FLUORESCENCE METHOD FOR SINGLE CELL EVALUATION O[1]F ELECTROPORATION YIELD

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Cellular electroporation (EP) represents a physical procedure to increase membrane permeability by applying controlled electrical pulses. Although many applications of EP have been developed in medicine and industry, experimental methods useful for evaluation of the effects of cellular electroporation are still investigated. Here, we propose a fluorimetric method for a real-time evaluation of the electroporation yield at single cell level. The novelty introduced by our work lies in the fact that quantitative information is provided in real time at cellular level, through non-intrusive optical measurements. The method consisting of the measurement of intensity of the emitted fluorescence of Propidium Iodide incorporated in cells during EP was applied to study the effect of Dimethyl Sulfoxide as modulator for the electropermeabilization of cell membrane. The method is considered to be useful for the experimental investigation of biophysical processes that underlie the EP efficacy.

Keywords: cellular electroporation, time-resolved fluorescence, propidium iodide, DMSO.

1. Introduction

Electroporation (EP) is a technique widely used in the biomedical field for molecules delivery (drugs, DNA/RNA, dyes, etc.) and non-thermal tissue ablation. EP is also employed in industrial applications for sterilization of food and water, non-thermal food processing, extraction of biofuels, recombinant protein production, etc. [1]. EP represents a physical procedure to increase membrane permeability to low and non-permeant molecules by applying high voltage electrical pulses for a short time. Depending on the number and characteristics of the pulses (amplitude, duration, repetition frequency, shape), at the end of EP, the cell can regain its initial selectivity of the transmembrane transport (reversible EP)

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or can remain altered leading to cell's destruction (irreversible EP) [2]. The general mechanism of permeabilization of the cellular membrane states that during the pulse application a re-arrangement of phospholipid bilayer creates "pores" in the cell membrane, which allow the passage of hydrophilic compounds. In short, strong electric field is applied to charge the cell membrane like a capacitor until it breaks down, creating transient, water-filled pores [2]. The aqueous "pores" are triggered when the total transmembrane electric potential is above a certain threshold (threshold value varying from one cell type to another) [3]. Once the voltage is off, the "pores" close but the membrane may remain permeable even for tens of minutes [4], period during which its phospholipids [5, 6] and proteins [7] modified due to chemical reactions triggered by the electric pulses [8] are replaced and the impermeability of the cell membrane restored. An electric-accelerated endocytosis process is considered the main ground of full membrane recovery, the more that endocytosis vesicles have been reported as main pathway for gene electrotransfer [9, 10], even there are reports debating their contribution to the transfection efficacy [11]. Some another mechanistic models argues that the high voltage applied to the cell membrane makes the phospholipid bilayer more mobile, enabling the extracellular molecule to slip by it into the cell [12]. Complexity of cell membrane structure and recovery process, and the fact that membrane permeabilization is obtained with pulses of different durations (from nanoseconds to milliseconds) makes the understanding of electroporation mechanisms a long running course.

Computational and experimental methods have been developed during the last 30 years for the investigation of EP mechanisms and effects [13]. Computational methods were designed as a combination of finite-element continuum models and image-based realistic cell geometries to study the relationship between field distribution and pore formation. Time-dependent finite-element models implemented in COMSOL were used to calculate induced transmembrane voltage and to simulate electroporation on irregular, image-derived cell shapes, showing how cell morphology, orientation and cell–cell packing alter local transmembrane voltage and predicted poration sites [14]. Recent works demonstrated how three-dimensional, realistic models of adherent cells reconstructed by quantitative phase microscopy, provide a more accurate estimation of local electric field heterogeneity and membrane polarization in electroporation simulations [15-17]. These approaches link imaging with modeling, allowing morphology and experimentally-quantified parameters to guide simulations of electric field distribution and effects at single-cell level. Computational models based on matrix optics have also been used in other contexts for the non-invasive assessment of oocyte maturity [18]. Regarding the experimental methods used for EP investigation, a first category relies on changes in electric conductivity of cell membrane and/or medium during and after pulses application (membrane conductance, impedance measurements) [19]. The second category is based on an

molecules exchange (fluorescent dyes, plasmids, radioactive compounds, etc.) across the membrane [20]. The characteristics of these molecules in terms of molecular weight, electric charge, fluorescence yield, chemical affinity, etc. are influencing the detection limit/monitoring power of the procedure. For example, propidium iodide (PI), a non-permeant dye whose fluorescence enhances 20− to 30−fold when linked to nucleic acids [21], has been successfully used for quantifying the ratio of electroporated to not-electroporated cells or the resealing time, by fluorescence microscopy visualization. Fluorescence signal–based methods were useful to evaluate photosensitiser kinetics and treatment delivery in real time, offering the advantages of being non-invasive, quantitative, and capable of providing immediate feedback on treatment dynamics [22]. Methods adapted for a real time quantification of membrane EP outcome are still expected [23, 24], such a method could be used, for instance, to assess the effect of different modulators of EP process.

