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INSTRUMENTS AND TECHNIQUES

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Single-cell electroporation

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Abstract Using modified patch-clamp methodology, we demonstrated that it is possible to insert genes or other compounds routinely into single cells by electroporation. When the cell is indented by a small-tipped microelectrode, a voltage of 10 V or less in the pipette is divided by the pipette resistance and the series resistance of the cleft between the pipette tip and the cell surface. The voltage at the cell membrane can be high enough to cause localized dielectric breakdown of the membrane and create pores that allow compounds in the pipette to enter the cell. Rectangular pulses from 20 μs to more than 300 ms are effective, as are frequencies from DC to 5 kHz. The most significant parameter was the total time for which the voltage was applied. Pipette voltages of 2–10 V were required, with larger genes requiring larger voltages. With optimal parameters, transfection rates in excess of 80% were also possible routinely. This approach offers an effective alternative to intracellular pressure injection and iontophoresis for placing genes, drugs, and other compounds in cells. Because of the small size of the electrode tips, substances can be inserted in cells from almost any location on their surfaces. In addition, the small tips electroporated only a limited area and so did little cell damage.

Keywords Electroporation · Single cell · Patch clamp · Gene insertion

Introduction

Electroporation has been used for over a decade for inserting plasmid DNA into bacteria. It has also been used more sparingly to insert genes and dyes into mammalian cells. While the molecular details of the mechanism of action of electroporation are not known, several principles are believed widely to be true. First, electroporation is believed to occur when the voltage applied across a cell membrane exceeds the dielectric breakdown voltage of the membrane [6, 14, 16, 17, 18]. The transmembrane voltage at which this occurs is somewhere between 200 mV and 1 V, with either polarity being effective [4, 6, 8, 12]. Second, the breakdown voltage is thought to produce pores across the membrane, with higher voltages producing larger pores. It is not known with certainty, but again believed widely, that higher voltages simply cause smaller pores to become larger rather than larger pores coming from a whole new population [6, 7, 14, 15]. The maximum-sized pores allow the movement of dyes, oligonucleotides, small peptides, and small-to-moderate-sized genes. Third, the formation or opening of the pores is fast [2, 6, 14]. Voltage pulses of less than 1 μs are sufficient to produce functional pores. The pores tend to close more slowly. Some appear to require only milliseconds for closure whereas others may take minutes [3, 11, 16].

Early work on electroporation utilized a large number of bacteria or mammalian cells in saline with electrodes dipped into the saline and between which the required fields were generated. Large voltages (hundreds to thousands of volts) were required and usually the fields were modulated repeatedly with sinusoids, rectangular pulses or exponential decay pulses [2, 3, 6]. The modulation frequencies were in the range from 1 Hz or so to several tens of kilohertz. Recently, several approaches have been used to electroporate smaller numbers of cells successfully. These include the use of small electrode arrays to electroporate cells in just one region of a Petri dish [13] or single, large-tipped electrodes to electroporate only a small population of cells in single embryos [1]. A very recent investigation has succeeded in inserting genes in single cells [5]. In that study, single microelectrodes were placed in the brains of living animals or in brain slices while 50–60 V were applied to the pipette.

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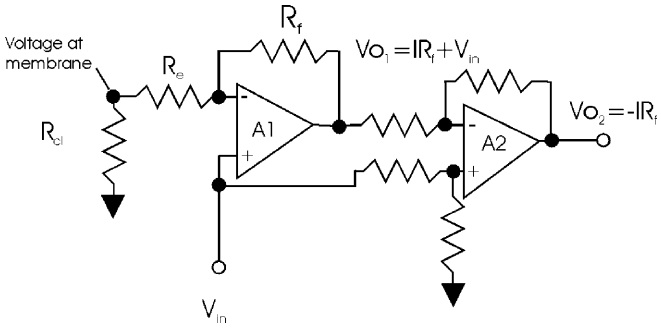

Fig. 1 Circuit diagram for electroporation apparatus [R_f feedback resistance, R_e electrode resistance, R_{cl} cleft resistance, V_{in} command voltage to be applied to the pipette interior, V_{O1} output voltage of the current-to-voltage converter (*A1*), V_{O2} output of the differential amplifier (*A2*)]

Since the technique was carried out under a dissecting microscope, particular single cells could not be seen and thus selected for electroporation. The transfection rate was relatively low (25–30%). Still, the study highlighted the exciting notion that single cells can be electroporated.

