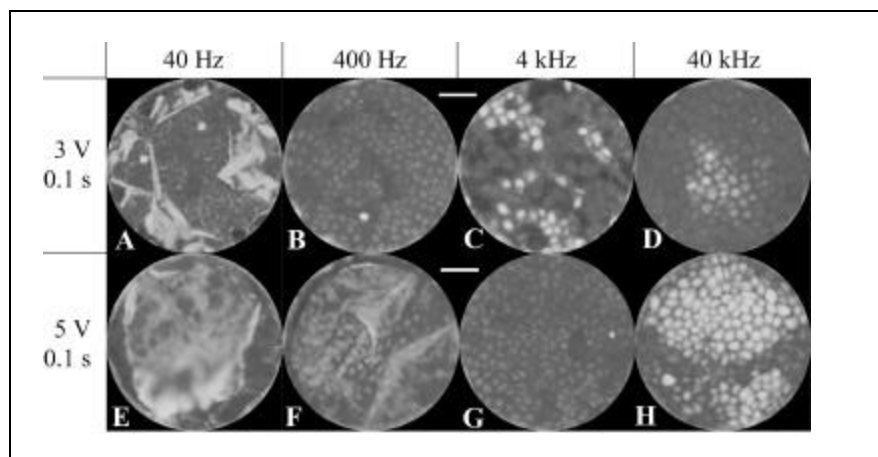


Figure 2. Uptake of Lucifer Yellow (2 mg/mL) for two different voltages as a function of frequency upon electroporation of confluent NRK cells. The frequency, amplitude, and duration of the electroporation pulses were as follows: (A) 40 Hz, 3 V, 100 ms; (B) 400 Hz, 3 V, 100 ms; (C) 4 kHz, 3 V, 100 ms; (D) 40 kHz, 3 V, 100 ms; (E) 40 Hz, 5 V, 100 ms; (F) 400 Hz, 5 V, 100 ms; (G) 4 kHz, 5 V, 100 ms; and (H) 40 kHz, 5 V, 100 ms. The scale bar represents 50 μ m.

lar uptake of Lucifer Yellow—a low molecular weight (457 Da), membrane-impermeable fluorophore with high quantum yield—during electrically induced membrane poration.

Variation of the Frequency of the ac Pulse

Figure 2 shows fluorescence micrographs of ECIS electrodes covered with confluent layers of NRK cells after the electroporation pulses had been applied with Lucifer Yellow in the extracellular fluid. Cells shown in the upper panel (Figure 2, A–D) were exposed to voltage pulses of 100 ms duration and 3 V amplitude [the given amplitude (rms) corresponds to the total voltage delivered to the system and is not the voltage that actually drops across the cell layer]. The cells in the lower panel also experienced pulses of 100 ms duration but with 5 V amplitude (Figure 2, E–H). In both panels, the frequency of the ac pulse was increased from left to right as indicated in the figure. It is apparent from panels E and F that the gold-film electrodes cracked for amplitudes of 5 V when the frequency of the pulse was set to 40 (Figure 2E) or 400 Hz (Figure 2F). When pulses of 3 V amplitude (upper panel) were applied, the electrodes again cracked for the lowest pulsing frequency of 40 (Figure 2A) but not 400 Hz (Figure 2B). In all other cases, the gold film remained intact, but only the cells pulsed at the highest frequen-

cy of 40 kHz and an amplitude of 5 V (Figure 2H) show a clear dye uptake in more than 50% of the cells. These findings become plausible when the frequency-dependent impedance of the ECIS electrodes covered with a confluent layer of NRK cells is inspected in closer detail. Figure 1B compares the impedance spectrum of a cell-covered ECIS electrode (open circles) to that of the same but cell-free electrode (filled circles). Apparently, the cell layer only contributes significantly to the overall impedance of the system above a threshold frequency of roughly 1 kHz. Below 1 kHz, the impedance of the system is dominated by the impedance of the electrode/electrolyte interface, regardless of whether the cells are attached to the electrode surface or not. From the impedance readings of both cell-covered and cell-free electrodes and the total voltage applied to the system, it is possible to calculate the voltage that actually drops across the cell layer (4). Figure 1C presents the individual voltage drops across the cell layer (filled circles), the electrode/electrolyte interface (open circles), and the bulk electrolyte (triangles) along the entire frequency range when a total voltage of 1 V is delivered to the system at each particular frequency. Figure 1C shows that the predominant fraction of the total voltage is actually delivered to the cell layer only for sufficiently high frequencies, whereas in the low-frequency regime, the voltage drops al-

