November 16 - December 4, 2020
Online Event, Ljubljana, Slovenia

Proceedings and workbook of the

Electroporation-based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

Edited by:

Peter Kramar
Damijan Miklavčič

Organised by:

University of Ljubljana
Faculty of Electrical Engineering

Institute of Oncology, Ljubljana

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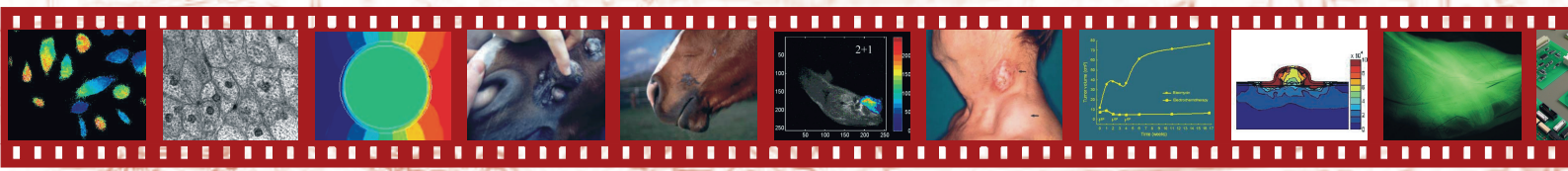

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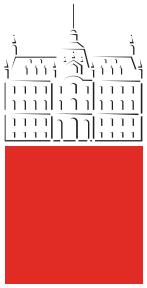

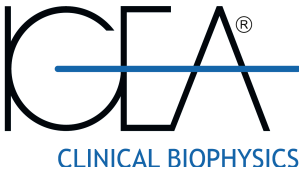

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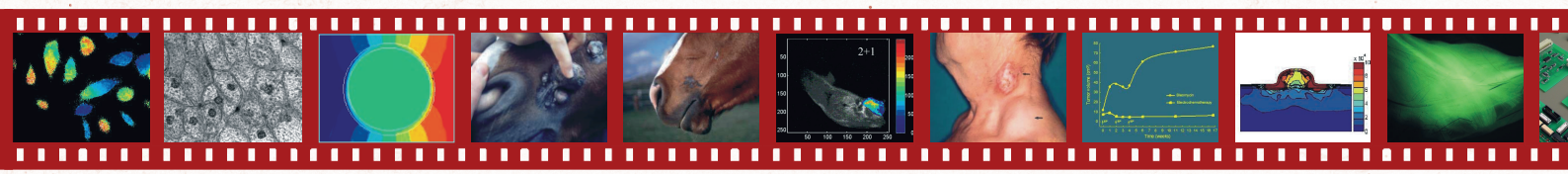

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Welcome note

Dear Colleagues, dear Students,

The idea of organizing the Workshop and Postgraduate Course on Electroporation-Based Technologies and Treatments (EBTT) at the University of Ljubljana had been developing for several years. After preliminary discussions, the Workshop and Course was organised for the first time in 2003. In 2020 the Course is organised for the 14th time! In these fourteen years, the Course has been attended by 844 participants coming from 41 different countries. And this year again – despite pandemics - we can say with great pleasure: "with participation of many of the world leading experts in the field" – unfortunately, as an online only event. The goals and aims of the Workshop and Course however remain unchanged: to provide the participants with sufficient theoretical background and practical knowledge to allow them to use electroporation effectively in their working environments.

It is a great pleasure to welcome you to the EBTT and in particular to the practical lab work taking place in the virtual space of University of Ljubljana, Faculty of Electrical Engineering organised as an integral part of the Interdisciplinary doctoral programme Biomedicine. From the very beginning we were aiming to prepare lab work for participants, which would complement the lectures. As preparing lab work takes more time than preparing and organizing lectures, we introduced lab work at the second workshop in 2005. Lab work covers different aspects of research: biological experiments taking place in the cell culture labs, microbiological lab, lab for tissue and planar lipid bilayer; numerical and molecular dynamics modelling, e-learning using computer classrooms, pulse generator development and electrical measurements using electronic laboratory workshop and magnetic resonance electrical impedance tomography.

This year we were faced with a new challenge as the Course is organised fully online due to Covid-19 pandemics. We have prepared in advance for this situation by prerecording wet lab practials and adapting those that can be conducted as on-line (*e.g.* modelling, computer simulations). The team here in Ljubljana (and Nancy) was working hard and will provide live webinars of the lab works so that you will be able to benefit most even if not actually being in the lab. We understand this is not perfect but we would nevertheless like to offer you the opportunity to learn as much as possible.

The biological experiments were pre-recorded in the labs of the network of research and infrastructural centre MRIC, University of Ljubljana, at the Faculty of Electrical Engineering in the Laboratory of Biocybernetics. Lab work would not be possible without extensive involvement and commitment of numerous members of the Laboratory of Biocybernetics and Igor Serša from Jožef Stefan Institute for what I would like to thank them all cordially.

It also needs to be emphasized that all written contributions collected in the proceedings have been reviewed and then thoroughly edited by Peter Kramar. We thank all authors and editors. Also, I would like to express our sincere thanks to the faculty members and invited lecturers for their lectures delivered during the course. Finally I would like to thank our sponsors who are making our EBTT possible: Educell (Slovenia), EnergyPulse Systems (Portugal), IGEA (Italy), Iskra Medical (Slovenia), Laboratorij-um (Slovenia), Medtronic (USA), Micro+Polo (Slovenia), Omega (Slovenia), and Pulse Bioscience (USA).

Thank you for participating in our Workshop and Course. We sincerely hope that you will benefit from being with us.

Sincerely Yours,

Damijan Miklavčič

In memoriam: Justin Teissié (1947–2020)

Justin Teissié, was a devoted scientist and a founding faculty member and still an active faculty member of the EBTT. Justin Teissié has dedicated his life to the study of biological membranes and the fundamental processes leading to membrane permeabilisation, and has played a major role in the development of its applications. He has been equally aware of the need and importance of developing fundamental knowledge and understanding of cell membrane electropermeabilisation, as well as of developing and promoting use of electroporation in medicine and biotechnology. For his work he received many honours: winner of the Prize for Medical Applications of Electricity awarded by the Institut Electricité Santé in 1995, CNRS honorary medallist in 2013 and laureate of the American Institute for Medical and Biological Engineering (AIMBE) in 2017. He became an Honorary Senator of the University of Ljubljana in 2015, and was Emeritus CNRS Research Director since 2012. Although retired, he never ceased to be an extremely involved and active researcher; Justin Teissié lived until his last days for Science. His passing creates an immense void in our community. His power of work, his integrity and scientific rigor, but also his humour, his great generosity, and his availability were exemplary. He was always posing questions, continuously reminding us that our knowledge is still incomplete, and even though we sometimes had an impression that we have done something completely new and complete, the literature search revealed Justin had at least addressed that specific question before. We, his students, colleagues, and friends, will never forget him, and will keep him in our memories. We will remember how invigorating scientific discussions with him were, and how he was capable of influencing our research with a simple question.

Marie-Pierre Rols and *Damijan Miklavčič*

INVITED LECTURERS

Nerve Impulses: Avoiding Them in and by Electroporation and Inducing Them with Injectable Microstimulators for Movement Restoration in Paralysis

Antoni Ivorra, *Department of Information and Communication Technologies, Universitat Pompeu Fabra, Carrer Roc Boronat 138, 08018, Barcelona, SPAIN*

INTRODUCTION

In this talk I will briefly present some recent studies conducted by the Biomedical Electronics Research Group of Universitat Pompeu Fabra that are about electroporation and electrical stimulation. In particular, I will focus on aspects that lie in the boundary between both phenomena.

Electrical stimulation of peripheral nerves is frequently an unwanted consequence of the delivery of currents to body tissues. This is, for instance, the case in conventional electroporation treatments; the delivery of relatively long (>10 μs) monophasic pulses causes pain and muscle contractions. In [1] we numerically explained how the delivery of bursts of short (≤ 5 μs) biphasic square pulses, in a technique referred to as H-FIRE, can cause irreversible electroporation (IRE) whilst minimizing stimulation. We then *in vivo* demonstrated that sinusoidal waveforms can also perform ablation by IRE and avoid muscle contractions [2]. Dwelling further on the delivery of high-frequency currents for therapeutics, we learnt about the non-ablative use of bursts of radiofrequency current for chronic pain treatment and we hypothesized that electroporation could somehow be related to their mechanism of action. In a recent publication we report multiple *in vitro* evidence of an electroporation mediated calcium uptake when high frequency (500 kHz) sinusoidal bursts are delivered and discuss how such uptake could explain pain relief [3].

Electrical stimulation of peripheral nerves would also be an adverse consequence of performing wireless power transfer (WPT) to implants by means of volume conduction at low frequencies. However, as we have shown recently [5], WPT based on volume conduction of high frequency (>1 MHz) currents is doable: powers above 1 mW can be obtained in very thin (diameter < 1 mm) and short (length < 15 mm) implants when ac fields that comply with safety standards are present in the tissues where the implants are located. This is one of the main operating principles of a technology we have proposed and *in vitro* demonstrated for the development of injectable passive sensors [7].

