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STRATEGY FOR INCREASED EFFICIENCY OF TRANSFECTION IN HUMAN CELL LINES USING RADIO FREQUENCY ELECTROPORATION

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ABSTRACT

Traditional electroporation devices use direct current electric fields to stimulate the uptake of oligonucleotides, plasmids, short peptides, and proteins into a variety of cell types. A variation of this widely used technique is now available which relies on radio frequency (RF) electrical pulses. This oscillating type of electrical field reportedly elicits greater uptake of plasmid DNA across the plasma membrane. We evaluated a protocol for RF electroporation of the a human embryonic kidney cell line and a Burkitt's lymphoma (BL) cell line for effeciency of transfection by RF electroporation. The plasmid EGFP, which codes for the widely used fusion protein, enhanced green fluorescent protein (EGFP), was used as a reporter of plasmid uptake after transfections.

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Transfection efficiency consistently increased approximately 30% from that typically obtained with conventional DC type electroporation and was accompanied by greater survivability of cells. Additionally, in some instances, percent transfection efficiency increased to over 70%. Thus, RF electroporation represents an improved methodology for transfection of human cell lines. Moreover, the RF protocol is simple to incorporate in laboratories already utilizing conventional electroporation devices and techniques.

INTRODUCTION

Electroporation is a gene transfer technique that is widely used for a number of investigative biological applications including transient or stable expression of genes in specific cell types ranging from bacterial to human origin. The technique utilizes an electrical field to enable the transfer of biological molecules such as oligonucleotides, plasmids, or proteins into a variety of different cell types.[4,13,14] Reversible permeabilization of the cell membrane and cell fusion is achieved through the application of this technique, and is thought to occur through the formation of volcano-shaped membrane openings as seen in freeze-fracture studies of electroporated cells. These pore structures in the plasma membrane rapidly open and expand to 20-20nm in diameter within 20ms of the delivery of the electrical pulse.[6] Several seconds later, the pores receed and the membrane returns to its initial structural integrity.

The technique is now routinely applied in numerous academic and biotechnology laboratories and commercial devices are now available for use in various biological applications. Conventional electroporators utilize direct current (DC) electrical field pulses with exponential decay characteristics.[10] This type of electroporator has been used with success for introducing plasmids and short oligonucleotides into mammalian, yeast, and a variety of other cell types.[1,2,9] Efficiency of transient transfection in these various cell lines has been shown to be dependant upon electrical field strength, cell type, electroporation buffer, state of the cells (adherent or in suspension), pulse duration, number of pulses delivered, and the timè interval between pulses.[10,12]

A modification of the convential DC electroporation technique was proposed by Chang and colleagues who suggested that electroporation might also be accomplished through the use of an oscillating electric field. In contrast to a DC field which relies solely on the dielectric breakdown of the plasma membrane for the induction of pore formation, an oscillating field is purported to have the advantage of creating a sonicating effect on the membrane which in addition to the dielectric effect might enhance structural fatigue.[3] These high frequency

oscillating electrical pulses can be superimposed over the waveform of a conventional DC pulse, creating an oscillating burst with a square waveform base (see Figure 1).

This type of electrical burst, referred to as radio frequency (RF) electroporation because its oscillations fall within the radio range, reportedly results in better transient permeation of the cell membrane. This allows larger numbers of biological molecules to cross and enter the cytosol with a reduction in cell lysis.[4,5] The greater survivability of cells is a distinct advantage of RF electroporation. Additionally, commercially available devices allow the user to modulate an increased number of electroporation parameters including voltage, burst duration, interval between bursts, number of bursts, and radio frequency of the oscillating portion of the waveform. This enables the user to more effectively tailor the electrical pulse characteristics to particular cell types in order to optimize the