It has been shown that cell EP is significantly influenced by various physical parameters related to the pulse sequences characteristics [25-27] and chemical modulators related to the composition of the environment in which the EP is performed. Optimized conditions for EP are often sought depending on the purpose of the experiment. For instance, osmotic agents and disaccharides such as trehalose, sucrose, or mannitol were found to help maintain the osmotic balance and stabilize the membranes during and after pulsing. Trehalose at concentrations between 100–250 mM in the electroporation buffer was shown improving the viability of the cells after the pulse application and cryopreservation [28-30]. Membrane-stabilizing copolymers such as Poloxamer-188 (Pluronic F-68) (typically 0.05–1 %) were used during the pulse application or shortly after pulsing to assist membrane resealing and improve cell survival without substantially reducing uptake efficiency [31-33].

For a long period, calcium ions present in the exterior medium were considered the “enemy” of cell recovery after EP (the intracellular calcium concentration increased by membrane permeabilization is triggering cellular apoptosis which was diminishing the efficacy of gene electrotransfer [34]) and buffers commercially available for eukaryotic cells or bacteria transfections are at very low concentration of calcium. In recent years, the role of calcium has been reconsidered and a new procedure called “calcium electroporation” turned into a well-established approach in clinical settings. When extracellular Ca^{2+} is higher than physiological concentrations (typically 0.5–5 mM) during the application of short, high-voltage pulses, a calcium uptake occurs leading to acute and severe ATP depletion associated with cancer cell death [35].

Changes in membrane cholesterol content influence cell sensitivity to electroporation. Methyl-β-cyclodextrin (MβCD) and related compounds commonly used for cholesterol depletion increase membrane fluidity and lower the field

intensity needed for permeabilization. Short incubations with 1–10 mM MβCD are typically sufficient, although excessive depletion can reduce cell viability [36, 37].

Dimethyl Sulfoxide (DMSO) is an aprotic, polar solvent that can dissolve polar and non-polar compounds, being miscible in water and in a wide variety of solvents. It has rapid penetration, and it increases the penetration of other substances through biological membranes. DMSO is commonly used as cryoprotectant [38] and as solvent for various compounds (dyes, peptides in NMR studies, drugs with skin penetration) [39, 40]. New applications for DMSO are under study, like modulation of the electroporation threshold. It was found that DMSO effect depends on its concentration [41, 42] having described three main modes of action: i/at low concentration, the membrane gets thinner, more fluid and displays undulations without being permeable to calcium ions or Yo-Pro-1 dye; ii/at intermediate concentrations, transient water pores occur and the cell swells, an exchange of ions and penetration of fluorescence dyes into the cells are observed; iii/ at even higher concentrations, the membrane integrity is lost and a disintegration of lipid bilayer occur. In cryopreservation, concentrations between 5 % v. to 20 % v. of DMSO, which preserve a good level of cell viability, are used [43]. In transfection protocols, low concentrations (1–5 % mol.) often enhance DNA or drug uptake, whereas high concentrations (> 20 % mol.) increase membrane leakage and cytotoxicity [44].

Here we propose a method for real-time evaluation of the electroporation yield at single cell level by using the intensity of the emitted fluorescence of PI incorporated by attached cells exposed to electric pulses. Further, the method efficacy was evaluated in case of cells electroporated in the presence of various concentrations of DMSO.

2. Materials and methods

Cells and chemicals

B16F10 murine melanoma cells (ATCC CRL-6475) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% L-glutamine (all produced by Sigma Aldrich), according to the American Type Culture Collection recommendations.

For cell electroporation procedure a sucrose-based buffer (85.58 g/l Sucrose, 1.125 g/L $Na_2H_2PO_4$, 0.283 g/L KH_2PO_4, 0.095 g/L $MgCl_2$ prepared with ultrapure water, with 1224 μS/cm, pH = 7.3, 286 mOsm/L) containing 10 μM propidium iodide (PI) (Fluka Biochemica, USA) was used. The DMSO – EP solutions were prepared at different concentrations by adding the corresponding amount of DMSO to the sucrose buffer to obtain final DMSO concentrations of 0, 0.15, 0.35, 0.50, 1.00, 1.50 % v/v. The effect of the DMSO addition on the buffer conductivity was negligible for all concentrations (aprox 0.5% for the maximum DMSO concentration), in good agreement with literature [45, 46].

Cell electroporation

Cells were grown for 24 h on round glass coverslips (2 cm diameter) (2×10^4 cells/ml). Cells were washed twice with 0.9% NaCl solution. The coverslip was then mounted in a lab-made EP device consisting of two L-shaped stainless-steel electrodes embedded in the wall of a chamber. The chamber was made of two threaded parts between which the glass coverslip with attached cells on was sealed, while the electrodes were in contact with the chamber's bottom (Fig. 1a). The sucrose-based buffer with 10 μM PI, without or with DMSO in different concentrations, was added (1 ml). Using the ELECTRO cell B10 pulse generator (Betatech, France) (Fig. 2), one rectangular bipolar pulse (800 V/cm amplitude), (a specific shape for electrochemotherapy procedures [47]) was applied (Fig. 1b).