most exclusively across the electrode/electrolyte interface. Thus, only for ac pulses with frequencies higher than 10 kHz do the cells experience a significant fraction of the applied voltage, which apparently creates a sufficiently strong electric field to induce membrane permeabilization. For lower frequencies, the applied voltage is mainly delivered to the electrode/electrolyte interface and induces electrochemical reactions that eventually lead to electrode rupture. Thus, these rather simple considerations explain the experimental observations that efficient electroporation is only achieved with high-frequency ac voltages. It is important to note in this context that the threshold frequency above which efficient electroporation can be achieved is dependent on the electrical properties of the cells. The curve for the cell-covered electrode as shown in Figure 1B has a somewhat different shape when, for instance, epithelial cells with tight intercellular junctions are cultured on the ECIS electrodes. Nevertheless, the cell-type-specific optimum of the pulsing frequency can be conveniently inferred from impedance spectra of the cell-covered and the cell-free electrode.

The frequency dependency of the voltage drop that is imposed on the adherent cells by the externally applied electric field was simply derived from the measured electrical properties of the different constituents of the system (cell layer, bulk electrolyte, and electrode/electrolyte interface). Holzapfel et al. (8) and most recently Gimsa and Wachner (4) have calculated the induced membrane potential difference as a function of frequency for cells exposed to external ac fields. These calculations reveal that for ac fields with frequencies higher than a few hundred kilohertz, the voltage drop that is induced across the cellular plasma membranes may strongly decline and even vanish (4). Thus, under these high-frequency conditions, electroporation may no longer be possible, even with high-voltage pulses.

Variation of the Pulse Amplitude

Figure 3 shows fluorescence micrographs of confluent NRK cells after an electroporation/dye-uptake assay, in

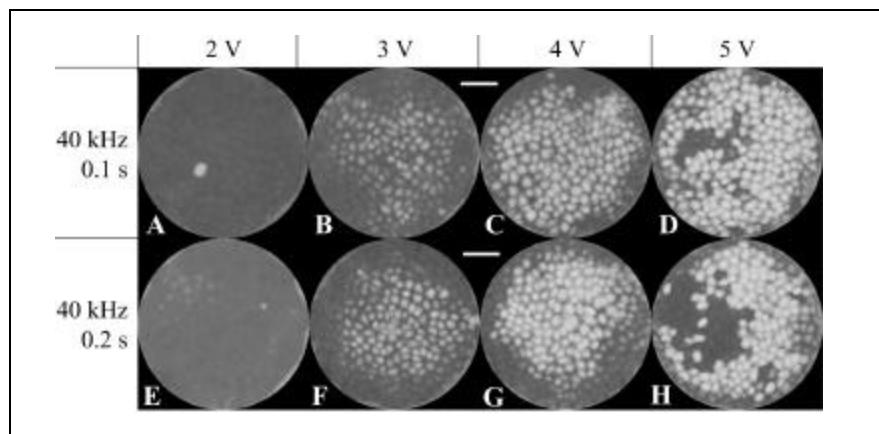


Figure 3. Uptake of Lucifer Yellow (2 mg/mL) for two different pulse durations as a function of applied voltage upon electroporation of NRK cells. The frequency, amplitude, and duration of the electroporation pulses were as follows: (A) 40 kHz, 2 V, 100 ms; (B) 40 kHz, 3 V, 100 ms; (C) 40 kHz, 4 V, 100 ms; (D) 40 kHz, 5 V, 100 ms; (E) 40 kHz, 2 V, 200 ms; (F) 40 kHz, 3 V, 200 ms; (G) 40 kHz, 4 V, 200 ms; and (H) 40 kHz, 5 V, 200 ms. The scale bar represents 50 μm .