Here, we report single-cell electroporation using modified patch-clamp techniques. Single cells under direct observation were indented with a microelectrode and electroporated using a few volts delivered from a simple voltage-clamp circuit. The technique, which is an alternative to intracellular pressure injection or iontophoresis, is simple, repeatable, highly efficient, and capable of inserting genes of up to at least 14 kb in size. Since large genes can be inserted in cells using this approach, it is reasonable to believe that dyes, drug molecules, oligonucleotides (like antisense oligos), ions and even small peptides can be inserted as well. Because of the small tip size used, compounds can be inserted in selected regions of single cells. Since the membrane patch permeabilized is about 1 μm^2, only a tiny fraction of the cell's surface is perturbed. Not only does this protect the cell's health after electroporation but it also means that local concentration increments will occur inside the cell in the vicinity of the electroporated patch. Therefore, for example, puffs of calcium might be achieved near particular synapses; microtubules might be disrupted only in spreading projections from single cells and nuclear regulatory peptides might be injected only over particular parts of the nucleus. The possibility for interesting single cell interventions seems very large.

Materials and methods

Electronics

Figure 1 shows the principle behind our approach. A simple current-to-voltage (*I-V*) converter (A1) is used to clamp the voltage at the negative terminal of the operational amplifier. A patch pipette is connected to this input and so the voltage in the electrode is also controlled. This simple circuit is mounted on a motor-driven micromanipulator so that the electrode tip could be pressed gently against the cell to be electroporated. The resistance (R_e) of the patch pipette in series with the resistance (R_{cl}) of the cleft between the patch pipette and the cell divides the voltage that exists between the pipette and the grounded bath. In this simple model, the cleft resistance increments as the space between the tip of the electrode and the cell surface narrows. For the operational amplifier to which the pipette is connected $V_o = V_{in}(1 + \frac{R_f}{R_e + R_{cl}})$, where V_o and V_i are the output and input voltages respectively, and R_f the feedback resistance. This configuration is stable for any ratio of R_f to the sum of the resistances at the negative terminal. A value of 1 MΩ for R_f with R_e+R_{cl} of 15 MΩ total ensures that ~15/16 of the amplifier's output voltage (up to about 12 V for an operational amplifier operating from a standard 15 V power supply) is available as the pipette's command voltage. Linear operation of the amplifier is also ensured. The voltage applied at the outer surface of the membrane is $V_{in}\frac{R_{cl}}{R_e + R_{cl}}$. In a typical example, V_{in}=–5 V, R_{cl}=3 MΩ and R_e=12 MΩ, resulting in a voltage at the outer surface of the membrane of about –1 V, 20% of the command voltage. A second operational amplifier (A2) is a simple differential amplifier and subtracts the command voltage from the *I-V* converter output, thus giving a voltage equal to $-IR_f$ and so also quantifies the current flowing through the pipette. At the V_{in} input, waveforms of various voltages, durations, and frequencies can be applied. For these studies we used negative voltages since all of the molecules we were trying to insert by electroporation were anticipated to have net negative changes.

The pulse trains were produced using a pulse generator that allowed independent setting of the duration and period of each pulse. This generator was gated by a second pulse generator that determined the total time for which a pulse train would be delivered. That included the ability to provide only one pulse if required. The pulses from the pulse generator and the current from the *I-V* converter were viewed simultaneously on two channels of a digital oscilloscope.

Electrode technology

The pipettes were constructed from thin-walled Schott 8250 glass (O.D. 1.7 mm, I.D. 1.3 mm) with an internal filament fused to the wall (Garner Glass, Claremont, Calif., USA). There is nothing special about this specific glass type beyond its thin wall and internal filament. The electrodes were pulled with a single-stage pull on a P-97 puller (Sutter Instruments, Novarto, Calif., USA) to ensure a sharp tip with a smooth conical taper. Figure 2 presents photographs of the tip taper (Fig. 2a) and the tip geometry viewed looking into the bore (Fig. 2b). These electrodes had resistances of 10–13 MΩ when filled with normal saline. The thin wall ensured a significantly lower resistance for a given O.D. than was possible with thicker-walled glass. This allowed small tips without producing excessively high-resistance electrodes. Since electroporation is caused by the voltage and not by the current flow out of the pipette, the resistance of the pipette should not matter so long as the same fractional increment in resistance can be produced when the electrode is pushed against the cell. The 0.5-μm tip opening ensured a small area for electroporation. Such electrodes are not required for this technique since more traditional patch clamp electrodes with 2- to 3-μm tip diameters also worked. However, an electrode tip of that diameter when approaching the cell at 45° (the angle of our micromanipulator), begins to indent when its lower rim first touches the cell and will indent a thin, tissue-cultured cell by almost 50% of the cell's thickness before the top rim of the pipette touches the cell and results in a fully occluded electrode tip. Smaller tips indented much less for the same fractional occlusion and were therefore much less damaging to the cells. At some pipette size, the area available for electroporation may become too small to allow gene transfer or the resistance may become too high for sufficient electrophoretic current to flow. Either would result in failure to insert enough DNA to see a fluorescing gene product. That point is reached with electrodes with a resistance of about 40 MΩ for the pulse parameters investigated here.