On the other hand, electrical stimulation of excitable tissues is intentionally performed for different clinical purposes. For instance, electrical stimulation of motor nerves is performed to restore motor functions in paralysis patients. A major limitation of electrical stimulation for paralysis is that it rapidly induces muscle fatigue. We recently *in vivo* demonstrated that such fatigue can be minimized or avoided if multiple portions of the muscles are stimulated asynchronously [6]. Such functionality is not feasible with existing technology for stimulation. Neuromuscular stimulation is typically performed with relatively large and invasive implants which contain bulky components (e.g. batteries or coils) for power. Each stimulation channel activates a whole muscle or a group of muscles. For being able to selectively activate portions of a muscle, we are developing a technology for the implementation of wireless networks of thin and flexible intramuscular implants to be deployed by injection [4,5].

ACKNOWLEDGEMENTS

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I gratefully acknowledge the financial support by ICREA under the ICREA Academia programme.

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Characterization of the Effects of Nanosecond Electric Pulses on Neuroendocrine Adrenal Chromaffin Cells

Gale Louise Craviso[1], Normand Leblanc[1], Thomas Gould[2], Josette Zaklit[3], Ji Hwan Yoon[3], Indira Chatterjee[3], *[1] Department of Pharmacology and [2] Physiology and Cell Biology, University of Nevada, Reno School of Medicine, Reno, Nevada, USA and [3] Department of Electrical and Biomedical Engineering, University of Nevada, Reno, USA*

INTRODUCTION

There is a great deal of interest in developing electrostimulation approaches that can selectively target specific regions of the body non-invasively and thus without the need for implanted electrodes. To work toward this goal, we have been conducting a comprehensive study of the interaction of electric pulses in the low nanosecond regime (sub-10 ns) with excitable chromaffin cells isolated from the adrenal medulla. Chromaffin cells, which mediate the "fight or flight" response by releasing catecholamines into the circulation, have served as a well-established model of neural-type/neuroendocrine cells. In our studies we employ numerical cell modelling and a variety of experimental approaches to elucidate the mechanisms by which neural cell excitability is altered at the cellular and molecular levels.

RESULTS

Exposing chromaffin cells to a single 5 ns pulse is sufficient to stimulate the release of the catecholamines epinephrine and norepinephrine via Ca^{2+}-dependent exocytosis [1], the same process used by neurons to release neurotransmitters. The source of Ca^{2+} is extracellular, where Ca^{2+} enters the cells via voltage-gated Ca^{2+} channels (VGCC). While much effort has gone into elucidating the mechanism underlying VGCC activation, which we found relies on Na^+ influx that leads to membrane depolarization [2,3], we have not yet established whether the plasma membrane pathway by which Na^+ enters the cell (Figure 1) is indicative of electropermeabilization and/or activation of a non-selective cation channel. Those studies are ongoing. Regardless, the sequence of events that leads to catecholamine release in cells exposed to a 5 ns pulse is similar to that evoked by acetylcholine, the physiological stimulus of chromaffin cells.

In other studies we also found that a 5 ns pulse differentially inhibits cell membrane macroscopic ion currents, which in chromaffin cells comprise a mixture of Na^+, Ca^{2+} and K^+ currents [4]. Thus, we have identified another way in which chromaffin cell excitability is affected by a single nanosecond pulse and are currently investigating the cellular processes by which this occurs.

While we will continue to use primary cultures of adrenal chromaffin cells for our studies, we recognize the need to investigate effects of nanosecond pulses on these cells in a more physiological setting. Consequently, our efforts now include exposing chromaffin cells in intact adrenal gland tissue, in particular, adrenal tissue from transgenic mice in which the cells are targeted to express specific genetically-encoded fluorescent reporters.

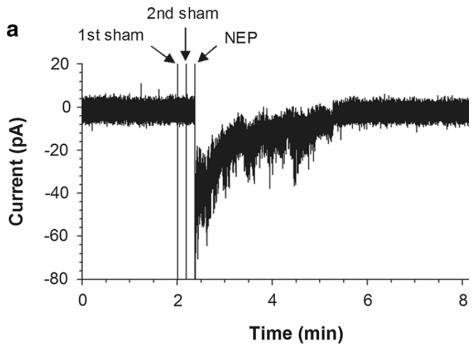

Figure 1: Typical whole-cell current recorded in a patch clamped chromaffin cell following exposure to a 5 ns pulse (NEP). The inward current, which is carried mainly by Na^+, is recorded with a delay of 8 ms after the pulse is delivered to the cell. From [2].

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Effect of Pulsed Electric Fields processing on plant-based foods

Indrawati Oey [1], [1] *Department of Food Science, University of Otago, Dunedin, NEW ZEALAND*

Pulsed electric field processing (PEF) technology has been used in food industries. This technique applies short pulses (microsecond ranges) of high voltages to samples placed between two electrodes. PEF processing could result in pore formation of cell membranes, a phenomenon called electroporation which can be irreversible or reversible - dependent on the treatment intensity applied. Under low or mild PEF processing intensities, the pores formed in the cell membrane can reseal after pulse application. Reversible electroporation can be used to induce stress reactions in plant tissues, stimulate metabolic activity and increase the biosynthesis of secondary metabolites such as production of antioxidants. While, at high PEF processing intensities, irreversible electroporation can occur and this phenomenon has been utilised as a processing aid by food industries to improve the mass transfer of metabolites prior to dehydration, extraction, or pressing of foods. Various studies have shown that PEF can enhance the extraction of juice, reduce the cutting force and improve the drying and freezing processes of plant based foods.

Most PEF studies have used liquid, semi-solid or solid plant materials that have been mechanically fragmented prior to PEF treatment. The damage caused by mechanical fragmentation (e.g. mashing, slicing, dicing or other mechanical breakdown) prior to PEF is likely to affect the nature of any PEF induced changes, which limits the widespread of PEF technologies for plant produce processing.

Plant-based foods such as fruits and vegetables are made up of many cell and tissue types (e.g. vascular tissues and ground tissues) with varying electrical and topological properties, which determine their sensitivity to electroporation. Hence, their heterogeneous structures need to be taken into consideration when investigating PEF-induced structural changes in plant cells, tissues and organs. Additionally, when placing plant materials with heterogeneous structures in a homogeneous electric field, the uniformity of the PEF effect across the plant materials can be disrupted and the heterogeneity of PEF induced structural changes in intact plant based foods could affect the subsequent processing.

Unfortunately, fruits and vegetables are often considered as being 'homogenous' plant structures. The potential for using PEF for industrial processing of solid plant produce has been underestimated due to a limited understanding of PEF effects on solid plant materials. For industrial applications, it is important to understand the relationship between PEF induced structure modification and the subsequent industrial applications (i.e., cutting, frying, extraction etc.). This lecture discusses different methodologies that have been used to assess the effect of PEF treatment on fruits and vegetables and the applications of PEF for fruits and vegetables processing.

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DNA Immunization Protocols by Electrotransfer

Emanuela Signori[1,2], [1]*CNR, Institute of Translational Pharmacology,* [2]*University Campus Bio-Medico of Rome, School of Medicine, Rome, ITALY*

INTRODUCTION

Important advances in understanding the biology of the immune system are leading to new therapeutic strategies for the treatment of infectious and cancer diseases, such as those based on gene therapy [1, http://www.abedia.com/wiley/]. Gene Electro-Transfer (GET) is an efficient method that involves the use of Electroporation (EP) after the injection of nucleotide sequences coding antigens or immunomodulatory molecules, delivered by different viral or non-viral vectors [1], to elicit or enhance an immune response into the host.

ADVANTAGES OF GET IMMUNIZATION

Application of controlled electric pulses at the injection site induces a transient perturbation of the cell membrane leading to a higher uptake of therapeutic nucleotides [2-4]. Mild local tissue damage induced by EP plays a role in enhancing both arms of the immune response, making EP a good approach for immunization protocols [5]. In the last two decades, GET strategy by employment of plasmid DNA demonstrated its efficacy and safety in pre-clinical and clinical vaccination protocols for infectious and cancer diseases [7]. Many anti-cancer GET therapies are based on the delivery of interleukin-12 [8].

CRITICAL POINTS OF GET IMMUNIZATION

Different routes of plasmid DNA administration (intradermal, intramuscular, intratumoral, peritumoral), different doses of plasmid DNA, combined treatments, as well as appropriate choice of electric parameters and electrodes, *in vivo* models and mathematical modeling for effective GET therapies [9-12], are essential steps of investigation for the translation of DNA EP pre-clinical protocols into clinical applications.