Research Report

which the influence of increasing pulse amplitudes was studied. According to the considerations discussed earlier, the pulse frequency was set to 40 kHz, and pulse durations of 100 ms (Figure 3, A–D) and 200 ms (Figure 3, E–H) were employed while the voltage amplitude was varied. Following micrographs A–D, it is obvious that the number of stained cells increases with increasing voltage amplitude. Grayscale analysis of Figure 3D reveals that for the pulsing conditions applied, more than 85% of the cells on the electrode have taken up the fluorophore. The brightness of cells, indicative for the amount of fluorophore that has been incorporated, also increases with increasing amplitude. However, note that when we used the highest amplitude of 5 V (Figure 3D), some cells close to the center of the electrode are almost unstained, although their direct neighbors show bright fluorescence. The same phenomenon is apparent in the lower panel that shows NRK cells electroporated with the same amplitudes as in the upper panel, but with 200 ms pulse duration. The number of stained cells and their individual brightness increases with increasing voltage up to 4 V amplitude (Figure 3, E–G). When the highest amplitude of 5 V was applied (Figure 3H), a considerable spot of unstained cells was visible. Comparing Figure 3, D and H, further reveals that the number of unstained cells increases when the pulse duration is doubled from 100 to 200 ms. Thus, we conclude that unstained cells within both populations were irreversibly damaged by the electroporation pulse and could not hold the fluorescence dye in the cytoplasm during the washing cycles that preceded microscopic inspection. Phase-contrast micrographs (data not shown) confirmed this conclusion because the cells that were unstained by the fluorophore appear irregular in morphology. Thus, the dependence of electroporation efficiency on the voltage amplitude shows, as expected, a maximum beyond which membrane permeabilization is no longer reversible.

In dc electroporation studies using suspended cells, the electric field strength (E) has often been used to correlate electroporation efficiency with a characteristic quantity of the applied

electric field. To make our data comparable, we calculated E from the voltages across the cell layers (U_{cl}) and the average cell height (d) using $E = U_{cl}/d$. U_{cl} was computed for each electrode shown in Figure 3 from the recorded impedance data for the cell-covered and the cell-free electrode, following the considerations given by Gosh et al. (5). The average cell height for the NRK cells is reported to be $d \approx 4 \mu\text{m}$ (17). The values for U_{cl} range 1–3 V for the experiments shown in Figure 3, A–D and E–H, which can be translated into an E between 2.5 and 7.5 kV/cm within the cell layer. These values compare favorably with field strengths that were reported to be effective for electroporation in suspension (15,21,22).

Variation of Pulse Duration

Figure 4 shows the results of a series of dye-uptake experiments with the ECIS setup, in which the duration of the pulse was varied while the pulse frequency was kept at 40 kHz, and amplitudes were set to 3 V (upper panel) and 4 V (lower panel), respectively. Following panels A–D, the number of stained cells gradually increases with increasing pulse duration, but only for the longest pulse duration of 500 ms were more than 50% of the cells clearly stained. With a voltage amplitude of 4 V, the dye uptake increases gradually for pulse durations from 50 to 200 ms. However, when the pulses last for 500

ms, the staining pattern again becomes irregular, with several clusters of cells that are unstained and presumably irreversibly damaged. Thus, under the experimental conditions applied here using ac voltages for electroporation, the pulses must last between 100 and 500 ms to achieve efficient electroporation and probe uptake.

In recent electroporation studies that use dc voltage pulses for membrane permeabilization, the electric field is only applied for a few microseconds (32). In our setup, ac voltage pulses have to be applied three orders of magnitude longer to achieve efficient electroporation. Initially, this seems to be controversial. However, as illustrated in Figure 1D, two features associated with the sinusoidal nature of the voltage pulses are important to recognize: (i) the polarity of the voltage drop induced across the plasma membranes alters continuously, and all polarization phenomena are thus reversed within half the period of one oscillation, and (ii) the high-voltage magnitudes are only present for a small fraction of the total pulse length. In Figure 1D, the times in which the amplitude exceeds a certain threshold value necessary for membrane permeabilization are indicated by gray shading. Thus, the ac pulses applied here may also be regarded as a fast sequence of dc voltage shots of alternating polarity. Each of these dc shots is then only effective for less than approximately 10 μs , since the time for