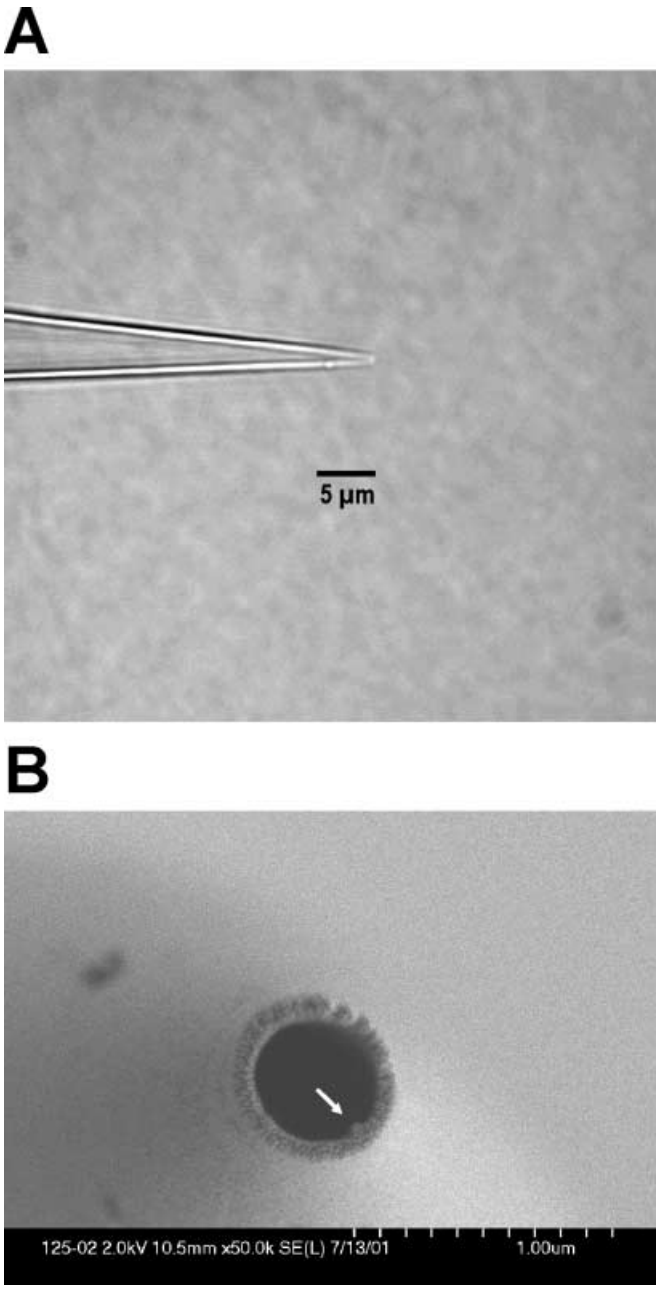

Fig. 2 a Light micrograph depicting tip taper of pipettes used. **b** Scanning electron micrograph looking into the pipette bore. The *arrow* shows the location of internal filament

The internal glass filament allowed the pipettes to be back-filled. A 1-µl volume injected into the back of the electrode fills the tip and 4 mm of the shank; 2 µl fills the entire shank. The remainder was back-filled using a 1-ml syringe and a 22-gauge needle that fitted easily into the bore of the glass. Since almost 1 cm of the electrode is filled with the appropriate filling solution, more than 1 h would be required before the tip solution would begin being diluted by diffusion of the back-filled solution [9]. This is more than enough time to electroporate dozens of cells. The pipettes were not fire-polished since their small size precluded fire-polishing to a visually distinct endpoint and so the final resistance was more consistently determined from the parameters on the electrode puller. The pipettes also were not coated with a capacitance-reducing elastomer (since such a coating is unnecessary in this application).