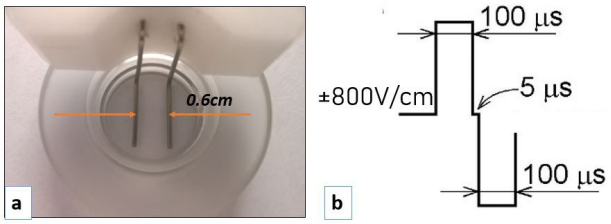

Fig. 1 (a) Lab-made EP chamber consisting of two threaded parts and two L-shaped stainless-steel electrodes positioned in contact with the coverslip of the bottom allows microscopic visualization of the cells attached to the coverslip simultaneously with application of electric pulses; (b) the parameters of electrochemotherapy-specific rectangular bipolar pulse applied to the cells

The pulses amplitude was calculated using the formula:

$$V_n = E_a \times d \tag{1}$$

where V_n is the nominal value of electric potential delivered by the pulse generator, E_a is the desired value for electric field intensity (800 V/cm) and d is the distance between the electrodes (0.6 cm). The cellular density on the glass coverslips was low, allowing us to select a cell that was parallel to the electrodes. No other cells were in close vicinity; thus, one may consider the cell to be exposed to a uniform electric field.

Real-time fluorescence recordings

The kinetics of PI cellular incorporation was measured using a RatioMaster D-104 C microscope-based spectrofluorometer, produced by Photon Technology International (PTI, Horiba Scientific, Japan) with the monochromator mounted to an inverted AxioObserver D1 fluorescence microscope (Zeiss, Germany, equipped with Canon EOS R10 camera and AxioVision™ v.4.9 software) (Fig. 2). The system allows us to select a part of the optical field (i.e., a cell) and to acquire the fluorescent signal from that area only. Dedicated FelixGX™ 4.2.1 software allows analyzing the signal in real time.

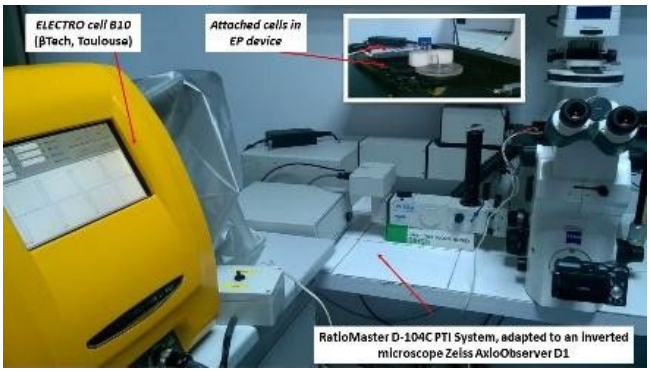

Fig. 2 Main components of the experimental setup: RatioMaster D-104 C PTI system mounted on AxioObserver D1 fluorescence microscope, pulse generator ELECTRO cell B10, and EP chamber that allows pulse application, cell visualization in bright field and fluorescence signal acquisition from a cell of interest (inset)

The main steps of the working protocol were the following: cells located between the electrodes were visualized in bright field with a 10× objective, a cell without any contact with other cells and orientated parallel to the electrodes was selected, then the magnification was changed to 100× and an image of the selected cell was acquired (Fig. 3). The fluorescence module of the microscope was initiated ($\lambda_{exitation}$ = 540 nm, Flter Set 20 Zeiss) and without any modification of the cell position, the diaphragms of the RatioMaster system were positioned to delimit an area centered on the cell of interest. The Timebased function of fluorescence signal acquisition module of FelixGX™ was turned on. The fluorescence intensity (I) was recorded for 10 minutes (one record per second). After 30 seconds, the electric pulse was delivered, without interrupting the fluorescence recording. The experiments were performed in a dark room to minimize the PI photo-bleaching.

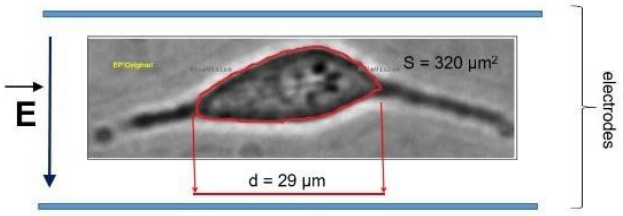

Fig. 3 Transmission image in bright field of a B16F10 cell (100× objective) oriented parallel to the electrodes and parameters: projected diameter (d) and area (S) of the cell body computed using the Measure function of AxioVision™ v.4.9 software