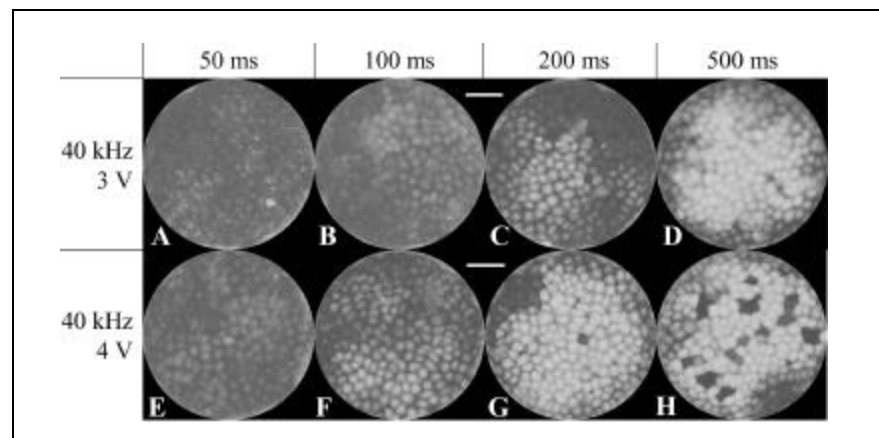


Figure 4. Uptake of Lucifer Yellow (2 mg/mL) for two different voltages as a function of pulse duration upon electroporation of NRK cells. The frequency, amplitude, and duration of the electroporation pulses were as follows: (A) 40 kHz, 3 V, 50 ms; (B) 40 kHz, 3 V, 100 ms; (C) 40 kHz, 3 V, 200 ms; (D) 40 kHz, 3 V, 500 ms; (E) 40 kHz, 4 V, 50 ms; (F) 40 kHz, 4 V, 100 ms; (G) 40 kHz, 4 V, 200 ms; and (H) 40 kHz, 4 V, 500 ms. The scale bar represents 50 μm .

one period with a frequency of 40 kHz equals 25 μ s. Examining our ac voltage profiles from this viewpoint means that the reduction of the frequency of the ac signal corresponds to an extension of the individual dc components in the sequence of dc voltage shots. Consistent with previous studies (32), the data in Figure 2 indicate that the reduction of the ac frequency—or extension of the dc components—is paralleled by a clear reduction of electroporation efficiency and cell survival.

Electroporation and Cell Recovery-ECIS Studies

The data presented in Figures 3 and 4 have already indicated that the cell membranes can be damaged irreversibly (on the time scale studied) when the parameters of the electroporation pulses are improperly selected. In these cases, we presume that the

cells could not hold the dye in the cytoplasm during the wash cycles after the pulse. Because it is our long-term objective to monitor the morphological response of adherent cells to the electroporative introduction of a foreign molecule into the cytoplasm, we studied cell recovery from the electroporation pulses in detail. To do this, NRK cells were equilibrated to incubator conditions in the same buffer as used in the dye-uptake studies, except that no dye was added. After recording baseline data in the 3f-ECIS mode, electroporation pulses were applied, and the cell response was subsequently followed using the 3f-ECIS mode. Figure 5A shows the time course of the electrode resistance (real part of the complex impedance) at the intermediate sampling frequency of 4 kHz before and after electroporation pulses (arrow) of 5 V/100 ms, but with varying frequencies applied to the cells. The resis-

tance of the electrodes at this frequency is the most sensitive measure for changes in cell shape and motility (9,11). The curves of Figure 5 clearly show that for voltage pulses consisting of 40 Hz, 400 Hz, or 4 kHz ac signals, the resistances do not recover to its pre-pulse values but instead drop to values that we typically recorded for cell-free electrodes. This observation supports our concept of an irreversible and intense membrane permeabilization. When the cells are electroporated with a 40 kHz voltage pulse, it takes only 30–40 min until the resistance has returned to its pre-pulse value. We conclude that changes in cell morphology induced by electroporation are fully recovered within this time range. When we studied the micromotion of the cells, which refers to the vertical motions of the cells relative to the substrate that is revealed in fluctuations of the resistance signal (11), only the

Research Report

motility of cells electroporated with high frequency recovered to normal activity. Cells pulsed with 40 Hz, 400 Hz, or even 4 kHz showed greatly reduced resistance fluctuations after the pulse had been applied. Since it has been shown previously (11) that the cellular micromotion as measured with ECIS is a direct indicator for the cell metabolism, we conclude that the high-fre-

quency voltage pulses do not disturb the latter significantly. Thus, these measurements support our finding that the cells on the ECIS electrode can fully recover in rather short time frames.