Optics and mechanical technology

Cells in a 60-mm Petri dish were viewed directly through a Zeiss IM35 inverted microscope through a 16× objective, 12.5× eyepieces, and a 2× Optivar. The electrodes were pushed against the cells using a Klinger micromanipulator. The final drive stage utilized a slow, high-resolution DC motor that allowed the final tip position to be set precisely. The tip was slowly advanced while the current passing through the electrode was monitored on the oscilloscope. When the current fell by 25% (meaning a 25% increase in resistance), the electrode forward movement was stopped and the pulse train delivered. With the electrode moving at 1 µm/s, the final positioning could be achieved so that the increment in resistance was always in the 20–30% range.

DNA and cell technology

The DNA used was in one of four plasmids obtained from Clontech (Palo Alto, Calif., USA). Cell-marking DNA was fused with the DNA for green, yellow or cyan fluorescent protein (GFP, YFP and CFP respectively) or red fluorescent protein-1 from *Discosoma* sp. (DsRed1) to produce a colored fluorescent product when the DNA was expressed. Each DNA stock was made in sterile water. The DNA stock had to be diluted with saline to produce a conductive fluid that would allow the passage of current. The mammalian saline used had the following composition (in mM): 149.2 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 5 HEPES. This is the same as the solution bathing the cells during the electroporation. Early experiments showed that the minimum DNA concentration required in the filling solution for electroporation to occur was 0.2 ng/µl, which is far less than the 1 µg/µl used in previously published single cell electroporation studies [5]. We did not use the 0.2 ng/µl minimal concentration for most of our experiments since it seemed sensible to use an excess of the DNA to ensure the best chance for electroporation and therefore highest transfection rate. Our usual procedure was to pipette 1 µl 1 µg/µl DNA stock into 29 µl saline in a 1-ml screw-cap tube, mix on a vortexer, and use 2 µl of the resulting solution to fill the tip of a single pipette. Again, that 2 µl was simply injected into the back of a filament-containing pipette and it filled the tip by running along the internal filament. The final DNA concentration was thus 33 ng/µl and the total DNA used was 66 ng. This approach, therefore, significantly conserves often precious DNA. A variety of different cell types were used although most studies were done on α-TN4 cells, a commonly used lens epithelial line [10] that we have used extensively for expression of lens and cornea ionic channels. Every cell type used was cultured in Petri dishes under ideal culture conditions for that cell. Before the electroporation, the culture medium was replaced with mammalian saline, different colored circles were drawn on the bottoms of the culture dish (for locating the electroporated cells later), and the Petri dishes were put into the apparatus for electroporation. For simplicity, the cells were at room temperature. After electroporation, the medium around the cells was replaced with ideal culture solution (containing antibiotics) and the cells cultured overnight under optimal conditions. Usually, the proteins produced by the expression of the genes could be observed within 4–5 h.

Confocal microscopy

Confocal micrographs were obtained from an Olympus (Melville, N.Y., USA) Fluoview 3 attached to an Olympus BX-50 upright microscope equipped with a 60× point-spread function objective. This water-immersion objective, NA1.2, is corrected for viewing through a cover-slip and up to 200 µm of saline. The cells imaged were electroporated on a cover-slip, incubated overnight, and placed in a special chamber on the BX-50 such that they were imaged through the cover-slip. The cells were alive. No fixatives or anti-bleaching compounds were used. A He-Cad laser line of 442 nm was used for ECFP probes, a 488 Ar laser line for EGFP, and a 543 green He-Ne laser line for pDsRed1 probes.

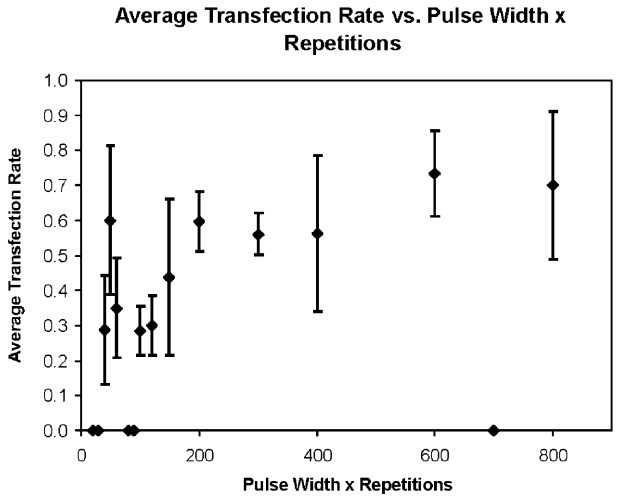

Fig. 3 Dependence of transfection rate of α-TN4 cells on the product of pulse width, frequency and duration of pulse train application (PWR). Pipette voltage was –5.6 V for all points. Means±SE