Data processing

For each DMSO concentration, 10 independent fluorescence recordings were acquired (using cells grown on 10 separate coverslips). The fluorescence signal was normalized using the equation:

$$Normalized\_I = \frac{I(t) - average(I_1 - I_{29})}{average(I_{30} - I_{600}) - average(I_1 - I_{29})} \quad (2)$$

where I(t) stand for the fluorescence intensity at a certain time t from the start recording moment (the indices indicate the seconds of recording). For each cell, the signal time course was fitted with a monoexponentially function, and the rate constant (k) was calculated (using R software v4.2.3). Each rate constant was normalized by division to a cell-related parameter obtained as follows: using the bright-field image of the cell (Fig. 3) the area (S) and the projected diameter with respect to the electrodes (d) of the cell body were computed using the Measure function of AxioVision™ v.4.9 software. The cell-related parameter consisted of multiplying S with d. Then, for each category of DMSO concentration, the k/Sd values were averaged and the standard deviation calculated. The statistical significance of the differences between the experimental categories was analyzed by one-way ANOVA test (R software v4.2.3).

3. Results and discussion

In Fig. 4, one may see examples of fluorescence intensities recordings for two cells, one electroporated in a buffer without DMSO (red line) and another electroporated in a buffer with 0.5 % v/v DMSO (black line) (no normalization of the signal was yet performed). Immediately after the EP pulse application (at 30 seconds), there is a fast signal increase. Once the cell membrane is permeabilized by the pulse, PI enters the cell, binds to nucleic acids (DNA, RNA) and emits a fluorescence signal. More molecules of PI bind to the nucleic acids, higher fluorescent signal. After about 250 seconds, the signal reaches a plateau, which is maintained until the end of the recording.

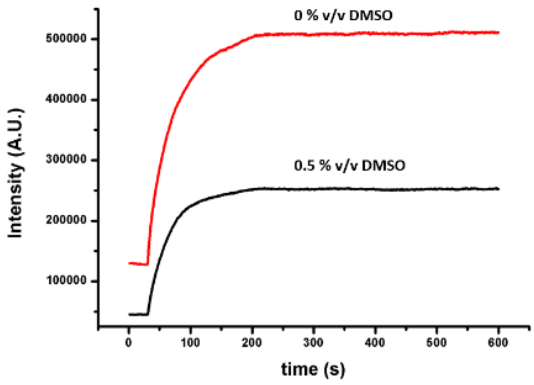

Fig. 4 Example of fluorescence intensity records (before normalization) for two cells in different experimental situations: cell electroporated in the buffer without DMSO (red line) and cell electroporated in the buffer with 0.5 % v/v DMSO (black line) (in both situations the PI concentration was 10 μM)

Fig. 5 presents an example of the fitting with a monoexponentially function of the normalized fluorescence signal and the values of fitting constants, including k.

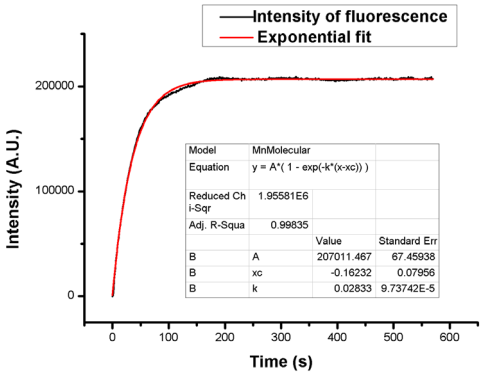

Fig. 5 Example of a normalized fluorescence intensity record fitted with a monoexponentially function, and a table with fit function constants

Fig. 6 presents the influence of the DMSO concentration on the k constant normalized to the cell body area (S) and diameter (d) projected on the electrode's direction (thus, perpendicular to the electric field direction). Fig. 7 presents the statistical significance of the differences between the experimental categories.

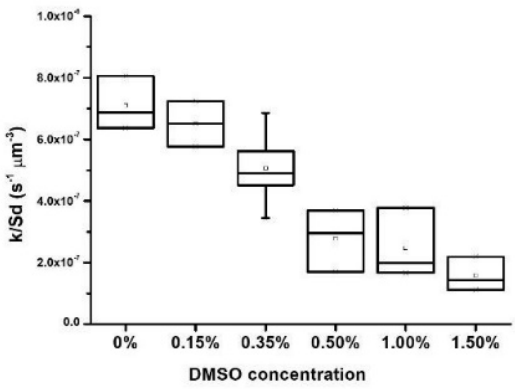

Fig. 6 Effect of DMSO concentration on the fitting k constant normalized to cell body area (S) and diameter (d) projected on the electrode's direction