Figure 5B shows the results of a 3f-ECIS experiment in which the pulse amplitude was systematically altered, while the frequency of the pulse was set to 40 kHz and pulse duration was kept at 100 ms. Times necessary to recover to pre-pulse resistance values obviously correlate with the voltage applied. With a 2-V signal, the cells are fully recovered within 20 min. However, it takes roughly 45 min for the cells pulsed with 5 V to regain their original shape, and they do not show any sign of irreversible damage. Figure 5C presents ECIS measurements of NRK cells after they were electroporated with pulses of 40 kHz frequency, 4 V amplitude, and increasing pulse duration. The curves do not show significant differences for pulse durations from 50 to 200 ms. Only the longest-lasting electroporation pulse of 500 ms duration impairs the cells such that recovery times are extended to about 60 min. It is important to note in this context that the resistance measurements presented here do not mirror the resealing of the plasma membrane but the recovery of cell morphology. Recent studies have reported that membrane resealing occurs on the time scale of a few seconds (6).

quency ac voltage pulses of several hundred millisecond duration are most suited to electroporate cells cultured on gold-film electrodes. For the NRK cells, we achieved the most efficient but still reversible permeabilization of the plasma membranes and the corresponding probe uptake when 40 kHz ac pulses of 4 V amplitude and 200 ms duration were applied. Under these conditions, we can introduce not only low molecular weight probes like Lucifer Yellow (457 g/mol) into the cell interior but also fluorescently labeled dextrans with molecular weights up to 250 kDa (data not shown). Nevertheless, the cells recover from these electroporation pulses in 45–60 min. When other cell types such as the epithelial cell line MDCK strains I and II or BSC-1 cells were examined in similar experiments, we found cell-type-dependent differences for the optimum electroporation parameters. However, these were only slightly different from those found for NRK cells. Figure 6 shows typical fluorescence micrographs of confluent cell layers of the specified kind after electroporation and Lucifer Yellow uptake. The observed differences with respect to the optimum electroporation parameters are given in the legend and can be readily explained by the cell-type-specific contribution to the overall impedance of the cell-covered ECIS electrodes.

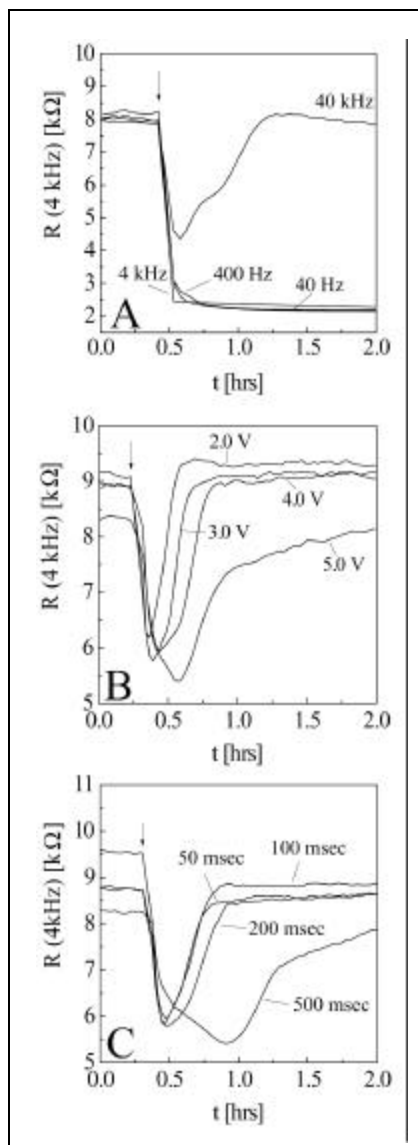


Figure 5. Time course of the electrode resistance at a sampling frequency of 4 kHz, when ac voltage pulses of the specified characteristics were applied to confluent NRK cell layers grown on ECIS electrodes. (A) Frequency as given in the figure, 5 V, 100 ms; (B) amplitude as given in the figure, 100 ms, 40 kHz; (C) duration as given in the figure, 4 V, 40 kHz. Data were recorded in the 3f-ECIS mode, but only the 4 kHz resistance readings are presented.