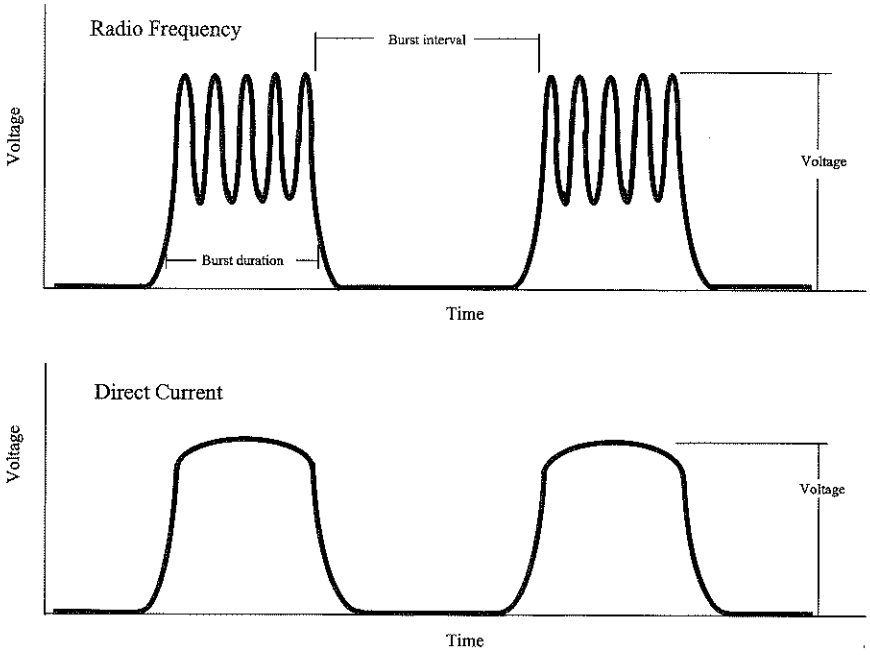

Figure 1. Comparison between radio frequency (RF) and direct current (DC) electroporation characteristics. The oscillating electric field provided by radio frequency electrical pulses reportedly enables better uptake of plasmid DNA through the cell membrane. Traditional square wave pulses utilize a direct current electrical burst. Figure adapted from BioRad technical bulletin 2076 and BioRad Gene Pulser® II RF Module instruction manual.

trade-off between increased transfection efficiency and decreased cell viability. RF electroporation uses protocols and equipment that are already familiar to users of traditional types of electroporation and consequently is simple to incorporate in place of traditional types of electroporation.

We describe a radio frequency electroporation protocol for a human embryonic kidney cell line and a BL cell line. The 27-kDa enhanced green fluorescent protein (EGFP) is a widely utilized protein fusion tag and reporter of gene expression and was used in our experiments to determine transfection efficiency.[8] It emits a characteristic fluorescence when excited by UV light, providing a simple method for verifying transfection using a fluorescence microscope. Radio frequency pulse electroporation could potentially be applied to a variety of cell types for expressing genes of interest with the advantage of significantly improved transfection efficiency.

EXPERIMENTAL

Plasmid

10μg quantities of plasmid EGFP (CLONTECH Inc., Palo Alto, CA, USA) were prepared and aliquoted from stock solution (4.9 μg/μL) and precipitated using cold ethanol/sodium acetate, washed with 70% ethanol then resuspended in 400μL aliquots of phosphate buffered sucrose electroporation medium (150mM sucrose, 27mM Na_2HPO_4, pH 7.5)[3]

Cells and Cell Culture

293 human embryonic kidney cells and the Burkitt's lymphoma, BJAB cell line were obtained from Elliott Kieff, Harvard Medical School, MA, USA. Cell lines were maintained in Dulbecco's Modified Eagle Medium or RPMI1640 Medium (Gibco-BRL, Life Technologies, MD, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 units/ml penicillin, 25μg/mL streptomycin, and 10μg/mL gentamycin. Adherent cells were allowed to reach 70% confluency, harvested by trypsin/PBS and uniformly resuspended by pipetting to break up cell clumps. Trypsinization was stopped by addition of complete media supplemented with fetal bovine serum.

Suspension cells (BJAB) were also resuspended by pipetting the culture to uniformly resuspend in media. An aliquot of the cell suspension was taken and viable cell counts were determined by trypan blue staining. Suspensions containing approximately 5 x 10^6 cells were transferred to 15mL centrifuge tubes and spun at 1800 rpm for 8 min. The supernatant was aspirated and the cells were washed in sterile PBS.

Electroporation

Cell pellets were resuspended in the previously prepared 400μL aliquots of phosphate buffered sucrose electroporation medium containing 10μg of EGFP plasmid and pipetted vigorously to ensure uniform suspension of single cells. This suspension was then immediately transferred to a sterile 0.4cm electroporation cuvette (Eppendorf, USA).