CONCLUSIONS

Several clinical trials based on GET have been started [7,13]. Due to the recent results in this field, we can be confident good improvements in electrogene immunotherapy will be reached soon, so opening new perspectives for therapies able to increase the patients' quality of life in the next future [14].

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The applications of IRE/ECT in clinical practice

L. Razakamanantsoa, MD[1, 2]; G. Srimathveeravalli, PhD[2]; O. Seror, MD, PhD[3]; F.H. Cornelis, MD, PhD[1] ; *[1]Sorbonne University, Paris, France, [2]University of Massachusetts, Amherst, USA, [3]University Paris 13, Paris, FRANCE*

Percutaneous irreversible electroporation (IRE) and electrochemotherapy (ECT) have been introduced in the past few years in Interventional Radiology. Various successful clinical applications have been reported so far.

The liver was found to be a suitable target for percutaneous IRE. In the case of small tumors efficacy varies widely (45.5-100%) among teams [1], but efficacy remains limited in case of larger tumors > 5 cm [2]. Compared to radiofrequency ablation (RFA) [3–5], IRE allows liver regeneration in patients without severe cirrhosis or previous chemoembolization and can potentially be mooted in selected patients unsuitable for other local treatments [6,7]. IRE efficacy may be limited in case of metallic implants [8]. ECT was also found to be effective to treat primary or secondary liver tumors that are unresectable because adjacent to portal vein or bile duct [9–12].

Regarding pancreatic cancer, which was thought to be an excellent indication for IRE/ECT, results are controversial with high morbidity and limited effectiveness, although some studies reported an improvement in long-term outcomes compared to other therapies [13]. A clinical trial in phase I/II using ECT with bleomycin for the treatment of metastatic unresectable pancreatic cancer is presently under investigation (NCT03225781).

IRE seems interesting for nephron, urinary-tract sparing treatment of renal tumors. It has been demonstrated in animals to safeguard urine-collecting system by preserving normal morphology and function, allowing urothelial regeneration without urine leakage or stenosis after treatment [14–16]. Therefore, IRE was used in case of small renal masses up to 4 cm [14,17]. However, more clinical evidence is still required to translate this technic to a standard option such as RFA. A case-report demonstrated tumor control after ECT with no evidence of residual disease [18].

Focal therapy for localized clinically significant prostate cancer (PCa) offers an alternative to radical treatment aiming to eliminate PCa while preserving benign prostatic tissue, bladder neck, sphincter, and adjacent neurovascular structures to minimize treatment-related toxicity [19]. A study supported the safety and feasibility of IRE as a primary treatment for localized PCa with effective short-term oncological control in carefully selected men [20]. ECT was used in a patient presenting with a PCa and infiltration of the urethral sphincter with low toxicity, mild adverse events and absence of tumor activity or impairment in the surrounding tissues or organs 6 months after treatment [21].

In bone, IRE might be an accurate treatment alternative close to sensitive structures such as nerves [22]. However, contrary to some animal studies, permanent neural function impairment can occur [23]. ECT seems more tolerate in well-selected patients with bone metastases of the spine, pelvis or appendicular skeleton with no intra- or post-operative complications and with an improvement of pain (>50%) in 84% of the cases [24,25]. The Italian Society of Orthopedics and Traumatology (SIOT) has included now this therapy in the guidelines for the management of unresectable tumors of the sacrum.

IRE was supposed to be effective in lung cancer treatment but clinical translation failed to demonstrate it, probably explained by high differences in electric conductivity between lung parenchyma and tumor tissue [26]. Animal studies demonstrated encouraging results for ECT [15][27].

Several other clinical trials are investigating the use of ECT for malignant melanoma (NCT03448666), vulvar carcinoma (NCT03142061), head and neck cancer (NCT 02549742). Finally, the use of calcium combined to electroporation unveiled to be a new, safe and inexpensive antitumor treatment demonstrating a response rate comparable to ECT with bleomycin (72% and 84%, p=0.5) with less ulceration (38% vs. 68%) in superficially cutaneous metastatic melanoma or breast cancer tumors [28].

To improve the evidence on safety and efficacy strengthening the use of IRE/ECT in clinical practice, prospective trials comparing them to other therapies such as radiation therapy or thermal ablation are needed. Unlike surgery, the additional ability to stimulate the immunogenic system to enhance tumor response, creating a potential immunogenic window remains a promise for IRE/ECT in clinical research [29–31].

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SHORT PRESENTATIONS

Differentiation of sublethal and lethal pulsed electric field effects on *E. coli* cells by using SYTOX™ Green

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INTRODUCTION

Depending on the intensity, pulsed electric field (PEF) can lead to reversible or irreversible electroporation [1] of bacterial cell membranes. This permeabilization can cause either cell death (lethal injury) or temporary damage (sublethal injury) [2]. The evaluation of bacterial cells by flow cytometry (FCM) shows excellent potential to differentiate those effects in addition to conventional culture-based analyses. The cell impermeant fluorescence stain SYTOX™ Green was already proposed for bacterial viability assessment [3], besides the typically used combination of the DNA-intercalating dyes, SYBR green and propidium iodide (SGPI).

MATERIAL AND METHODS

In this study, SYTOX™ Green is tested to detect PEF induced stress on gram-negative *E. coli* DSM 1116 and to differentiate between sublethally and lethally injured cells by the combined use of FCM and plate count technique on selective and non-selective media. Plate counts were presented as log ($N\ N_0^{-1}$), while for FCM data was grouped in dot plots. For PEF treatment, a static parallel plate chamber is used. Monopolar square wave pulses are generated at field strengths between 6 to 25 kV cm^{-1} and ten pulses with a total specific energy input of 1.3 up to 55.0 kJ kg^{-1}.

RESULTS

For FCM analysis, two subpopulations of *E. coli* cells are clustered in dot plots within four quadrants (R1, R2, R3, and R4): Intact, non-stained (R1: SYTOX-, FL1- & FL3-) and SYTOX-stained cells (R2: SYTOX+, FL1+, FL3- and R3: SYTOX+, FL1+, FL3+) (s. Figure 1).

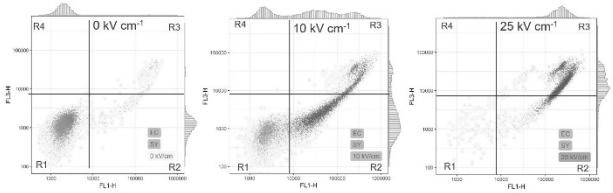

Figure 1: Flow cytometry dot plots of FL-1 (green fluorescence) vs. FL-3 (red fluorescence) of PEF-treated (0, 10 and 25 kV cm^{-1}) *E. coli* cells. Cells were stained directly before PEF treatment. The symbols R1 - R4 refer to the quadrant denotations.

After PEF treatment with 10 kV cm^{-1}, a prominent amount of cells shifted from quadrant R1 to R2, and after 25 kV cm^{-1}, a noticeable amount moved from R2 to R3. This shift indicates increased membrane permeability and cell damage induced by electroporation and further could suggest the influence of PEF intensity on the membrane functionality and integrity.

The culture-based analysis showed a maximum inactivation of -2.95 ± 0.38 log *E. coli* cells at 25 kV cm^{-1} (s. Figure 2). At 10 kV cm^{-1} only -1.0 ± 0.4 log cycles are inactivated. Additionally, the highest sublethal injury, with more than 90%, is found at 10 kV cm^{-1}, while at 25 kV cm^{-1}, the sublethal fraction makes up less than 65%.

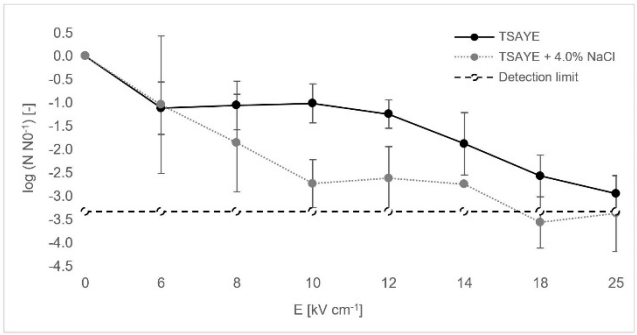

Figure 2: Inactivation of *E. coli* cells after PEF treatment between 6 and 25 kV cm^{-1}. To distinguish between sublethally and lethally injured cells, *E. coli* cells were plated on non-selective (= TSAYE) and selective (= TSAYE + 4.0% NaCl) media. Difference between the two inactivation lines are considered as sublethally injured cells.

CONCLUSION

This approach confirms the successful use of the less studied dye SYTOX™ Green for the rapid detection of PEF induced stress on *E. coli* cells. However, as SYTOX Green™ only indicates cell membrane integrity, further research should focus on the distinction of sublethally and lethally injured cell fractions by other staining methods without additional culture-based analysis.

