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Variable Electric Fields for High Throughput Electroporation Protocol Design in Curvilinear Coordinates

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ABSTRACT: The mathematical solution to the electric field equation in cylindrical coordinates, has suggested to us a new experimental methodology and device for reducing experimental effort in designing electroporation protocols. Using a new cylindrical electroporation system, we show, with an *Escherichia coli* cell model, how key electroporation parameters emerge precisely from single experiments rather than through interpolation from numerous experiments in the conventional Cartesian electroporation system.

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The permeabilization of the cell membrane using electric fields applied across the membrane is known as electroporation (Neumann et al., 1982) or electropermeabilization (Stopper et al., 1985). Electroporation is reversible when cells survive the electropermeabilization and irreversible when they do not. Reversible electroporation is commonly used in biotechnology and medicine for such applications as gene or drug delivery into cells (Dev et al., 2000). Irreversible electroporation is important for non-thermal sterilization in the food industry, biotechnology and medicine, and for tissue ablation in medicine (Pakhomov et al., 2010; Rubinsky, 2010).

The outcome of an electroporation protocol, whether reversible or irreversible, depends on the parameters of the electric field such as strength, pulse length, number of pulses, time interval between pulses, frequency; on solution composition, pH, temperature and on cell type, shape and size. Because electroporation depends on so many parameters, designing optimal electroporation protocols requires tedious and lengthy efforts. To illustrate the complexity of protocol design, Figure 1 shows a theoretical curve adapted from (Dev et al., 2000), which correlates electric field strength, single pulse length and the biophysical phenomenon that occurs when the particular parameters are applied across a cell. One of the most important features of the figure is the line that separates between the reversible and irreversible electroporation domains, which is critical in designing optimal electroporation protocols. In optimal reversible electroporation it is desirable to be close to and below that line while in optimal irreversible electroporation it is desirable to be close to and above that line. Conventional methods for the systematic development of optimal electroporation protocols employ experimental systems made of two parallel electrodes, bounding the media of interest, in a one-dimensional Cartesian configuration (e.g., Sale and Hamilton, 1967; Hamilton and Sale, 1967). The solution to the simple Laplace equation ($\nabla^2\varphi = 0$; where φ is the potential) for a homogeneous Cartesian system, subject to constant voltage boundary conditions on the electrodes, V_2 and V_1, gives an expression for the electric field between the planar electrodes. It is, $(V_2 - V_1)/L$ where L is the distance between the electrodes. It is evident that the Cartesian configuration produces a constant electric field in the treated medium between the electrodes. Identifying the electric field parameters that separate between reversible and irreversible electroporation requires numerous constant electric field experiments, in which the electric field strength is continuously changed in separate experiments until the interface is detected approximately, through interpolation between experiments (Rubinsky et al., 2008).

Several approaches were introduced for multiparameter optimization of in vitro and in vivo electroporation. Heiser (1999) published an extensive review on electroporation parameters for various cell lines and general guidelines for electroporation protocol optimizations in vitro (Heiser, 1999). A review and guidelines for optimization of in vivo

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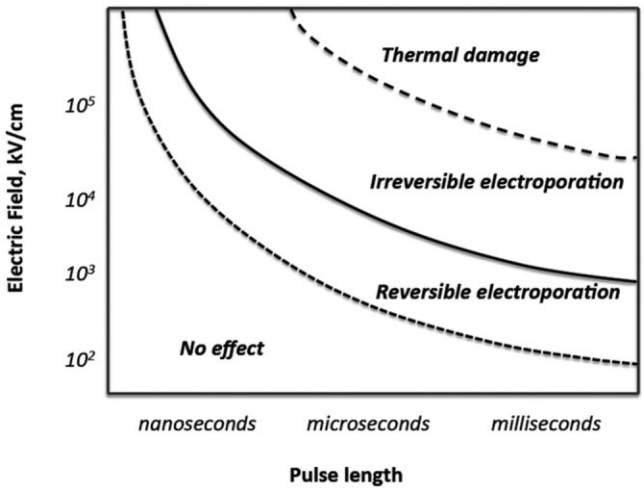

Figure 1. Theoretical mapping of biophysical effects experienced by cells as a function of electroporation parameters.

electroporation applications was reported by Gehl (2003). Furthermore, several statistical methodologies were proposed to reduce the number of experiments required for protocol optimizations. Multifactorial experimental design for optimizing transformation protocols was introduced by (Marciset and Mollet, 1994). Keng-Shiang et al. (2007) used the Taguchi Method for the optimization of gene electrotransfer (Keng-Shiang et al., 2007). Recently, a central composite design was used to optimize electroporation protocols (Madeira et al., 2010).