Cells were pulsed at various voltage (200-240V) and burst duration combinations (3-5ms) using the Gene Pulser II RF Module® with the Gene Pulser II® Electroporation System (BioRad, Hercules, CA). Several electroporation parameters were held constant for all experiments: 100% modulation, 40kHz radio frequency, 5 bursts, and 1 second interval between bursts. Following delivery of shock, the cell suspension was immediately transferred to 10mL of complete culture medium (as described above) in a 100mm culture dish (Corning, USA). These cell cultures were then placed in a 37°C, 5% CO_2 incubator overnight. Cells transfected with the EGFP plasmid were identified with fluorescence microscopy and counted in multiple fields to determine transfection efficiency.

Determination of EGFP Expression and Transfection Efficiency

After a 24 hour incubation period, cells were again harvested, washed in media, and resuspended in 500μL PBS. At 24 hours the electroporated cells had not doubled. Counting the cells by 24 hours minimized the problem of population doubling which could potentially skew the results. A 100μL aliquot of the cell suspension was also taken for counting and determination of cell viability by trypan blue staining.

30μL of suspension was placed and spread on a 25 x 75mm glass slide to which 10μL of antifade was added to minimize loss of fluorescence. Transfection efficiency was detected by fluorescence microscopy utilizing an Olympus IX70 microscope and 1X-FLA Fluorescence Observation® attachment. A 405, 490, 570nm triple filter was used for optimum excitation of EGFP. Percent transfection was measured as the average of three one hundred cell fields counted manually under low power and is defined as the percent of cells exhibiting EGFP fluorescence.

RESULTS AND DISCUSSION

293 cells transfected at voltages in the range of 210-240V with varying burst durations had relatively high transfection efficiencies (see Table 1). Specifically, we obtained an average of 48% transfected cells at 210V and 4ms burst duration, and at 220V and 4ms burst duration the transfection efficiency

Table 1. Comparative Transfection Efficiency at Various Electroporation Settings*

Electroporation Type	Voltage	Transfection Efficiency (Percent Transfected Cells)
RF	200	39 ± 2.5%
DC	*200*	*18 ± 3.5%*
RF	210	48 ± 6.0
DC	*210*	*20 ± 3.5*
RF	220	41 ± 4.0
DC	*220*	*30 ±2.5*
RF	230	56 ± 16
DC	*230*	*20 ± 0.5*
RF	240	34 ± 4.5
DC	*240*	*24 ± 3.0*

* Presented as the average of independent transfections. Three separate 100 cell fields were counted manually for each independent trial and transfection efficiency is expressed as percent of cells expressing EGFP fluorescence. Direct current electroporation at the corresponding voltages was done at 975uF and RF electroporation was held at an RF burst duration of 4ms.

was 41%. The highest efficiency of transfection was obtained at 230V and 4ms, with an average of 56% of the cells transfected. Moreover, the range was as high as 70% or greater for most experiements at this specific parameter. However, taking all transfections at 230V and 4ms single experiments resulted in lower than average transfection efficiency so that the overall average dropped to 56%. Results are based on the average of multiple independent transfection experiments. Equivalent transfections were carried out at each voltage setting using both radio frequency and standard pulse electroporation.

In preliminary trials, transfection efficiency decreased at voltage and burst duration combinations outside of this range, with efficiencies from 15-2% based on the specific parameters used. The best results were consistently obtained at 210V and 4ms (48% average), as well as at 230V and 4ms burst duration (56% average). For a given voltage setting, increased burst duration starting from a low value (2ms) generally resulted in a peak in transfection efficiency at 4 to 5ms, followed by diminishing efficiency at higher burst duration above 5ms and increasing to 10ms.