Results and Discussion

Sodium fluorescein studies

Initially, we used dilute solutions of sodium fluorescein in mammalian saline to establish minimal electroporation parameters. With the microscope in fluorescence mode, it was possible to watch the dye enter the cells in real time. Sine waves and triangle waves required much higher voltages than simple rectangular pulses and so all subsequent experiments were done using rectangular pulses. For fluorescein, a pipette voltage of –2 V was sufficient. Unfortunately, the voltage and pulse parameters yielding entry of fluorescein did not ensure entry of the genes we tried. In general, electroporation for genes required larger voltages so subsequent studies were done without sodium fluorescein in the pipette.

Determining pulse parameters

Given the approach described here, there was no a priori reason, other than perhaps precedent, to believe that pulse width or frequency would have optimal values. In the simplest voltage-divider model, the divided voltage at the membrane would have to be large enough to open the pores to the extent required to allow passage of the DNA of interest. The pulse width (PW) would have to be of sufficient duration to allow the pores to open and allow some DNA molecules to enter. The pulses would then have to be repeated sufficiently often to allow sufficient DNA molecules to enter the cell for reasonable expression. The simplest pore behavior would be for the pores to open rapidly following a voltage pulse, to stay open during the voltage pulse, and to close rapidly after the pulse. With this behavior, a wide range of pulse protocols would result in electroporation. Likewise, if larger voltages produce larger pores, as is thought, then the pore open-time required for the flux of a given number of gene molecules would decrease as the voltage increased. Larger voltages should also mean more flux (current) from the pipette.

The idea behind the electrical procedures reported here was to try to find the minimum product of PW and the number of repetitions (hereafter referred to as the PWR product, i.e., the total time that the perturbing voltage was applied to the pipette) that would result in observable fluorescence of our gene product. Note also that the number of repetitions of the pulse is the product of frequency (pulses/s) and duration of the pulse train in seconds. In total, we studied 1124 cells, 840 of which were used for gene insertion. The remainder were used with Na fluorescein. We did most of our studies using the Clontech pDsRed1-Mito probe since it has brilliant fluorescence when expressed and its 4.7 kb size is similar to the size of many natural genes. We used 5.6 V in the pipette for most studies. We already knew that this plasmid was harder to get into cells than was pEYFP-Peroxi, a Clontech plasmid of similar size or several GFP probes of our own design. Figure 3 shows, as expected from our simple pore model, that the transfection rate is dependent on the PWR product. Clearly, the gene entered and was expressed when the PWR product exceeded 50–100 ms. While this appeared to be a threshold, it is probably only a threshold of our ability to detect a fluorescing gene product visually through a fluorescence microscope. It is unlikely to be a threshold for gene entry. By 300 ms or so, the transfection rate exceeded 60% and did not increase much with further increases in PWR product. In some experiments, transfection rates were as low as 20–60%, even at PWR products with which 100% transfection rates had been achieved in previous experiments. Therefore, the procedure as implemented here does not ensure 100% transfection rates. In fact, the total transfection rate for all cells exposed to a PWR product >200 ms was 61% at a pipette voltage of 5.6 V. Even so, this percentage is much better than most other means of transfecting genes into cells, albeit one cell at a time. In general, our experiments were done in groups of five cells. At least one of a five-cell group was transfected in 86% of experiments in which the PWR product exceeded 100 ms. Transfection rates of 100% (all five of five cells expressing the gene) occurred often in individual experiments using PWR products of 300 ms or above.

Figure 4 shows that there was no obvious optimal pulse width or frequency for achieving transfection. When the PWR was sufficient to cause electroporation, the transfection rate was about the same at all pulse widths (Fig. 4a). While the data scatter somewhat, they do not support a preference for short, pulses. One anecdotal observation about pulses longer than 2 ms is that on several occasions, long pulse widths caused a fusion-like event between the pipette tip and the membrane, particularly if the command voltage was as large as 10 V. The adherence of the tip and membrane was sufficiently strong that the cell could be lifted from the Petri dish as