ACKNOWLEDGMENT

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Irreversible electroporation and radiofrequency ablation: A computational modelling comparison of lesion morphology in cardiac tissue

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INTRODUCTION

Radiofrequency (RF) ablation is the most extended non-pharmacological treatment for atrial fibrillation (AF). However, it can cause thermal damage in surrounding organs. Irreversible electroporation (IRE) is a new non-thermal ablation technique that preserves the extracellular matrix, which favors its recellularization, avoiding the negative effects of RF. IRE traditionally applies monophasic pulses of 100 µs. However, it requires the use of sedation or anesthesia because of the occurrence of muscle stimulation. As an alternative to reduce it, high-frequency biphasic pulses have been proposed, allowing a monopolar catheter configuration. The objective is to compare the injury morphology of both techniques by using a computational model.

METHODS

The 3D models follow the geometry described in [1]. They consist of a fragment of a cardiac chamber filled with circulating blood, with a catheter inserted 0.5 mm deep and perpendicular to the tissue, and a dispersive electrode at the base of the tissue to simulate a monopolar catheter configuration. Materials are those used in the same study. The models are based on triple coupling of electric-thermal problem and fluid dynamics. Electrical and thermal conductivity of cardiac tissue are considered temperature-dependent [2]. In IRE model, electrical conductivity with electric field dependence is included [3]. In RF model, constant voltage ablation method has been used, setting an arbitrary voltage for 60 s. The IRE protocol uses 20 bursts of high-frequency biphasic pulses at 150 kHz. Duration of the burst is 100 µs. Amplitudes were adjusted to obtain a depth of injured tissue of 2 mm from the catheter tip. Blood velocity is set to 3 cm/s.

To evaluate lesion morphology, RF model considers tissue temperatures above 50 ºC. IRE model considers the affected tissue by an electric field greater than 700 V/cm. This value has been arbitrarily chosen due to the lack of experimental data that offer a threshold value for irreversible electroporation in cardiac tissue.

RESULTS

Figure 1 shows the volume within the white line as injured tissue in both models. The volume of damaged tissue in RF (9.74 mm^3) is 2.6 times smaller than in IRE (25.33 mm^3). With the settings established in this study, to cause a lesion 2 mm deep, the RF voltage obtained is 17.5 V while for IRE is 780 V. Regarding lesion geometry, the RF model predicts a more irregular geometry than in the IRE model due to the influence of blood flow. Interestingly, for this catheter geometry, the IRE lesion is wider than the RF lesion.

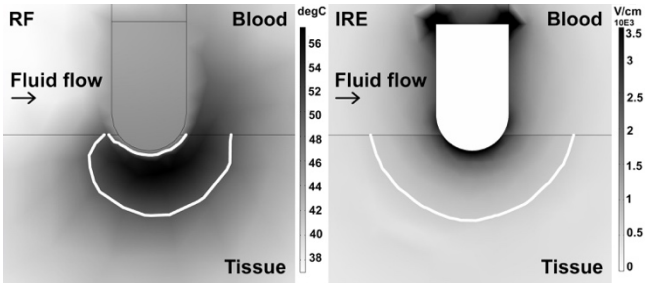

Figure 1: (RF) Temperature solution of radiofrequency model. White line shows the boundary at 50 ºC. (IRE) Electric field solution of irreversible electroporation model. White line shows the boundary at 700 V/cm.

CONCLUSIONS

To obtain a lesion depth of 2 mm (thickness of the atrium), a 17.5 V amplitude needs to be applied in RF and 780 V in IRE. The blood flow generates an irregular lesion in RF. In IRE the influence of flow is minimal, obtaining a regular geometry of the lesion. Our results suggest that IRE may have lower spatial resolution than RF with wider lesions for equal depth. This may be translated in fewer application sites for IRE compared to RF to cause a continuous lesion of the same size. Further studies should be performed to verify this statement.

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High-Voltage Generator for Delayed Bipolar and Paired Nanosecond Pulsed Electric Fields (nsPEF)

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INTRODUCTION

Cancellation or increase of the classical bio-effects induced by unipolar nanosecond pulsed electric fields (nsPEF) have been observed when bipolar –a negative polarity pulse follows a positive polarity pulse– [1] or paired –two identical pulses with positive polarity– [2] nsPEF are applied, respectively. However, the precise mechanism responsible for these phenomena remains unknown. The possibility to explore different pulse durations as well as different delays between a positive and a negative polarity or two positive polarities might be of interest to provide new insights for the understanding of the above-mentioned events. The aim of this work is to introduce a novel versatile high-voltage generator able to provide 10-ns bipolar or paired pulses with very short interphase intervals between 17 and 360 ns.

METHODS

The generator proposed in this study exploits the frozen-wave concept fully detailed in [3]. The main components are: 1) a high-voltage DC power supply (SR20kV-300W, Technix, France), 2) two photoconductive switches (PCSSs) embedded in a 2- and 3- port coaxial boxes filled with oil, 3) a high-energy 3-ns pulsed laser (PL2241A, Ekspla, Lithuania), 4) a tap-off (Model 245-NMFFP-100, Barth Electronics, Inc, NV, USA), connected between the generator and the electrodes, for the real-time measurement of the generated pulses, and 5) a 12-GHz oscilloscope (DSO, TDS6124C, Tektronix, USA) for the visualization of the pulses. The energy delivered by the high-voltage DC power supply is stored into a transmission line (coaxial cable) and conveyed to the load (biological cells) through a pair of electrodes when the laser optically triggers, simultaneously, the two PCSSs. The 3-port box is connected to the high-voltage DC power supply, the tap-off, and the 2-port box. Depending on the termination at the output of the 2-port coaxial box it is possible to generate unipolar, bipolar, or paired pulses by using a 50 Ω terminator, short circuit, or open circuit, respectively. The duration of the interphase interval is established by the length of the transmission line at the output of the 2-port coaxial box. In this study, interphase delays within the 17–360 ns range were generated using a coaxial cable whose length went approximately from 0.5 up to 35 m. In addition, the proposed generator allows to obtain pulses of different duration by varying the length of the coaxial cable connecting the 2- and 3- port boxes, i.e., for two 10 ns-duration pulses, a 2-m cable was used.

RESULTS

Figure 1 shows an example of bipolar and paired pulses with the interphase interval of 17 ns, obtained with a ~0.5-m cable connected to the output of the 2-port box. Note that by varying the bias voltage from 4 to 8 kV, it is possible to vary the amplitude of the pulses from about 2 to 4 kV.

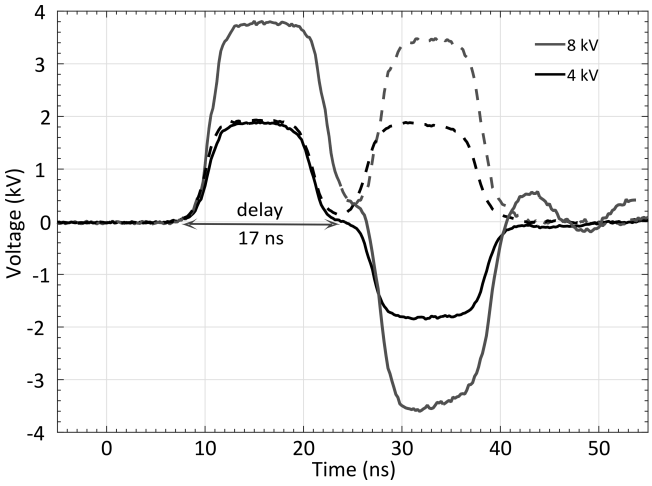

Figure 1: 10-ns bipolar (solid lines) and paired (dashed lines) pulses with a delay of 17 ns. Bias voltage is set to 4 (black lines) and 8 kV (gray lines).

CONCLUSIONS

In this study, a new flexible generator for *in vitro* bioelectric experiments has been introduced. The main novelty consists in the possibility to obtain bipolar and paired pulses with different delays between the polarities in the ns range (17 – 360 ns) by varying the length of the transmission line at the output of the 2-port box. Complementary information can be found in [4].

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Detection of electroporation using bioluminescence of biological cells

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INTRODUCTION

Electroporation is the methodology which is applied to increase and control cell membranes permeability based on pulsed electric field (PEF) [1]. The combination of electroporation phenomenon with drugs, which is known as electrochemotherapy, is the highly effective method in cancer treatment [2][3]. The effectiveness and result of electrochemotherapy depends on electric field amplitude, pulse shape, duration, repetition rate and other physical factors.

In this work, we propose a cell membrane permeabilization and electroporation efficiency in vitro detection model based on Sp2/0 bioluminescent myeloma cells.

METHODS

SP2/0 cells were maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% of fetal calf serum (FCS). All cell culture reagents were obtained from Gibco, Thermo Fisher Scientific, USA. Initially the cell were electro-transfected (4 x 100 µs x 1.2 kV/cm) with Luciferase-pcDNA3 plasmid (Adgene plasmid #18964, a kindly gift from William Kaelin) linearized with Bgl II.