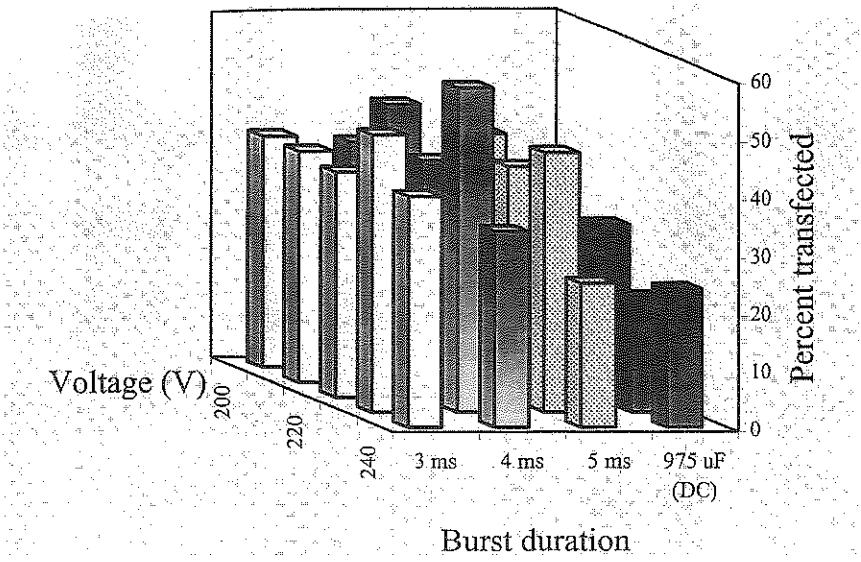

Figure 2. Transfection efficiency at various electroporation parameters. Voltages from 200-240V were used in combination with burst durations of 3, 4, and 5ms indicated on the x-axis. The z-axis represents percent transfected cells. The 975 on the x-axis for the standard method represents the capacitance. A direct current pulse electroporation with capacitance setting 975μF is provided for comparison at each voltage. Results are based on three independant trials.

The same trend appeared at constant burst duration and increasing voltage settings (see Figure 2). Cell viability after electroporation was varied, but was consistently higher using the RF electroporation. Viability decreased at higher voltage and increased burst duration settings. In the most effective range of settings (210V and 4ms, or 230V and 4ms) approximately 15-5% of cells were killed by the radio frequency electroporation as measured by cell counts and trypan blue staining 18 hours after shock (Table 2). Cell killing following standard pulse electroporation was consistently higher, usually greater than 40%, while sometimes over 50% of of the cells were killed.

Consistent with previous reports, we found that the choice of electroporation buffer (as described in Experimental) was crucial to achieving efficient transfection. In preliminary trials, two electroporation media were used with little success. Poor transfection efficiency was achieved with D10 complete culture medium alone (DMEM supplemented with 10% fetal bovine serum;10mg pEGFP/400μL complete medium), as well as 360μL D10 plus 40μL 150mM

Table 2. Viability of Cells After Various Electroporation Conditions Using 10 Million Cells per Transfection*

Electroporation Type	Voltage (V)	Burst Duration (ms) or Capacitance Setting (uF)	Viability
RF	200	4	75%
RF	210	4	70%
RF	220	4	68%
RF	230	4	67%
RF	240	4	61%
DC	200	975	42%

* Results presented as percent of cells surviving as determined by trypan blue staining. Both cells expressing and not expressing the green fluorescent protein are included in the surviving fraction indicated by percent viability.

sucrose (10μg pEGFP/400μL medium). In fact, these media tended to electrically overload the RF module when pulses higher than 200V were used. Consistent results were achieved with phosphate buffered sucrose medium pH 7.5 (see materials and methods) across a large range of voltage settings (190-260V) and burst durations (1-5ms) without the problem of electrical overload of the device.

We obtained optimal results at settings of 230V and 4ms burst duration using approximately 5-10 x 10^6 293 or BJAB cells and 10μg pEGFP suspended in 400μL phosphate buffered sucrose medium (Figure 3). High percentages of transfected cells, generally greater than 50% efficiency (and even up to 73%) were also obtained at settings of 210V/4ms and 220V/4ms with this electroporation buffer.

In conclusion, RF electroporation of 293 cells resulted in better transient transfection efficiency and greater survivability of the cell population. Studies in our laboratory indicates that improved results can be expected in other cell lines using this protocol with some adjustment of pulse characteristics. A consistently greater than 50% transfection efficiency was seen in the BL cell line, BJAB, on multiple occassions using similar parameters (210V/4-5ms or 230V/4ms) to those used with the 293 cells (data not shown). These cells line are used in numerous laboratories carrying out transient transfections and reporter assays. Therefore, radio frequency electroporation provides a simple and familiar