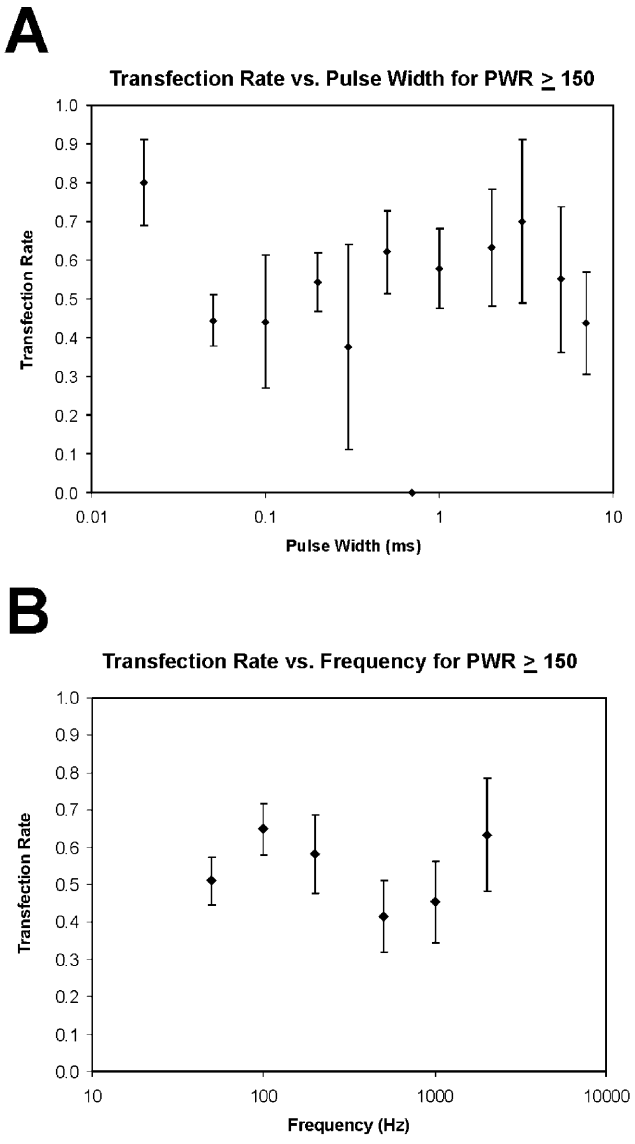

Fig. 4 Lack of dependency of transfection rate of α-TN4 cells lack on (**a**) pulse width and (**b**) frequency for all experiments in which PWR>150 ms. The pipette voltage was –5.6 V. Means±SE

the electrode was withdrawn. Since this never happened with shorter pulses, we decided to do most experiments with pulse widths of 1–2 ms and a frequency of 100 Hz. Figure 4b shows likewise that there was no obvious optimal frequency for electroporation by these methods. The transfection rate was the same at all frequencies tried within experimental error so long as the same PWR product was achieved at any effective voltage.

To quantify the effect of voltage, we attempted to insert DsRed1-Mito by electroporation using a PWR of 50 ms, very near our previously determined "threshold" for gene product detection when the pipette voltage was 5.6 V. Here we tried 30 cells at 4 V and 30 other cells at 10 V. For the 4-V group, the transfection rate was 28% and many of the cells showed very dim fluorescence. For the 10-V group, the fluorescence was generally brighter and the transfection rate was 77%. Therefore, as expected, transfection can occur at lower PWRs when the voltage is higher.

To investigate further the voltage effects, we attempted to electroporate 45 α-TN4 cells using a PWR of 300 ms using 10 V in the pipette instead of the 5.6 V used for the majority of our experiments. Thirty-eight of the cells expressed the DsRed1-Mito gene, a transfection rate of 84.4%. Therefore it is clear that higher transfection rates occur as expected when the voltage is increased.

DNA and cells electroporated

To date, polycystin-1 fused to GFP, a construct of about 20 kb, is the only DNA we tried for which the gene product could not be visualized in the cells following electroporation. This does not necessarily mean that the electroporation failed since the cell might simply have been unable to express the protein with a sufficiently high copy number for fluorescence to be detected by our crude means. Fibronectin fused to GFP, a construct of about 14 kb, could be inserted but required 10 V and 60% was the maximum transfection rate achieved in three experiments of five cells each. The BK potassium channel alpha-subunit fused to GFP, with a size of almost 9 kb, was inserted easily into electroporated cells, but again 60% was the maximum transfection rate achieved. It was not difficult to insert multiple genes simultaneously by electroporation. In one experiment on five cells, we were able to transfect simultaneously with Clontech's pDsRed1-Mito, pEYFP-Peroxi, and pCFP-Nuc. The ECFP plasmid (from previous work done during these studies) takes 10 V to insert, making it much more resistant to insertion by electroporation than ECFP, EYFP, or EGFP constructs. In this tri-gene experiment, each of the five cells had both the EGFP and EYFP plasmids but only three had the ECFP. Figure 5 shows confocal micrographs of three typical electroporations 24 h after the transfection occurred and demonstrates that the technique works, is specific, and does not damage the cells transfected. Successful electroporation and gene insertions were obtained with pDsRed1-Mito in CHO cells, HEK293 cells, α-TN4 cells (used for most experiments) and in primary cultures of chicken lens epithelial cells. Consequently, we expect the technique to be generally useful in a number of cell types.