Up to 3 kV, 100 ns – 1 ms square wave high voltage and high frequency pulse generator and electroporation cuvette with 1 mm gap aluminum electrodes (Biorad, Hercules, USA) was used for electroporation. Electroporation has been studied using the 100 µs x 1–8 pulsing protocols in 1–2.5 kV/cm PEF range.

After electroporation 40 µL of the cells (each data point) were transferred into the white plates with 96 wells. D-Luciferin (Promega, USA) was added to the cells at final concentration of 150 µg/ml. The luminescence of SP2/0 cells was evaluated using a Synergy 2 microplate reader and Gen5 software (BioTek, USA).

RESULTS

The changes in bioluminescence were detected after using microsecond range (100 µs x 1–8) PEF treatment and the summary of the data is presented in Fig. 1.

In all cases, (1–2.5 kV/cm) when the electroporation was predominantly reversible a statistically significant increase of RLU was observed. By increasing electric field to 1.5 kV/cm (which corresponds to partly irreversible electroporation) RLU significantly decreased after 4 pulses indicating loss in cell viability.

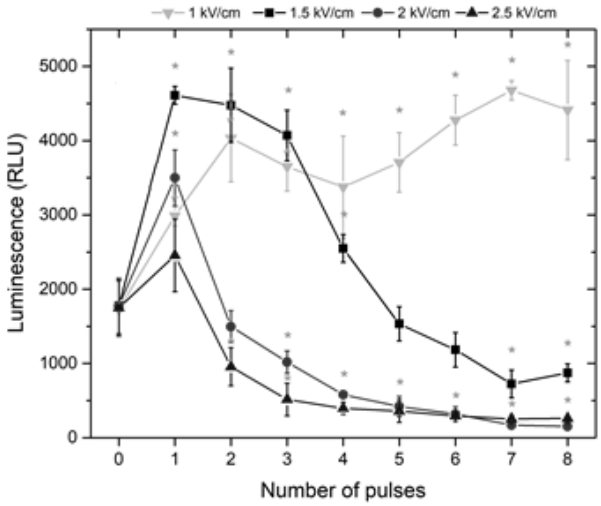

Figure 1: Changes in cell bioluminescence depending on pulsed electric field treatment.

CONCLUSIONS

The bioluminescence response is dependent on the cell permeabilization state and can be effectively used to detect permeabilization. During saturated permeabilization the methodology accurately predicts the losses of cell viability due to irreversible electroporation.

ACKNOWLEDGEMENTS

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Bacterial inactivation with combination of electroporation and antibiotics with different targets

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INTRODUCTION

The growing number of antibiotic-resistant microorganisms has increased the demand for alternative bacterial inactivation methods. One of the most efficient such methods is irreversible electroporation [1], however, alone it often induces insufficient inactivation, motivating the investigation into synergistic treatments [2]. In such treatments, electroporation is combined either with physical methods or antimicrobial agents [1], which become more effective if the pathogens are previously sublethally damaged by electroporation [2].

Our aim was to assess the inactivation of a non-resistant strain of *Escherichia coli* using synergistic treatment of electroporation and antibiotics with different modes of action. Here we report our first results.

METHODS

Working concentrations were determined based on MIC (minimal inhibitory concentration in an overnight culture) concentrations of antibiotics for our non-resistant *E. coli* strain (K12 ER1821, New England BioLabs). Antibiotics used in this study were ampicillin (#A9518), tetracycline (# T3383) and ciprofloxacin (#17850).

For electroporation, a single rectangular electric pulse was used, with a duration of 1 ms and an amplitude (voltage-to-distance ratio, serving as an approximation of the electric field) of up to 20 kV/cm. For each antibiotic, 25 different combinations were tested: five different concentrations of the antibiotic and five different pulse amplitudes. Samples were incubated for 2 hours 40 minutes at room temperature before plating. CFU counts were made the next day and inactivation rate was determined as log (N/N_0).

RESULTS

With electroporation alone, we achieved up to -1.8 log inactivation at 20 kV/cm. When only antibiotics were used, increasing the concentration from MIC to 30xMIC increased bacterial inactivation, but with tetracycline this difference was small (0.25 log). When a combination of electroporation and antibiotic were used, bacterial inactivation increased with increasing electric field and antibiotic concentration. However, with tetracycline, this increase in inactivation was small for electric field below 10 kV/cm. Highest inactivation was achieved with the highest concentration of ampicillin and electric field of 20 kV/cm (-4.02 log). The results are summarized in Table 1.

CONCLUSIONS

Highest inactivation was achieved with ampicillin which targets cell wall synthesis. Ciprofloxacin had similar inactivation rates without electroporation as ampicillin, but when combined with electroporation, the inactivation rates were lower than the corresponding ones obtained with ampicillin. The reason could be in the location of the antibiotics' target, which is intracellular for ciprofloxacin, which must thus traverse the membrane fully in order to inhibit DNA replication. Tetracycline alone had very little effect even at the highest concentration investigated. Electroporation increased its effect, but with lower electric fields this difference was small. Tetracycline must get across the membrane to inhibit protein synthesis and it is a bit larger than ciprofloxacin, so that could explain why lower electric fields are not sufficient for its internalization.

Table 1: Inactivation rates (log+-sd) for *E. coli* with electroporation and three different antibiotics. E=electric field (5, 10, 15 or 20 kV/cm), MIC=minimal inhibitory concentration in overnight culture.

Parameters	Ampicillin	Ciprofloxacin	Tetracycline
MIC	-0.55 +-0.07	-0.50+-0.1	-0.07+-0.02
E5-MIC	-0.80+-0.28	-0.67+-0.25	-0.23+-0.03
E10-MIC	-1.64+-0.22	-1.23+-0.41	-0.75+-0.06
E15-MIC	-2.20+-0.25	-1.90+-0.53	-1.28+-0.07
E20-MIC	-2.65+-0.22	-2.18+-0.37	-1.78+-0.12
30xMIC	-2.17+-0.14	-2.10+-0.3	-0.32+-0.01
E5-30xMIC	-2.86+-0.13	-2.33+-0.24	-0.73+-0.03
E10-30xMIC	-3.31+-0.04	-3.08+-0.15	-2.05+-0.17
E15-30xMIC	-3.80+-0.16	-3.44+-0.13	-2.71+-0.23
E20-30xMIC	-4.02+-0.15	-3.47+-0	-3.45+-0.15

ACKNOWLEDGEMENTS

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Advanced numerical model of a continuous PEF treatment chamber

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INTRODUCTION

Pulsed electric field (PEF) treatment is a promising novel technique in the field of food processing, biotechnology, and environmental engineering. It is based on applying short, high-intensity electric pulses. One of the main components of a PEF treatment system is a treatment chamber, in which the product is exposed to electric pulses. Numerical modelling provides a unique insight into the distribution of the electric field, fluid flow, and temperature inside the treatment chamber, which cannot be provided experimentally.

Existing literature is reporting only on stationary state simulations that do not account for the influence of individual electric pulses, but rather employ a time-averaging of the voltage applied to electrodes.

In the study we present a time-dependent numerical model of a continuous PEF treatment chamber where the influence of each individual pulse is accounted for.

MATERIALS AND METHODS

Experiments were performed using 0.18 % NaCl solution, since its electrical conductivity is similar to that of fruit juices. Saline solution was pumped with a syringe pump Aladin-1000 (World Precision Instruments, USA) and commercial 50 ml plastic syringe. Three different continuous treatment chambers were used; a co-linear treatment chamber [1], a large parallel plate treatment chamber [2], and a small parallel plate treatment chamber (University of Ljubljana, Slovenia). Unipolar square wave pulses were applied using a prototype pulse generator [1]. Voltage and current were recorded with an oscilloscope, a high voltage differential probe, and a current probe (all Teledyne LeCroy, USA). During the application of electrical pulses, the temperature within the outlet channel of the chamber was recorded using an optical thermometer (OpSens Solutions INC, Canada).

In the study protocols with fixed number of pulses applied (e.g. N = 10) and continuous pulse application were used for each treatment chamber. Pulse lengths (τ) of 10 µs and 100 µs were used in combination with repetition frequencies (f) of 10.3 Hz and 1.1 Hz, respectively. Flow rates (F) and peak voltage (U_m) for each chamber were chosen within limitations of experimental equipment.

Time-dependent numerical model was built in COMSOL Multiphysics. In the numerical model, geometries of treatment chambers were constructed in 3D and constituent materials were chosen from the Material Library. A stationary study step was used to solve the fluid flow profile. Two separate time-dependent study steps were used to model conditions in a chamber during and after pulse application. Electrical pulses were described with experimentally recorded voltage and *Events* interface was used to accurately model each individual pulse.

RESULTS AND DISCUSSION

The model was validated with experimentally measured electric current and changes in temperature of the fluid within the outlet channel of the treatment chambers during pulse application. Good agreement between measured and simulated values was obtained (Figure 1).