As reported in the literature, the cellular EP effect depends to a great extent on the size of the cell and on the orientation of the membrane curvature toward the electric field lines [48]. Bigger cells are electroporated easier than smaller cells. [49]. The higher exposure of the membrane to the electric field, the higher values of transmembrane potential is induced, leading to a more efficient permeabilization process [27]. We mention here that attached murine melanoma cells have high variability in size (250 to 500 μm^2) and, although it was quite impossible to find cells

in a perfect parallel position, in our experiments only single cells, with their longest axis parallel to the EP electrodes, were selected for fluorescence reading. In consequence, to increase the accuracy of our method in observing the variation of fluorescence intensity, we normalized the time constant k to cell size-related parameters: cell body area (S) and diameter (d) projected on a direction perpendicular to the EP field. In Fig. 6, one can observe that the addition of low concentrations of DMSO in the electroporation buffer leads to a decrease of normalized k. For DMSO concentrations of 0.15 and 0.35 % v/v, the decrease of k was not statistically significant with respect to the control. When the concentration of DMSO was higher than 0.5 % v/v, the inhibitory effect was significant with respect to 0% v/v DMSO and even with respect to low concentrations of DMSO (k for 1.5% v/v DMSO was significantly lower than 0.15 and 0.15 and 0.35 % v/v DMSO).

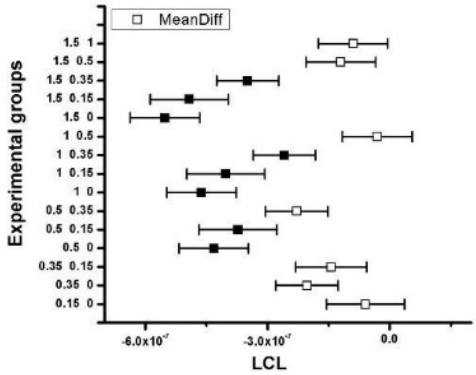

Fig. 7 Statistical significance evaluated with one-way ANOVA non-parametric test (R software v4.2.3) of various experimental groups presented as concentrations of DMSO: 0, 0.15, 0.35, 0.50, 1.00, and 1.50 % v/v; black squares - significant differences; open squares - non-significant differences

Our finding suggests an inhibitory effect of DMSO on PI incorporation and is in agreement with [50]. Notman and col. found that at this range of concentrations, DMSO makes the membrane significantly floppy, enabling cells to accommodate stresses more readily. Moreover, DMSO may promote the resealing process, which was predominate in our experimental conditions. There are literature reports leading to the hypothesis that DMSO may enhance the membrane repair processes after electroporation through complex and context-dependent mechanisms. During electroporation, transient aqueous “pores” are created in the cell membrane, causing cellular stress that can compromise viability if not rapidly repaired [51, 52]. Membrane resealing is the complex, multi-stage process by which a cell restores its barrier function. The time required for a cell to reseal is highly variable and depends on the intensity of the electroporation pulse and the resulting damage, occurring rapid (miliseconds) for small pores and much slower (seconds to minutes) for large pores. Factors influencing resealing time include the pulse strength and duration

(which determine initial pore size), temperature (affecting lipid fluidity), and cell type (different cells have different repair capacities) [2]. The rate of membrane resealing is also critically influenced by membrane tension [53]. DMSO could directly target this mechanism by reducing membrane tension and, consequently, facilitating pore closing. DMSO's role in membrane repair may be also linked to its influence on the cytoskeleton. DMSO may indirectly support the resealing by fostering an environment favorable to actin reorganization as described in [54]. Some other compounds, like surfactants, are reported to enhance the resealing by modifying the membrane itself; DMSO may have a similar, direct effect on the membrane like poloxamers [55]. Moreover, DMSO could enhance resealing rates post-electroporation not only by modulating membrane integrity, but acting on calcium signaling pathways [56]. Specifically, DMSO could facilitate the calcium influx required to trigger the membrane repairing processes [57].

In summary, DMSO appears to promote membrane resealing in our experimental conditions. These observations were possible due to the versatile method proposed for this study, method for studying membrane permeabilization at single-cell level, by recording a fluorescence signaling before, during and after the pulse delivery. This method makes possible to analyze relevant phenomena for electroporation (i.e., membrane resealing) over extended time scales of several hundred seconds in various conditions (e.g., DMSO). Various modulators of membrane permeabilization may thus be analyzed in controlled single-cell conditions.

4. Conclusion

In this work, we proposed a real-time fluorimetric method to measure the electroporation yield at single cell level based on the kinetics of the PI incorporation after the pulse delivery. We validate this method by studying the effect of DMSO presence in the EP buffer.

Membrane electro-permeabilization process may be modulated by addition of DMSO. Our work will continue with extended range of DMSO concentrations, reporter molecules with various molecular weights and electric pulse parameters (amplitudes, number of pulses, frequency of pulses, etc.) for unveiling details of DMSO effects on membrane structure when exposed to electric pulses.

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REFERENCES

[1] *M.L. Yarmush, A. Golberg, G. Serša, T. Kotnik, D. Miklavčič,* Electroporation-based technologies for medicine: principles, applications, and challenges, Annu Rev Biomed Eng, vol. **16**, Iss. July, 2014.

[2] *T. Kotnik, L. Rems, M. Tarek, D. Miklavčič,* Membrane Electroporation and Electropermeabilization: Mechanisms and Models, Annu Rev Biophys, vol. **48**, Iss. 2019.