The conclusion from this study is that single cells can be electroporated easily and gene expression achieved reliably in 60–100% of cells when a proper combination of pulse parameters is used. The fact that in many of our experimental groups of five cells 100% transfection rates were achieved, raises the question of why the overall transfection rate was only 61%. One factor is that the 5.6 V pipette voltage used for most of our experiments was not optimal for producing the highest transfection rate. A PWR of 300 ms and 10 V increased the transfection rate to over 84%. Another possibility is that failure of gene expression might sometimes be due to cell dam-

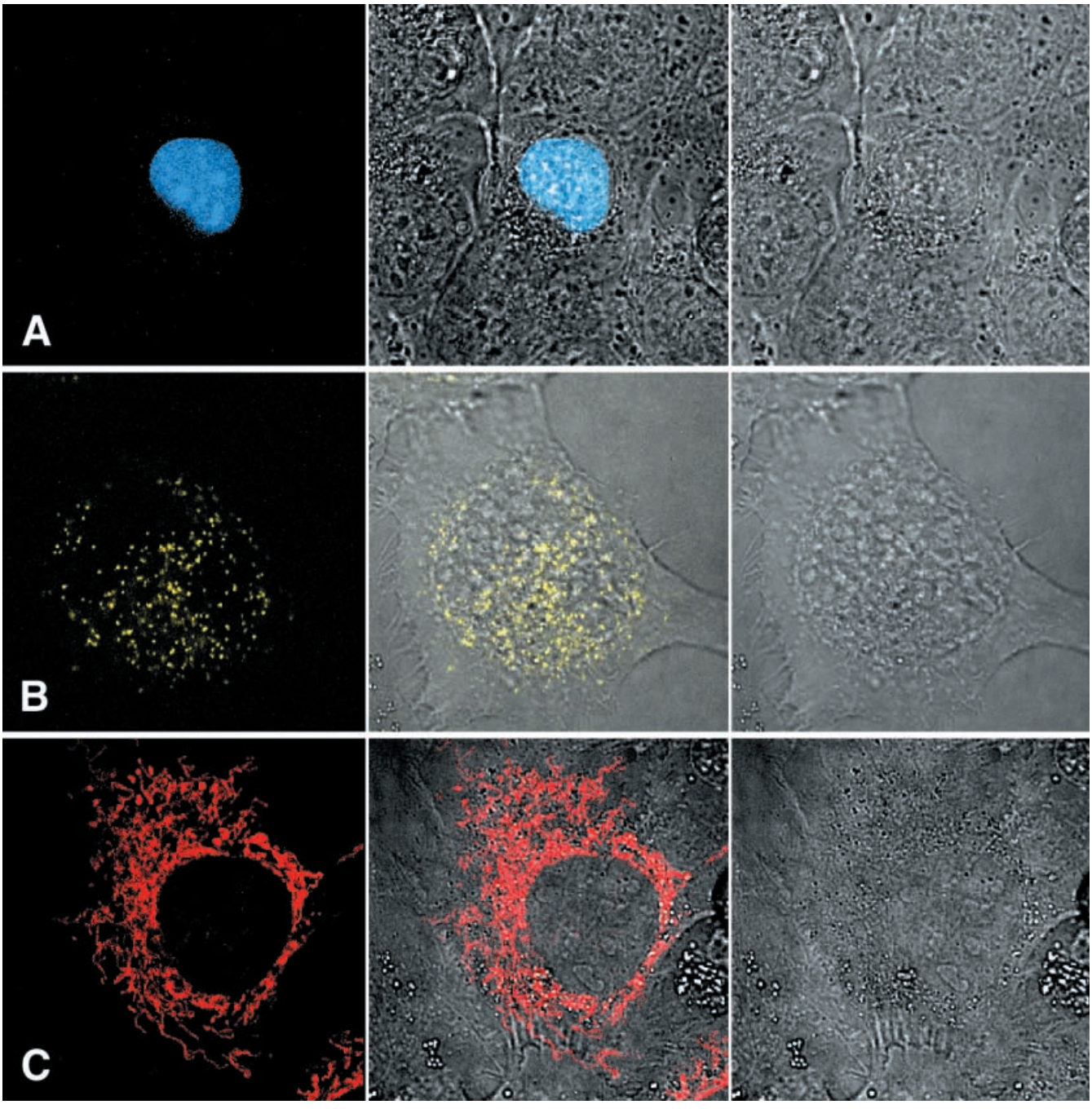

Fig. 5a–c Confocal micrographs of gene products in three different cellular organelles. **a** Nucleus (cyan fluorescent protein probe). **b** Peroxisomes (yellow fluorescent protein probe). **c** Mitochondria (DsRed1 probe). In each, *left* is fluorescence mode only, *middle* is bright-field and fluorescence merged and *right* is bright-field only

age. We filled our pipette with the same solution used for bathing the cells to simplify the expected composition of the solution in the cleft between pipette tip and cell. Future studies might better be done with a filling solution that more closely mimics the intracellular solution of the cell being electroporated. That would ensure that the cell would not be loaded with either sodium or calcium during the electroporation process and might decrease the expected toxicity from opening large non-selective pores in the plasma membrane. A third possibility is that because we must indent the cell to produce a voltage divider to achieve an adequate voltage at the cell membrane, debris on the cell surface might clog the tip. The resistance increment noted on pressing against the cell might then be a simple increase in the electrode resistance from clogging rather than an increase in the cleft resistance from close apposition of tip and membrane. One should not lose sight of the fact, however, that our 61–84% overall transfection rates are largely unprecedented with respect to other transfection techniques.

Cells can be electroporated with pipette voltages of 4–10 V using simple operational amplifier circuitry and commonly available power supply voltages. Unfortunately, as simple as this circuitry is, there is no commercial version presently available, given that this is the first report of the use of this circuit for electroporation purposes. The pulse parameters required can be varied so long as the PWR product equals or exceeds 150–300 ms. This can be achieved by single pulses of 300 ms or 15,000 pulses of 20 μs. Even this relatively brief PWR time can be decreased if higher voltages are used or extended if lower voltages are used. We settled on 5.6-V, 1-ms pulses at 100 Hz for 3 s for DsRed1-Mito only because longer pulses or higher voltages caused the tip to adhere to the cell membrane in about