Electroporation and Dye-Uptake for Different Mammalian Cell Lines

Summarizing the results shown in Figures 2–5, we observed that high-fre-

CONCLUSION

Combining the technical merits of the ECIS biosensor to monitor mammalian cells with the option to intro-

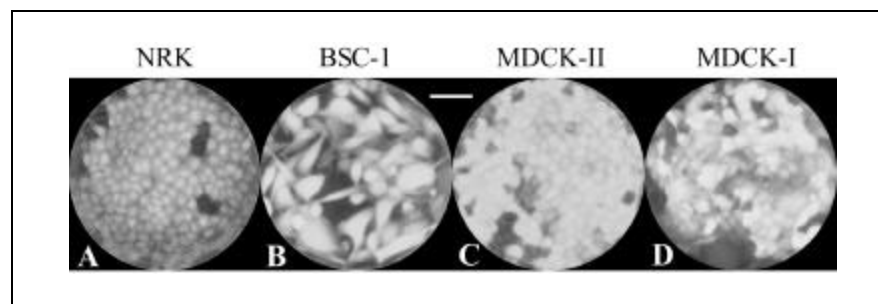


Figure 6. Uptake of Lucifer Yellow (2 mg/mL) upon electroporation of different mammalian cell lines after they had been grown to confluence on the ECIS electrodes. (A) NRK cells; (B) BSC-1 cells; (C) MDCK-II cells; and (D) MDCK-I cells. The electroporation parameters that provided the most efficient dye uptake were to some degree dependent on the cell type. Results given in A–D were recorded with pulse parameters as follows: (A) 40 kHz, 4 V, 200 ms; (B) 40 kHz, 4 V, 200 ms; (C) 40 kHz, 3 V, 200 ms; and (D) 40 kHz, 3 V, 200 ms. The scale bar represents 50 μ m.

duce biologically active probes into the cytoplasm by means of in situ electroporation provides a new and versatile tool with value in many areas of cell biology. Based on the experiments presented here, it should be possible to introduce metabolic key substrates, peptides, antibodies, or even nucleic acids into the cells and to follow their immediate response by noninvasive ECIS readings. The entire experiment is highly automated and can be performed on several samples in parallel. With this combined technique and the powerful capabilities of molecular biology available today, certain subcellular structures can be specifically addressed and probed for their particular contribution to cell morphology, differentiation, or motility. The ECIS electroporation technique may thus make significant contributions in the emerging field of functional proteomics.

ACKNOWLEDGMENTS

This work was performed in part pursuant to a contract with the National Foundation for Cancer Research. A stipend to J.W. granted by the German Academic Exchange Service (DAAD) is gratefully acknowledged. The authors thank George Edick (Rensselaer Polytechnic Institute) for access to the fluorescence microscope and his valuable assistance.