[3] *T. Kotnik, G. Pucihar, D. Miklavcic,* Induced transmembrane voltage and its correlation with electroporation-mediated molecular transport, J Membr Biol, vol. **236**, Iss. 1, 2010.

[4] *B. Gabriel, J. Teissié,* Control by electrical parameters of short- and long-term cell death resulting from electropermeabilization of Chinese hamster ovary cells, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, vol. **1266**, Iss. 2, 1995.

[5] *J. Garrec, A. Monari, X. Assfeld, L.M. Mir, M. Tarek,* Lipid Peroxidation in Membranes: The Peroxyl Radical Does Not "Float", The Journal of Physical Chemistry Letters, vol. **5**, Iss. 10, 2014.

[6] *I. Tivig, M.G. Moisescu, T. Savopol,* Changes in the packing of bilayer lipids triggered by electroporation: real-time measurements on cells in suspension, Bioelectrochemistry, vol. **138**, Iss. April, 2021.

[7] *A. Azan, V. Untereiner, C. Gobinet, G.D. Sockalingum, M. Breton, O. Piot, L.M. Mir,* Demonstration of the Protein Involvement in Cell Electropermeabilization using Confocal Raman Microspectroscopy, Scientific Reports, vol. **7**, Iss. 1, 2017.

[8] *P.T. Vernier, Z.A. Levine, Y.-H. Wu, V. Joubert, M.J. Ziegler, L.M. Mir, D.P. Tieleman,* Electroporating Fields Target Oxidatively Damaged Areas in the Cell Membrane, PLOS ONE, vol. **4**, Iss. 11, 2009.

[9] *C. Rosazza, H. Deschout, A. Buntz, K. Braeckmans, M.P. Rols, A. Zumbusch,* Endocytosis and Endosomal Trafficking of DNA After Gene Electrotransfer In Vitro, Mol Ther Nucleic Acids, vol. **5**, Iss. 2, 2016.

[10] *C.C. Chang, M. Wu, F. Yuan,* Role of specific endocytic pathways in electrotransfection of cells, Mol Ther Methods Clin Dev, vol. **1**, Iss. January, 2014.

[11] *M. Pavlin, G. Pucihar, M. Kandušer,* The role of electrically stimulated endocytosis in gene electrotransfer, Bioelectrochemistry, vol. **83**, Iss. February, 2012.

[12] *P. Marracino, L. Caramazza, M. Liberti, F. Apollonio,* Electroporation Mechanisms: The Role of Lipid Orientation in the Kinetics of Pore Formation, Annu Int Conf IEEE Eng Med Biol Soc, vol. **2020**, Iss. July, 2020.

[13] *T. Batista Napotnik, D. Miklavčič,* In vitro electroporation detection methods – An overview, Bioelectrochemistry, vol. **120**, Iss. April, 2018.

[14] *G. Pucihar, D. Miklavcic, T. Kotnik,* A time-dependent numerical model of transmembrane voltage inducement and electroporation of irregularly shaped cells, IEEE Trans Biomed Eng, vol. **56**, Iss. 5, 2009.

[15] *A.M. Pleava, R.D. Negoita, M.A. Ilisanu, M. Mihailescu, M. Morega, V.L. Calin, E.N. Scarlat, I.A. Paun,* Holographic microscopy of cell compartments to build realistic models for electric field simulations, Romanian Reports in Phisics, vol. **75**, Iss. 1, 2023.

[16] *A.M. Sandu, M.A. Ungureanu, M. Morega, V.L. Calin, M.G. Moisescu, I.A. Paun, M. Mihailescu*, "Realistic models of cultured cells for electroporation simulations starting from phase images," in Conference on Advanced Topics in Optoelectronics, Microelectronics and Nanotechnologies X, Constanta, Romania, 2020, Spie-Int Soc Optical Engineering

[17] *R.D. Negoita, A.M. Pleava, M.A. Ilisanu, M. Mihailescu, E.N. Scarlat, V.L. Calin,* Influence of cells malignancy on pores formation under bipolar electric pulse, Romanian Reports in Physics, vol. **77**, Iss. 4, 2025.