In this study we developed a different approach to multiparameter optimization, based on the use in a single experiment of a well-defined variable electric field topology in the curvilinear coordinate system. The concept will be illustrated with a simple to implement cylindrical coordinate system. The electric field calculated from the solution to the one-dimensional Laplace equation in cylindrical coordinates, in a medium between two cylinders of radiuses R_1 and R_2 on which electric potentials of V_1 and V_2 are imposed, respectively, is given by,

$$\frac{V_1 - V_2}{r \cdot \ln\left(\frac{R_2}{R_1}\right)}$$

where r is the variable radius inside the domain of interest. Obviously, the electric field varies continuously as an inverse function of the radius. (In one-dimensional spherical coordinates the electric field varies as one over the radius squared.) Therefore, in a single experiment in one-dimensional cylindrical or spherical electrode systems, the cells between the electrodes will experience a *continuously* variable electric field, that is, nevertheless, well defined as a function of the radius. The response of the cells to any electroporation protocol can be evaluated as a function of their relative location (defined by radius) and thereby correlated to the electric field. Therefore, when an experiment is performed with cylindrical (or spherical) electrodes, the results of a single experiment produce *continuous* information on the effect of a wide range of electric fields, which are quantified by the radius at which they are produced. In contrast, to produce similar information, the conventional Cartesian electrode system requires a very large number of experiments and the interpolation of results between the studied discrete data points.

Figure 2 illustrates results obtained from a study performed with a one-dimensional cylindrical system, using *Escherichia coli* BL21 (D13) PSJS1244, an ampicillin stable strain. The Figure 2d shows the electric field at the reversible/irreversible interface as a function of the number of pulses. The microorganisms were spread on a Petri dish and a constant pulsed electric potential was imposed on two concentric metal cylinders, in contact with the surface on which the microorganism was plated. In the one-dimensional cylindrical electrode system used, the outer diameter of the inner cylinder was 1.18 mm and the inner diameter of the outer cylinder was 22.15 mm. The electric pulse was delivered by a BTX (BTX ECM 830, Harvard Apparatus, Holliston, MA). Four sites were treated in each Petri dish, after which the samples were incubated for 18 h at 37°C and examined. Figure 2a and d reports on results with a pH buffered plate, at which pH did not change after the application of electric field, and in which 2,200 V pulses were applied between the concentric electrodes in 40 μs pulse

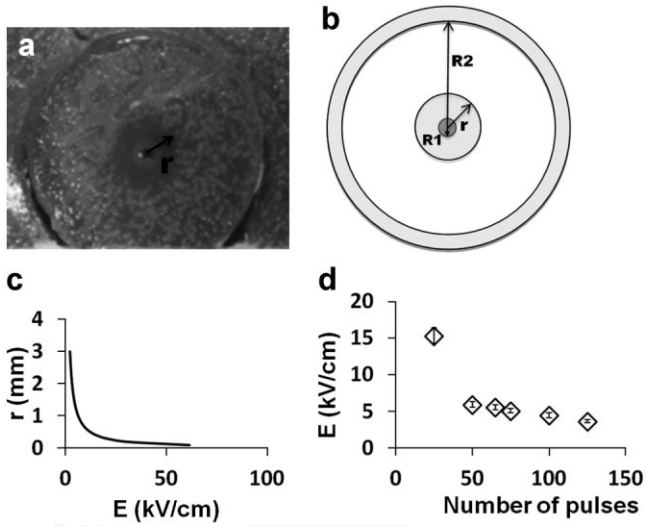

Figure 2. Electroporation parameters analysis: (**a**) image of a plate electroporated in one-dimensional cylindrical coordinates, after incubation. The central part has no cell colonies, (**b**) a schematic view of the analyzed cylindrical system. Two cylindrical electrodes with radiuses R_1 and R_2 were used. The radius (r) of irreversible electroporated zone was measured. **c**: Calculated electric field-radius (r) correlation for the experiment, (**d**) electric parameters that identify the conditions in which irreversible electroporation occurs during electroporation of *E. coli* in pH buffered medium (error bar 1 standard deviation).

duration at 1 Hz frequency. Five repeats were performed for each condition. It should be emphasized that each data point on the curves was obtained from a single experiment (with five repeats). In contrast, obtaining such a single data point with Cartesian electrodes would require numerous single electric field experiments and interpolation.

Figure 2b and c shows how the plot in Figure 2d was obtained. Figure 2a shows the appearance of a treated cylinder after incubation. It is evident that the cells in the central area did not survive the electric fields to which they were exposed and did not form colonies. To determine the radius of cell death we measured the innermost radius of the colonies that survived electroporation, as described in the Materials and Methods Section. Figure 2b shows the model of the analyzed system. Two cylindrical electrodes, with radiuses of R_1 and R_2, and a measured radius (r) of a zone where irreversible electroporation takes place, are shown on Figure 2b. Then, the mathematical expression for the electric field as a function of radius in cylindrical coordinates was used to produce Figure 2c. Figure 2c is used to correlate the radius of cell death in Figure 2a with the electric field at that radius. The electric field at the radius of cell death is than plotted as a function of number of pulses in Figure 2d. This plots the electric parameters at which irreversible electroporation begins.