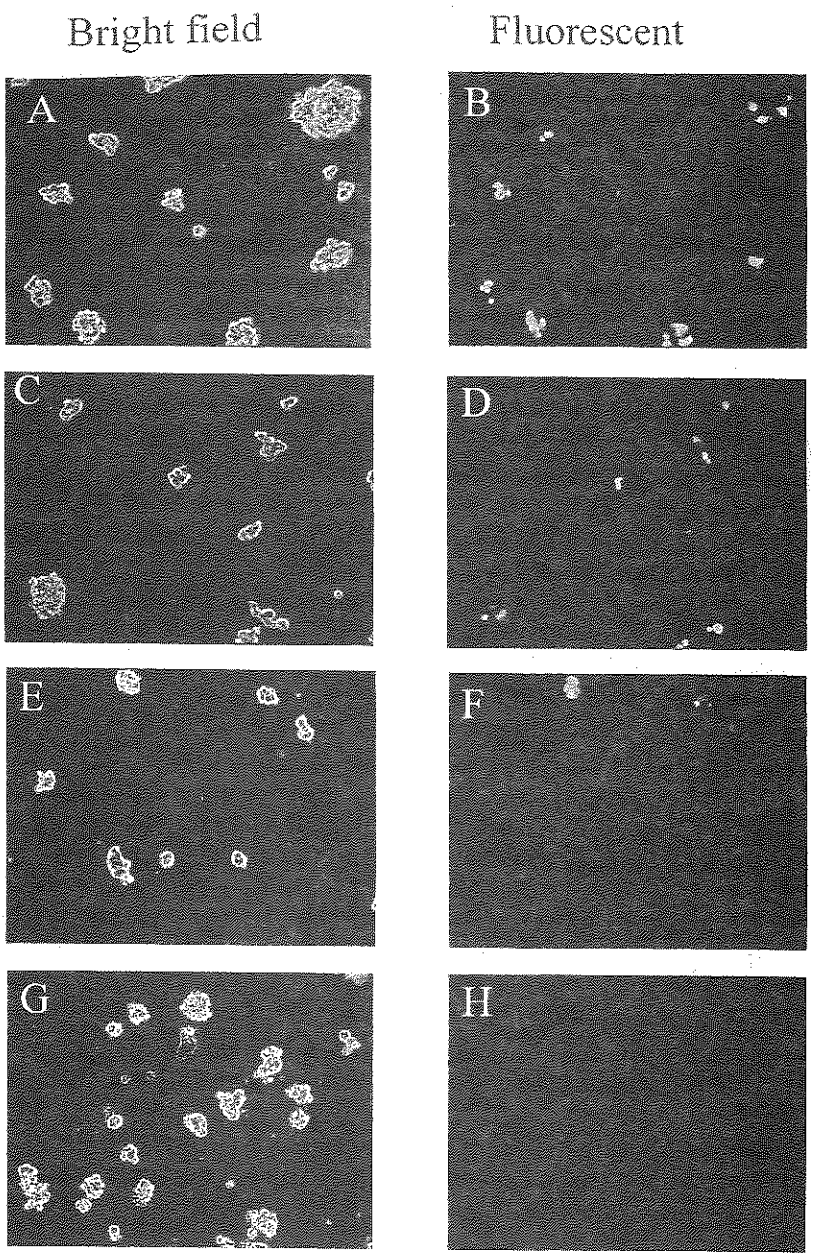

Figure 3. Bright field and fluorescence microscopy images of transfected 293 cells. Image pairs A and B (RF, 230V, 4ms), C and D (RF, 210V, 4ms), E and F (DC, 220V, 975μF), and G and H (Mock) were obtained 24 hours after electroporation. The left panel represents the brightfield and the right panels are fluoresence images. Photographs were taken by 24 hours prior to adherence of 293 cells to dishes. (RF = radio frequency electroporation, ST = standard type electroporation, Mock = Without GFP plasmid).

method for increasing transfection efficiency with wide applicability. The ability to increase yield of transfected cells in experimental culture models is of potential interest to a wide variety of investigations.

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Roles of Authors: ESR proposed the work. PBZ and MAC maintained cell lines, performed transfections, and collected cell counts. Fluoresence microscopy images were obtained by MAC and PBZ. ESR provided evaluative support and critical analysis. Manuscript, table, and figures were produced by PBZ, MAC, and ESR.

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