[18] *G. Vasile,* Refractive index determination of canine oocytes using matrix-optics, UPB Scientific Bulletin, Series A: Applied Mathematics and Physics, vol. **82**, Iss. 2020.
[19] *Q. Castellví, B. Mercadal, A. Ivorra*, "Assessment of Electroporation by Electrical Impedance Methods," in Handbook of Electroporation, D. Miklavcic, Ed., ed Cham: Springer International Publishing, 2016.
[20] *P. Kramar, D. Miklavčič*, Book of the Electroporation-based Technologies and Treatments: Založba FE, 2023.
[21] *M.M. Sadik, J. Li, J.W. Shan, D.I. Shreiber, H. Lin,* Quantification of propidium iodide delivery using millisecond electric pulses: Experiments, Biochimica et Biophysica Acta (BBA) - Biomembranes, vol. **1828**, Iss. 4, 2013.
[22] *E. Petrescu, M. Calin, B. Marinescu, C. Cirtoaje,* In vivo study of mal-pdt using fluorescence spectra, UPB Scientific Bulletin, Series A: Applied Mathematics and Physics, vol. **77**, Iss. 2015.
[23] *V.L. Calin, M. Mihailescu, N. Tarba, A.M. Sandu, E. Scarlat, M.G. Moisescu, T. Savopol,* Digital holographic microscopy evaluation of dynamic cell response to electroporation, Biomedical Optics Express, vol. **12**, Iss. 4, 2021.
[24] *I. Tivig, T. Savopol, E. Kovacs, M.G. Moisescu,* An experimental system for real-time fluorescence recordings of cell membrane changes induced by electroporation, Eur Biophys J, vol. **49**, Iss. 1, 2020.
[25] *V. Novickij, N. Rembiałkowska, W. Szlasa, J. Kulbacka,* Does the shape of the electric pulse matter in electroporation?, Frontiers in Oncology, vol. **12**, Iss. September, 2022.
[26] *A. Silve, A. Guimerà Brunet, B. Al-Sakere, A. Ivorra, L.M. Mir,* Comparison of the effects of the repetition rate between microsecond and nanosecond pulses: electropermeabilization-induced electro-desensitization, Biochim Biophys Acta, vol. **1840**, Iss. 7, 2014.
[27] *J.C. Weaver, K.C. Smith, A.T. Esser, R.S. Son, T.R. Gowrishankar,* A brief overview of electroporation pulse strength-duration space: a region where additional intracellular effects are expected, Bioelectrochemistry, vol. **87**, Iss. October, 2012.
[28] *P. Simonis, A. Linkeviciute, A. Stirke,* Electroporation Assisted Improvement of Freezing Tolerance in Yeast Cells, Foods, vol. **10**, Iss. 1, 2021.
[29] *H. Mussauer, V.L. Sukhorukov, U. Zimmermann,* Trehalose improves survival of electrotransfected mammalian cells, Cytometry, vol. **45**, Iss. 3, 2001.
[30] *B. Dovgan, D. Miklavčič, M. Knežević, J. Zupan, A. Barlič,* Intracellular delivery of trehalose renders mesenchymal stromal cells viable and immunomodulatory competent after cryopreservation, Cytotechnology, vol. **73**, Iss. 3, 2021.
[31] *I. Tsoneva, I. Iordanov, A.J. Berger, T. Tomov, B. Nikolova, N. Mudrov, M.R. Berger,* Electrodelivery of drugs into cancer cells in the presence of poloxamer 188, J Biomed Biotechnol, vol. **2010**, Iss. August, 2010.
[32] *J.G. Moloughney, N. Weisleder,* Poloxamer 188 (p188) as a membrane resealing reagent in biomedical applications, Recent Pat Biotechnol, vol. **6**, Iss. 3, 2012.
[33] *A. Crabtree, N. Boehnke, F. Bates, B. Hackel,* Consequences of poly(ethylene oxide) and poloxamer P188 on transcription in healthy and stressed myoblasts, Proceedings of the National Academy of Sciences, vol. **120**, Iss. 18, 2023.
[34] *Y.G. Zhao, H.L. Lu, J.L. Peng, Y.H. Xu,* Inhibitory effect of Ca2+ on in vivo gene transfer by electroporation, Acta Pharmacol Sin, vol. **27**, Iss. 3, 2006.
[35] *S.K. Frandsen, M. Vissing, J. Gehl,* A Comprehensive Review of Calcium Electroporation -A Novel Cancer Treatment Modality, Cancers (Basel), vol. **12**, Iss. 2, 2020.
[36] *I. Park, S. Choi, Y. Gwak, J. Kim, G. Min, D. Lim, S.W. Lee,* Microfluidic Electroporation Arrays for Investigating Electroporation-Induced Cellular Rupture Dynamics, Biosensors (Basel), vol. **14**, Iss. 5, 2024.