one-third of the α-TN4 cells tried. This adherence problem could largely be solved by using shorter pulses (0.1–0.2 ms) but of course then many more of them have to be delivered to reach the total of 300 ms PWR. Literally thousands of different combinations of voltage, pulse width, frequency, and total time could be used and would probably be as effective as the parameters we used. We did not try frequencies higher than 5 kHz simply because the briefer pulses inherent in higher frequencies were about the length of the capacity transient and made viewing the resistance increment on the oscilloscope screen more difficult. This problem could be solved easily by incorporating a capacity compensation circuit like those used in most commercial patch clamps. There is no obvious reason why higher frequencies could not be used. These results suggest, in keeping with the literature, that the electroporated pores open in less than 20 μs and do not inactivate over a 300-ms period. The fact that larger DNA molecules require larger electroporation voltages supports the hypothesis that the pore size increases with voltage.

The amount of DNA required per electrode is at least 0.2 ng and usually between five and ten cells can be electroporated with a single electrode. The number of cells is that low only because repeated use of a single electrode can result in the accumulation of foreign material in the tip which is likely to create a diffusion barrier to gene movement. We suspect that at least some failures to transfect, when pulse parameters were used that should produce transfection, were due probably to a tip diffusion barrier. If, after touching the cell, an electrode upon withdrawal did not recover its initial electrical resistance, subsequent attempts to electroporate with that electrode usually failed. DNA up to 14 kb can be used but the technique has so far failed with genes larger than that. The gene products generally began to fluoresce within 4–5 h of electroporation of the cell. The product was visible in daughter cells after up to four cell divisions and so clearly the electroporated cells recover well from any damage the procedure might have produced. We have not studied later generations of cell divisions.

While we used mostly DNA for these studies, it is clear that pores large enough for DNA permeation are large enough for most other molecules, apart from large peptides, to permeate also. Since the electroporation can occur at positive or negative voltages, compounds of either charge should be able to be inserted into a cell from any location on its surface. This opens possibilities for many types of single-cell experiments that to date have been either difficult or impossible. It is anticipated that when more investigators have had experience with this technique using additional genes and other compounds, technique enhancements will be found that result in even greater efficiencies.

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