In summary, we propose that the use of cylindrical one-dimensional electrodes will substantially reduce the number of experiments needed to design optimal electroporation protocols, over those obtained with the use of traditional Cartesian electrodes. In this report we have shown the use of the concept for obtaining the reversible/irreversible interface. Obviously a similar experiment with fluorescence dies or genes can be used to determine the parameters at the interface between reversible and no effect electric fields. Furthermore, this method provides a means to examine in a single experiment, various colonies that have undergone electroporation with a wide range of well-defined electroporation parameters. The relative location of each colony of interest identifies the electroporation conditions it has experienced. It should be noted that the idea of a well-defined topological space of variable electric fields could be extended through further research in topology to the design of systems of more complex surfaces than the cylinder or sphere, which may produce in a single experiment complex ranges of parameters of interest.

Materials and Methods

Experimental Device

The cylindrical one-dimensional electroporation electrodes were manufactured using a Perspex "square" (3 cm by 3 cm) basis. A half cm notch carved in the side of the square was attached to the top of a brass ring using a heated glue gun. The brass ring had an inside diameter of 22.15 mm, an outside diameter of 25.40 mm, and a height of 4 mm. The tip of an 18 gauge steel needle (Precision Glide needle, Becton Dickinson & Co, Franklin Lakes, NJ) was cut 1 cm from the top, to form the inner, 0.6 mm radius cylinder. The needle was then inserted through the center of the plastic square in the middle of the brass ring forming two concentric cylinders.

Electroporation Procedure

The study was performed using *E. coli* BL21 (D13) PSJS1244 an ampicillin stable strain. A single *E. coli* colony was used to inoculate 50 mL of sterile LB Broth (Ditco, Saratoga Springs, NY) containing 100 μg/mL of ampicillin (American System, Chantilly, VA). The sample was placed in a Thermo Scientific MaxQ 4450 shaker-incubator. The temperature was maintained at 37°C. The shaker speed was 200 rpm to allow aeration for optimal growth. The sample was kept in the shaker-incubator for 14 h to reach stationary phase. The final concentration of approximately 10^6 CFU/mL was determined by viable count method. After 14 h in the shaker-incubator a 100 μL sample was removed and diluted in 10 mL of sterile water (100× dilution) 100 μL of the diluted sample was plated on to each pre-prepared agar plate and spread using glass beads (Novagen, Madison, WI). The glass beads were removed and the electroporation device was inserted into the agar in one quadrant of the Petri dish. The device was pushed into the agar plate until the ring and needle touched the Petri dish bottom in order to ensure they were at the same depth. Alligator clips were attached to the brass ring and the 18G needle. The alligator clips were never in direct contact with the agar to ensure no direct discharge into the gel. This allowed the field to be equally distributed around the needle. The clips were hooked up to the BTX (BTX-model 610, BTX ECM 830 square-wave electroporator, Harvard Apparatus). The electroporation parameters used were 2,200 V, 40 μs pulse duration, 1 Hz frequency. The numbers of pulses were changed between experiments. Statistical analysis was done with the final parameters recorded from the BTX device.

Following the electroporation the needle and the ring were removed from the agar gel. (A similar experiment was than performed in another quadrant. A total of four experiments were performed per dish.) A total of five experiments per parameter were performed. After the experiment the Petri dish was incubated at 37°C for 18 h. Following the incubation period the dishes were removed and IRE curve radius was measured.

Plate Preparation

Agar Petri plates were prepared to maintain pH 7 after the application of electric pulses. The mixture was composed of 0.1 g/L NaCl (Spectrum Chemical, Mfg Corp, Gardena, CA), 10 g/L Bactotryptone, 5 g/L Yeast Extract, 15 g/L Bacto Agar (Becton Dickinson & Co, Franklin Lakes, NJ), 0.5 g/L glucose was dissolved in distilled water and heated at 121°C in an autoclave for 15 min. After cooling down and reaching

50°C, 23.83 g/L HEPES (Sigma-Aldrich, Selma, CA) and ampicillin (American Bioanalytical, Natick, MA) at 10 mg/mL was added to 100 μg/mL final concentration. The buffered agar was then poured into a 100 mm Petri dish and the drying time between the pouring and the closing of the plates was 6.5 min. In fact, the evaporation of water during storage must be taken into account because it changes the NaCl concentration and of course the conductivity of the medium.

Radius Measurement and Statistical Analysis

Electroporated plates were removed from the incubator after 18 h. Digital images of the plates and scale reference were taken and then used to determine the death zone diameter. The error on the electric field estimate includes the diameter measurement errors (precision of 0.05 mm) and the BTX device output error (20 V). The reported radius is an average of five repeats with a standard deviation calculated from the five measurements.

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