REFERENCES

- Giaever, I. and C.R. Keese. 1993. A morphological biosensor for mammalian cells. *Nature* 366:591-592.
- Giaever, I. and C.R. Keese. 1992. Toxic? Cells can tell. *Chemtech*. 116-125.
- Giaever, I. and C.R. Keese. 1991. Micromotion of mammalian cells measured electrically. *Proc. Natl. Acad. Sci. USA* 88:7896-7900. Erratum published in *Proc. Natl. Acad. Sci. USA* 90:1634.
- Gimsa, J. and D. Wachner. 2001. Analytical description of the transmembrane voltage induced on arbitrarily oriented ellipsoidal and cylindrical cells. *Biophys. J.* 81:1888-1896.
- Gosh, P.M., C.R. Keese, and I. Giaever. 1994. Morphological response of mammalian cells to pulsed ac fields. *Bioelectrochem. Bioenerg.* 33:121-133.
- Gosh, P.M., C.R. Keese, and I. Giaever. 1993. Monitoring electroporation in the plasma membrane of adherent mammalian cells. *Biophys. J.* 64:1602-1609.
- Hapala, I. 1997. Breaking the barrier: methods for reversible permeabilization of cellular membranes. *Crit. Rev. Biotechnol.* 17:105-122.
- Holzapfel, C., J. Vienken, and U. Zimmermann. 1983. Rotation of cells in an alternating electric field: theory and experimental proof. *J. Membr. Biol.* 67:13-26.
- Keese, C.R. and I. Giaever. 1994. A biosensor that monitors cell morphology with electrical fields. *IEEE Eng. Med. Biol. June/July*:401-408.
- Keese, C.R., N. Karra, B. Dillon, A.M. Goldberg, and I. Giaever. 1998. Cell-substrate interactions as a predictor of cytotoxicity. *In Vitro. Mol. Toxicol.* 11:183-192.
- Lo, C.-M., C.R. Keese, and I. Giaever. 1993. Monitoring motion of confluent cells in tissue culture. *Exp. Cell Res.* 204:102-109.
- Lo, C.-M., M. Linton, C.R. Keese, and I. Giaever. 2001. Correlated motion and oscillation of neighboring cells in vitro. *Cell Adhes. Commun.* 8:139-145.
- Meldrum, R.A., M. Bowl, S.B. Ong, and S. Richardson. 1999. Optimization of electroporation for biochemical analysis in live cells. *Biochem. Biophys. Res. Commun.* 256:235-239.
- Miller, D.L., S. Bao, and J.C. Morris. 1999. Sonoporation of cultured cells in the rotating tube exposure system. *Ultrasound Med. Biol.* 25:143-149.
- Neumann, E., K. Toensing, S. Kakorin, P. Budde, and J. Frey. 1998. Mechanism of electroporative dye uptake by mouse B cells. *Biophys. J.* 74:98-108.
- Orlowski, S. and M. Lluís. 1993. Cell electroporation: a new tool for biochemical and pharmacological studies. *Biochim. Biophys. Acta* 1154:51-63.
- Parak, W.J., M.G. Domke, A. Kardinal, M. Radmacher, H.E. Gaub, A.D.G. de Roos, A.P.R. Theuvsen, G. Wiegand, et al. 1999. Electrically excitable normal rat kidney fibroblasts: a new model system for cell-semiconductor hybrids. *Biophys. J.* 76:1659-1667.
- Raptis, L. and K.L. Firth. 1990. Electroporation of adherent cells in situ. *DNA Cell Biol.* 9:615-621.
- Raptis, L.H., S.K.-W. Liu, K.L. Firth, C.D. Stiles, and J.A. Alberta. 1995. Electroporation of peptides into adherent cells in situ. *BioTechniques* 18:104-114.
- Reddy, L., H.S. Wang, C.R. Keese, I. Giaever, and T.J. Smith. 1998. Assessment of rapid morphological changes associated with elevated cAMP levels in human orbital fibroblasts. *Exp. Cell Res.* 245:360-367.
- Rols M.-P. and J. Teissié. 1990. Electroporation of mammalian cells—quantitative analysis of the phenomenon. *Biophys. J.* 58:1089-1098.
- Rols, M.-P. and J. Teissié. 1998. Electroporation of mammalian cells to macromolecules: control by pulse duration. *Biophys. J.* 75:1415-1423.
- Soughayer, J.S., T. Krasieva, S.C. Jacobson, J.M. Ramsey, B.J. Tromberg, and N.L. Allbritton. 2000. Characterization of cellular electroporation with distance. *Anal. Chem.* 72:1342-1347.
- Teruel, M.N. and T. Meyer. 1997. Electroporation-induced formation of individual calcium entry sites in the cell body and processes of adherent cells. *Biophys. J.* 73:1785-1796.
- Tiruppathi, C., A.B. Malik, P.J. DelVecchio, C.R. Keese, and I. Giaever. 1992. Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier functions. *Proc. Natl. Acad. Sci. USA* 89:7919-7923.
- Tsong, T.Y. 1991. Electroporation of cell membranes. *Biophys. J.* 60:297-306.
- Wegener, J., A. Hakvoort, and H.-J. Galla. 2000. Barrier function of choroid plexus epithelial cells is regulated by cAMP-dependent pathways in vitro. *Brain Res.* 853:115-124.
- Wegener, J., C.R. Keese, and I. Giaever. 2000. Electric cell-substrate impedance sensing as non-invasive means to follow the kinetics of cell spreading on artificial surfaces. *Exp. Cell Res.* 259:158-166.
- Wegener, J., S. Zink, P. Rösen, and H.-J. Galla. 1999. Use of electrochemical impedance measurements to monitor β -adrenergic stimulation of bovine aortic endothelial cells. *Pflügers Arch.* 437:925-934.
- Xie, T.-D. and T. Tsong. 1990. Study of mechanisms of electric field-induced DNA transfection. Transfection by low-amplitude, low frequency alternating electric fields. *Biophys. J.* 58:897-903.
- Zheng, Q. and D.C. Chang. 1991. High-efficiency gene transfection by in situ electroporation of cultured cells. *Biochim. Biophys. Acta* 1088:104-110.
- Zimmermann, U. and G.A. Neil. 1996. *Electromanipulation of Cells*. CRC Press, Boca Raton, FL.

Received 21 November 2001; accepted 25 April 2002.

Address correspondence to:

Prof. Dr. Ivar Giaever
Rensselaer Polytechnic Institute
School of Science
Troy, NY 12180, USA
e-mail: giaevi@rpi.edu

For reprints of this or
any other article, contact
Reprints@BioTechniques.com