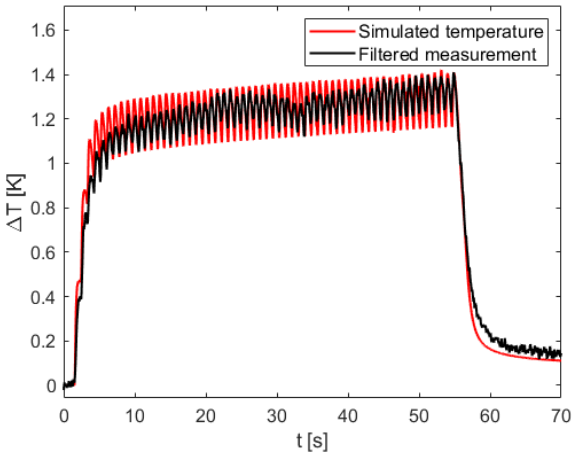

Figure 1: Comparison of simulated and measured temperature change within the outlet channel of large parallel plate treatment chamber. Pulse protocol: U_m = 1000 V, τ = 100 µs, f = 1.1 Hz, F = 20 ml/min.

Presented numerical model allows for the analysis of variables in the treatment chamber (temperature, electric field, etc.) during each individual electrical pulse, between pulses and after pulse application. It can be used as a complement to experiments or as a substitution, to study the effect of different values of PEF parameters that could not be performed due to experimental limitations.

ACKNOWLEDGEMENTS

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Calcium electroporation as a potential ovarian cancer treatment modality

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INTRODUCTION

Epithelial ovarian cancer is one of the most fatal estrogen-dependent gynecological malignancies. The high mortality rate is associated with the fact, that almost 75% of women are not diagnosed before the disease has progressed to stage III or IV [1]. This is because, in most cases, cancer has no or very vague symptoms. The conventional treatment strategy is mainly based on the combination of the tumor resection surgery and chemotherapy with cisplatin or its derivatives. In the early stages of the disease, this approach is initially effective. Unfortunately, in a large number of patients, the disease relapse without responding to re-treatment. Therefore, there is a necessity to develop new therapeutic methods, that also have less toxicity to normal cells.

Calcium electroporation (CaEP) is a novel promising cancer treatment based on conventional electrochemotherapy (ECT), where the chemotherapeutic agent is replaced by calcium ions (Ca^{2+}) [2]. Intracellular calcium (Ca^{2+}) is an essential second messenger involved in a wide range of cellular processes, from fertilization to cell death [3]. In the eukaryotic cell, there is a concentration gradient of calcium ions across the cell membrane. Depending on the location, this gradient is 10-20,000-fold and it is tightly controlled using several mechanisms [5].

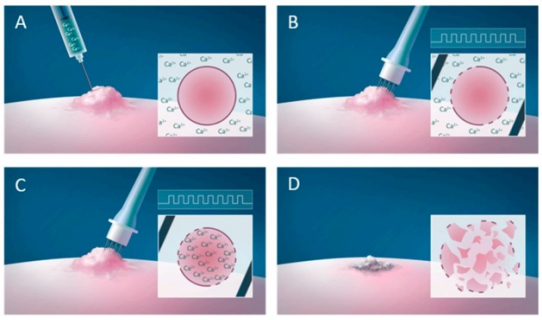

Figure 1: Calcium electroporation (CaEP). (**A**) 5-225 mM $CaCl_2$ injection into the tumor cell (**B**) electroporation and membrane permeabilization (**C**) influx of Ca^{2+} (**D**) cell necrosis [2][6][7].

The formation of pores in the cell membrane as a result of electroporation increases its permeability, which allows the influx of Ca^{2+} into the tumor cell and an increase of its intracellular concentration [4]. The cell extrudes the excess of Ca^{2+} outside through increased activity of plasma membrane calcium ATPase (PMCA). The energy needed for its functioning comes from adenosine triphosphate (ATP) hydrolysis. A high concentration of Ca^{2+} leads to the amplified activity of Na^+/K^+-ATPase [5]. The loss of the electrochemical gradient of mitochondrial membranes has also been noted, which results in the inhibition of ATP production. The depletion of ATP resources leads the cell to necrosis. Moreover, it has been shown that this disturbance of calcium homeostasis can induce the production of reactive oxygen species and the activation of proteases and lipases [3]. The mechanism of the CaEP procedure is shown in Fig. 1.

So far, CaEP has been tested *in vitro* and *in vivo* in five human tumors grown subcutaneously in immunocompromised mice. CaEP induced tumor cell death in all tested tumors with different sensitivity to treatment [5]. Unfortunately, there are no literature reports about the effectiveness of this treatment method in ovarian cancer.

AIM OF THE FUTURE WORK

Our future work will focus on investigating the effectiveness of calcium electroporation in ovarian cancer as a treatment modality.

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Gene therapy of murine melanoma B16F10 using a combination of interleukin 2 and 12 gene electrotransfer

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INTRODUCTION

Immunotherapy has become an important pillar of cancer treatment improving the outcome of many patients [1]. One approach is the use of interleukin 2 (IL-2) and 12 (IL-12) that are potent cytokines that exhibit antitumor effect [2]. These two cytokines use separate signaling pathways to induce complementary biological effects, thus the aim of our study was to determine the anitumour effects of gene therapy of murine melanoma B16F10 using a combination of IL-2 and IL-12 gene electrotransfer (GET).

METHODS

The cytotoxic effect of GET coupled with both plasmids individually and combined was determined in B16F10 murine melanoma cell line. Two pulse protocols were used, called ECT (8 pulses with a voltage to distance ratio of 1300 V/cm, duration of 100 µs and frequency of 1 Hz) and GET (8 pulses with a voltage to distance ratio of 600 V/cm, duration of 5 ms and frequency of 1 Hz). Cell viability was determined using PrestoBlue assay and the mRNA expression profile of *Il2* and *Il12* in cells was determined by qRT-PCR. In vivo gene electrotransfer of pIL-2 and pIL-12 using GET protocol was performed in C57BL/6 mice bearing subcutaneous B16F10 melanoma tumours. Tumour growth delay was calculated and the IL-2 and IL-12 protein expression in tumour and serum samples was determined using ELISA. The experimental procedures were performed in compliance with the guidelines for animal experiments of the EU directive (2010/63/EU) and the permission from the Veterinary Administration of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. U34401–1/2015/38).

RESULTS

The viability of cells (*in vitro*) treated with both plasmids individually and combined coupled with ECT pulses was notably higher compared to the viability after treatment with GET pulses ($p<0.05$). However, the expression of mRNA coding for Il2 and Il12 in cells treated with ECT pulses was lower than in cells treated with GET pulses. In vivo, prolonged tumour growth delay was observed in the GET pIL-12 and GET combination group and in some mice also complete response (Figure1). Additionally, no tumour growth after rechallenge was observed in mice from the GET combination group. Increased protein expression of IL-2 and IL-12 was detected only in the respective groups (GET pIL-2 or GET pIL-12) and in GET combination group.

CONCLUSIONS

To conclude, we successfully transfected B16F10 cells with pIL-2 and pIL-12 *in vitro*. Treatment of cells with plasmid DNA and thereafter with ECT pulses yielded higher cell viability but lower expression than treatment with GET pulses. The treatment with GET pulses coupled with pIL-12 alone and in combination with pIL-2 resulted in complete responses of tumours. In addition, all mice in the combination group developed also immune memory, as they remained tumours free 100 days after the rechallenge.

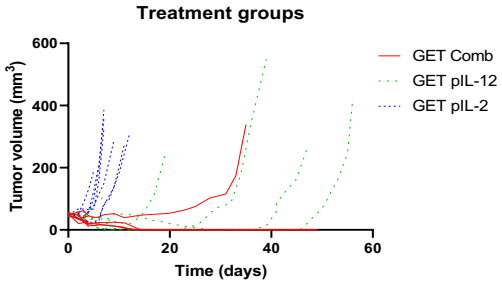

Figure 1: Growth curves of tumours in the therapeutic treatment groups.

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Electroporation based modifications of plasma membrane organization in cancer cells for targeted therapy

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INTRODUCTION

Pharmacotherapy has always been the most frequently chosen therapeutic option to treat a patient. Currently, the biggest problem is not so much finding a drug for a specific disease, but looking for an effective method of its administration, so that it has a justified clinical effect. Many active substances have been tested for pharmacological activity and have often obtained spectacular results in vitro, but their final effect in the subsequent stages of the tests was far from the expected. One of the main reasons is the imperfection of the absorption and distribution of drugs in the human body. Effective drug action is also related to the concentration at the site of action, which is often only a fraction of the starting concentration. Even if the pathway of the drug in the body is shortened, the question of the direct effect of the drug on target cells remains [1].