[37] *S.N. Wu, C.C. Yeh, H.C. Huang, W.H. Yang,* Cholesterol depletion with (2-hydroxypropyl)- β-cyclodextrin modifies the gating of membrane electroporation-induced inward current in pituitary tumor GH3 cells: experimental and analytical studies, Cell Physiol Biochem, vol. **28**, Iss. 5, 2011.
[38] *W.F. Rall, G.M. Fahy,* Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification, Nature, vol. **313**, Iss. 6003, 1985.
[39] *K. Marren,* Dimethyl sulfoxide: an effective penetration enhancer for topical administration of NSAIDs, Phys Sportsmed, vol. **39**, Iss. 3, 2011.
[40] *K. Capriotti, J.A. Capriotti,* Dimethyl sulfoxide: history, chemistry, and clinical utility in dermatology, J Clin Aesthet Dermatol, vol. **5**, Iss. 9, 2012.
[41] *M.A. de Ménorval, L.M. Mir, M.L. Fernández, R. Reigada,* Effects of dimethyl sulfoxide in cholesterol-containing lipid membranes: a comparative study of experiments in silico and with cells, PLoS One, vol. **7**, Iss. 7, 2012.
[42] *A.A. Gurtovenko, J. Anwar,* Modulating the Structure and Properties of Cell Membranes: The Molecular Mechanism of Action of Dimethyl Sulfoxide, The Journal of Physical Chemistry B, vol. **111**, Iss. 35, 2007.
[43] *C.B. Morris*, "Cryopreservation of Animal and Human Cell Lines," in Cryopreservation and Freeze-Drying Protocols, J. G. Day and G. N. Stacey, Eds., ed Totowa, NJ: Humana Press, 2007.
[44] *H. Melkonyan, C. Sorg, M. Klempt,* Electroporation Efficiency in Mammalian Cells is increased by Dimethyl Sulfoxide (DMSO), Nucleic Acids Research, vol. **24**, Iss. 21, 1996.
[45] *T. Prem Kumar, P.V.S.S. Prabhu, A.K. Srivastava, U. Bejoy Kumar, R. Ranganathan, R. Gangadharan,* Conductivity and viscosity studies of dimethyl sulfoxide (DMSO)-based electrolyte solutions at 25 °C, Journal of Power Sources, vol. **50**, Iss. 3, 1994.
[46] *L.J. Yang, X.Q. Yang, K.M. Huang, G.Z. Jia, H. Shang,* Dielectric properties of binary solvent mixtures of dimethyl sulfoxide with water, Int J Mol Sci, vol. **10**, Iss. 3, 2009.
[47] *M. Marty, G. Sersa, J.R. Garbay, J. Gehl, C.G. Collins, M. Snoj, V. Billard, P.F. Geertsen, J.O. Larkin, D. Miklavcic, I. Pavlovic, S.M. Paulin-Kosir, M. Cemazar, N. Morsli, D.M. Soden, Z. Rudolf, C. Robert, G.C. O'Sullivan, L.M. Mir,* Electrochemotherapy – An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study, European Journal of Cancer Supplements, vol. **4**, Iss. 11, 2006.
[48] *B. Valic, M. Golzio, M. Pavlin, A. Schatz, C. Faurie, B. Gabriel, J. Teissié, M.P. Rols, D. Miklavcic,* Effect of electric field induced transmembrane potential on spheroidal cells: theory and experiment, Eur Biophys J, vol. **32**, Iss. 6, 2003.
[49] *B. Henslee, A. Morss, X. Hu, G. Lafyatis, L. Lee,* Electroporation Dependence on Cell Size: Optical Tweezers Study, Analytical chemistry, vol. **83**, Iss. June, 2011.
[50] *R. Notman, M. Noro, B. O'Malley, J. Anwar,* Molecular Basis for Dimethylsulfoxide (DMSO) Action on Lipid Membranes, Journal of the American Chemical Society, vol. **128**, Iss. December, 2006.
[51] *G. Blumrosen, A. Abazari, A. Golberg, M.L. Yarmush, M. Toner,* Single-step electrical field strength screening to determine electroporation induced transmembrane transport parameters, Biochimica et Biophysica Acta (BBA) - Biomembranes, vol. **1858**, Iss. 9, 2016.
[52] *L. Rubinsky, E. Guenther, P. Mikus, M.K. Stehling, B. Rubinsky,* Electrolytic Effects During Tissue Ablation by Electroporation, Technology in Cancer Research & Treatment, vol. **15**, Iss. 5, 2016.
[53] *T. Togo, T.B. Krasieva, R.A. Steinhardt,* A Decrease in Membrane Tension Precedes Successful Cell-Membrane Repair, Molecular Biology of the Cell, vol. **11**, Iss. 12, 2000.

[54] *D.L. Perrier, A. Vahid, V. Kathavi, L. Stam, L. Rems, Y. Mulla, A. Muralidharan, G.H. Koenderink, M.T. Kreutzer, P.E. Boukany,* Response of an Actin Network in Vesicles Under Electric Pulses, Scientific Reports, vol. **9**, Iss. 1, 2019.

[55] *S.E. Burgess, Y. Zhao, A. Sen, S.W. Hui,* Resealing of Electroporation of Porcine Epidermis Using Phospholipids and Poloxamers, International Journal of Pharmaceutics, vol. **336**, Iss. 2, 2007.

[56] *Y. Zhou, J. Shi, J. Cui, C.X. Deng,* Effects of Extracellular Calcium on Cell Membrane Resealing in Sonoporation, Journal of Controlled Release, vol. **126**, Iss. 1, 2008.

[57] *T. Togo,* Disruption of the Plasma Membrane Stimulates Rearrangement of Microtubules and Lipid Traffic Toward the Wound Site, Journal of Cell Science, vol. **119**, Iss. 13, 2006.