MULTIDRUG RESISTANCE OF NEOPLASTIC CELLS

Due to numerous abnormalities, neoplastic cells lose their self-control mechanisms, proliferate, are resistant to apoptosis, tend to expand and invade, and are characterized by specific metabolism. Due to a large number of divisions, they also quickly acquire new features that make it easier for them to adapt to the prevailing conditions. One of them is the development of multidrug resistance (MDR), which is mainly based on the altered membrane composition. Such cells often contain more cholesterol in it, making it more rigid and less permeable to various substances, including drugs [2].

Another type of multi-drug resistance in neoplastic cells is the overexpression of membrane transporters. These proteins limit the supply of the drug inside the cell or even actively pump it out of the cell. The best-known membrane transporters are ABC transporters, which, even against the concentration gradient, can excrete the chemotherapeutic agent toxic to the cell beyond the cell membrane, preventing its accumulation [3].

ELECTROCHEMOTHERAPY

One of the possibilities of modulating biological membranes is the use of electroporation. This method is aimed at increasing the membrane permeability by disrupting its integrity under the influence of an electromagnetic field. This effect is reversible and, depending on the pulses used, it may last several minutes. The created pores allow free movement of particles of larger size into the cell, which allows a better pharmacological effect to be obtained, without an overall increase in the concentration of the drug and surgical intervention. Temporary damage to the regular structure of the membrane will also disrupt the placement of drug transport proteins, thus their localization in the membrane and activity [4].

miRNAs MODULATE VAROIUS DRUG RESISTANS MECHANISM

Considering the significant role of proteins in multidrug resistance, a method was developed that allows miRNA to regulate their expression. The prospect of influencing the number of synthesized membrane transporters in the cell membrane has opened the possibility of using miRNAs in the pharmacotherapy of neoplastic diseases. Another mechanism that can be modulated by miRNAs is autophagy. This process maintains homeostasis in cells by breaking down damaged cellular elements that can later be reused as nutrients. Inhibiting this process with miRNA allows disturbing the further development and proliferation of the neoplastic cell, which loses the possibility of its repair and rearrangement [5]. Some miRNAs are designed to inhibit the apoptosis of cancer cells, so they indirectly contribute to their immortality. Therefore, scientists are interested in substances that will limit the action of such miRNAs [6]. Physical and mechanical methods, i.e. electroporation and microgravity, that will be used in the research, are aimed at sensitizing cancer cells to the chemotherapeutic agent used. Cells with an altered structure of the cell membrane and its protein elements will be assessed in terms of their ability to accumulate the drug after the applied treatments concerning the control sample.

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Percutaneous Electrochemotherapy of Hepatocellular Carcinoma: A case report

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INTRODUCTION

Electrochemotherapy (ECT) is a local nonthermal treatment of cancer that combines chemotherapy and the application of electric pulses. ECT is already recognized as safe and effective treatment of cutaneous tumors and skin metastases [1-3]. It has also proved to be safe and effective treatment of deep-seated tumors during open surgery [4-6]. Percutaneous ECT has also already been described, but not on HCC [7,8].

CASE DESCRIPTION

We report a case of a 66-year old man with Child A liver cirrhosis with single HCC with diameter of 18mm in liver segment 3 and no signs of extrahepatic disease. His documentation was reviewed on hepatopancreaticobiliary (HPB) multidisciplinary team meeting, which recognized this lesion to be suitable for minimally invasive percutaneous electrochemotherapy.

The detailed technological procedure has already been reported in another article [9]. Treatment was performed under general anesthesia and deep muscle relaxation. Because the lesion was not visible on ultrasound the insertion of four long needle electrodes was performed under contrast enhanced cone-beam computed tomography (CBCT) guidance. Before the electric pulses were delivered, bleomycin was administrated intravenously. After electrode extraction, control CBCT with contrast injection through microcatheter showed no bleeding or hematoma and also that the lesion was avital. There were no complications after the procedure and the patient was discharged the day after the procedure with analgesics and antithrombotic prophylaxis.

RESULTS

In this case, percutaneous ECT showed to be an effective and safe treatment of single small HCC, considering the complete response immediately after the procedure, absence of disease relapse after 18 months and absence of adverse events.

CONCLUSION

Percutaneous ECT could be an effective technique for the treatment of HCC, especially for tumors not suitable for surgery or percutaneous ablation. However, more studies are needed to confirm our results.

ACKNOWLEDGEMENTS

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Treatment of lymph node metastases from colorectal cancer with percutaneous image guided electrochemotherapy: a case report

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INTRODUCTION

Electrochemotherapy is effective in treatment of a variety of tumors. Intraoperative electrochemotherapy of colorectal liver metastasis proved to be efficient treatment modality and could be translated into percutaneous treatment of colorectal lymph node metastasis. The aim of this case presentation is to demonstrate the feasibility, safety and effectiveness of electrochemotherapy with percutaneous approach on colorectal lymph node metastasis.

PATIENT AND METHODS

A 47-year-old man was presented at multidisciplinary team meeting with a diagnosis of lymph node metastasis from recurrent adenocarcinoma of the ascending colon in May 2020. FDG-PET/CT from two months earlier showed pathological FDG uptake form enlarged retroperitoneal lymph node, indicating metastasis (Figure 1). Subsequent abdominal CT scan demonstrated an enlarged right para-aortic lymph node measuring 3.5 cm in diameter. At the time the patient has just finished first cycle of FOLFOXIRI regimen in combination with bevacizumab. According to the multidisciplinary team meeting, the patient was eligible candidate for percutaneous electrochemotherapy.
after the procedure (A, B). Position of the electrodes in relation to metastasis on cone-beam CT (C, D).

Treatment was performed under general anesthesia and deep muscle relaxation.

Five electrodes with 3 cm active length were placed percutaneously around the tumor under stereotactic CBCT guidance. In keeping with European Standard Operating Procedures on Electrochemotherapy (ESOPE) recommendations, 28.500 IU of bleomycin in 20 ml of physiological saline was administered intravenously in bolus and two trains of 4 electric pulses (duration 100 µs, pulse repetition frequency 1 kHz) of opposite polarity with voltage-to-distance ratio of 1000 V/cm were delivered between all electrode pairs starting 8 minutes after the bleomycin injection (total number of pulses = 48).

RESULTS

Only a slight increase in inflammatory parameters was observed during hospitalization, which was attributed to the inflammatory reaction of the treated tissue to the procedure.

Six weeks after percutaneous electrochemotherapy, control PET/CT scan showed marked reduction of FDG uptake from lymph node metastasis, with only peripheral rim of enhancement remaining. The patient was feeling well, in good physical condition and pain-free. On the second follow-up three months after the procedure, a control CT scan with intravenous contrast showed centrally necrotic treated lesion, however new metastasis occured in other locations.

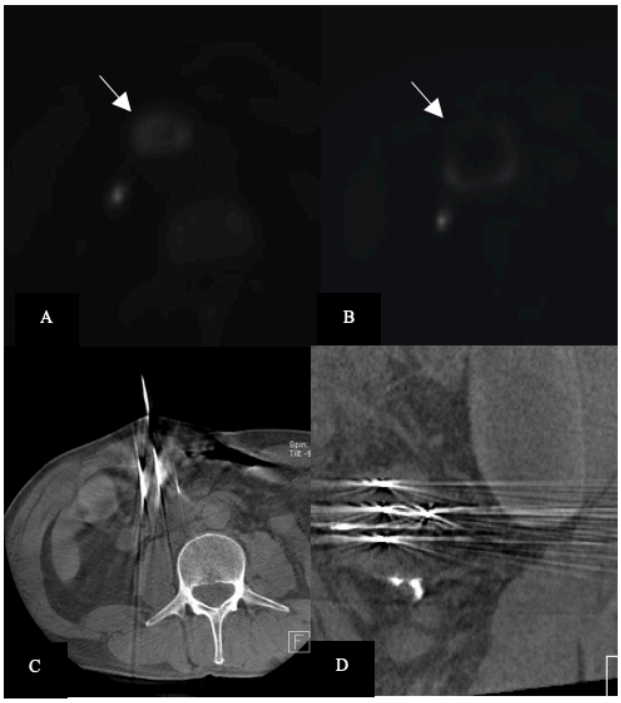

Figure 1: A 47-year old male with colorectal lymph node metastasis. PET/CT demonstrating the lesion before and

CONCLUSIONS

We describe the first case of percutaneous electrochemotherapy of coloretal lymph node metastasis. This case shows that percoutaneous electrochemotherapy could provide an effective treatment for a selected group of patients with unresectable colorectal lymph node metastasis. A prospective cohort study is needed to confirm our findings.

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Comparable effectiveness of electrochemotherapy with cisplatin and oxaliplatin, but not bleomycin in B16F10, CT26 and 4T1 murine cell lines *in vitro*

Urša Kešar[1,2], Katja Uršič[1], Tanja Jesenko[1,2], Maja Čemažar[1,3], Primož Strojan[2,4], Gregor Serša[1,5]; *[1] Institute of Oncology Ljubljana, Department of Experimental Oncology, Zaloška cesta 2, SI-1000 Ljubljana, SLOVENIA [2] Faculty of Medicine, University of Ljubljana, Vrazov trg 2, SI-1104 Ljubljana, SLOVENIA [3] Faculty of Health Sciences, University of Primorska, Polje 42, SI-6310 Izola, SLOVENIA [4] Institute of Oncology Ljubljana, Department of Radiation Oncology, Zaloška cesta 2, SI-1000 Ljubljana, SLOVENIA [5] Faculty of Health Sciences, University of Ljubljana, Zdravstvena pot 5, SI-1000 Ljubljana, SLOVENIA*

INTRODUCTION

In electrochemotherapy (ECT), electric pulses increase the uptake of cytostatics in the cells which causes stronger cytotoxic effect [1]. Furthermore, ECT can induce immunogenic cell death (ICD), apoptosis or necrosis in tumor cells [1–3]. The aim of the study was to compare the sensitivity of three immunologically different cell lines to ECT with cisplatin (CDDP), oxaliplatin (OXA), or bleomycin (BLM) *in vitro* in order to determine the IC30, IC50 and IC70 concentrations of cytostatics. These concentrations will later be used for the evaluation of immunological effects of ECT.

METHODS

We used three different murine tumor cell lines: B16F10, CT26 and 4T1, which are known to induce tumors with different immunological profiles *in vivo*. We treated them either with electric pulses alone or with CDDP, OXA and BLM alone, as well as in combination with electric pulses. For electroporation, we used plate electrodes with 2 mm distance and ECT electric pulses (1300 V/cm, 100 µs, 1 Hz, 8 pulses). We compared the cytotoxicity of the three cytostatics among cell lines using a clonogenic assay.

Additionally, we determined the concentration of tested drugs that induce death of 30 %, 50 % and 70 % of cells (IC30, IC50, IC70 values).

RESULTS

All three cell lines were comparably sensitive to electric pulses alone and to ECT with CDDP and OXA at IC30, IC50 and IC70 (IC50 results shown in Figure 1: A, B and C). However, a statistically significant difference ($p < 0.05$) between CT26 and 4T1 cell lines at IC50 (but not at IC30 and IC50) for ECT with BLM was demonstrated (Figure 1: D).

CONCLUSIONS

We have shown that all three cell lines are equally sensitive to electric pulses. However, 4T1 cells were more sensitive to ECT with BLM (IC50) than CT26 cells. The determined IC30, IC50 and IC70 values for all cytostatics in all three cell lines will enable us further exploration of immunological effects of ECT.

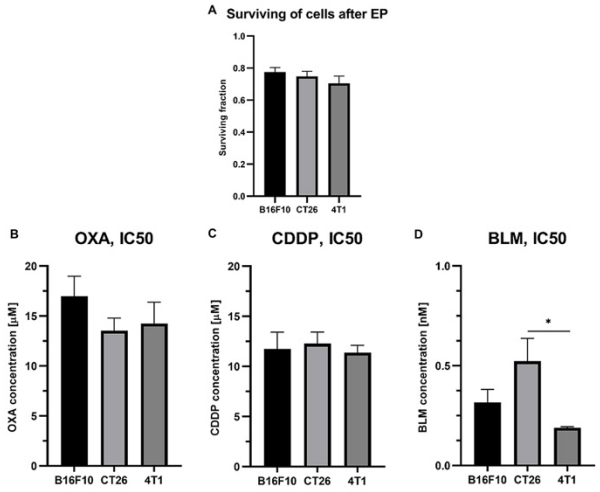

Figure 1: **A** Surviving of cells after electroporation (EP) alone (n = 9); **B** IC50 concentration for ECT with OXA (n = 3); **C** IC50 concentration for ECT with CDDP (n = 3); **D** IC50 concentration for ECT with BLM (n = 3).

ACKNOWLEDGEMENTS

This work received funding from Slovenian Research Agency (ARRS): research programme P3-0003.

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Adjuvant electrochemotherapy in canine oral melanoma – Case Report

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INTRODUCTION

The oral cavity is a common location for the development of neoplasia in small animals [1]. As the most common malignant oral tumor in dogs, oral melanoma typically occurs on the gingiva and is characterized by rapid growth, local invasion and early metastasis. In general, oral tumors can be difficult to manage surgically because of their close association with local periodontal structures including oral bones [1,2] and frequently are resistant to chemotherapy and radiation therapy [1,3] being the electrochemotherapy (ECT) a valuable indication [4].

CASE REPORT

Chokito, a 13-years old dachshund, attended under tutor's complaint of halitosis was referred for treatment of periodontal disease. During orotracheal intubation, the presence of a neoformation mass of 3cm in the soft palate was detected (Figure 1a). Through histopathological analysis it was confirmed as melanoma. The indicated treatments were tumor debulking + ECT (8 pulses/ 1300 V/cm/ 100 µs/ 5 kHz) with I.V. bleomycin (15 UI/m²) in the surgical site as well as right submandibular lymphadenectomy due to fine-needle aspiration cytology (FNAC) indicating lymph node metastasis. Six sessions of carboplatin 300 mg/m² and melphalan (2mg/m²) was also administered. Oral cavity assessments under anesthesia never revealed recurrence, yet a second ECT session was performed 20 days after the first ECT. A new biopsy three weeks after the 2nd ECT was performed and indicated the absence of tumor cells in the surgical site. The animal was discharged from treatment follow-up 135 days after treatment without signs of tumor relapse (Figure 1b), and after 1 year and 8 months it remained healthy and without local tumor recurrence or metastasis.

DISCUSSION

Oral tumors in companion animals often arrive at an advanced stage in the veterinary clinic, due to the difficulty or lack of habit of visualizing the oral cavity by the animal's tutors. Due to the impossibility of a surgical resection with safety margins in this case, debulking of the macroscopic mass and cleaning of residual tumor cells with ECT was performed. Due to the early metastasis profile of positivity in lymph node FNAC, systemic therapy (conventional chemotherapy) was applied. The disease-free survival of 1 year 8 months indicates successful treatment and highlights the role of ECT as adjuvant therapy in incompletely resected tumors.

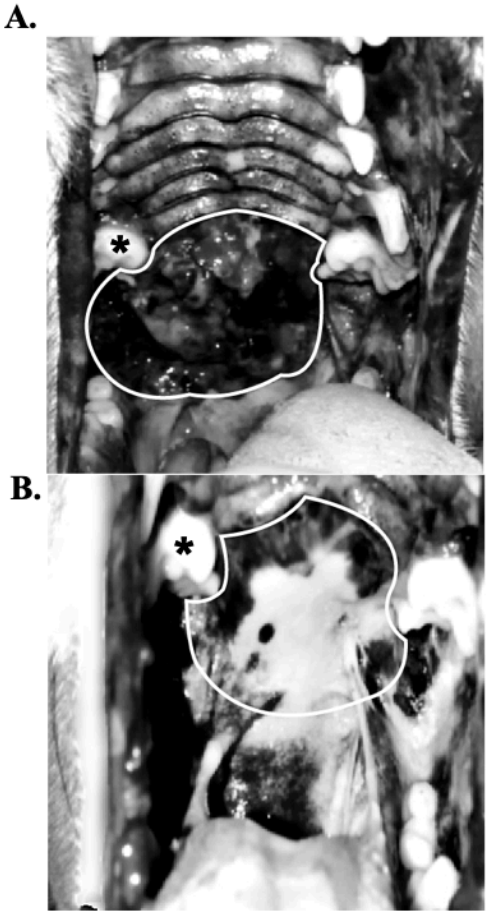

Figure 1: A. Tumor before first treatment (white line), 30th day after biopsy. **B.** Tumor site (white line), 135th day after treatment, with visualization of the caudal region of the soft palate (light area) and no signals of tumor relapse. *Arrow:* epiglottis. *Asterisk:* right molar.

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LABORATORY EXERCISES

The influence of Mg^{2+} ions on gene electrotransfer efficiency L1

Saša Haberl Meglič, Mojca Pavlin
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiment: day 1: 60 min; day 2: 60 min
Max. number of participants: 4
Location: Cell Culture Laboratory 3
Level: Basic

PREREQUISITES

Participants should be familiar with the Safety rules and Rules for sterile work in cell culture laboratory. No other specific knowledge is required for this laboratory practice.

THEORETICAL BACKGROUND

Gene electrotransfer is a non-viral method used to transfer genes into living cells by means of high-voltage electric pulses. An exposure of a cell to an adequate amplitude and duration of electric pulses leads to transient increase of cell membrane permeability for molecules which are otherwise deprived of membrane transport mechanisms. This allows various nonpermeant molecules, including DNA, to be transported across the membrane and enter the cell. Although mechanisms of the process are not yet fully elucidated, it was shown that several steps are crucial for gene electrotransfer: interaction of plasmid DNA (pDNA) with the cell membrane, translocation of pDNA across the membrane, migration of pDNA towards the nucleus, transfer of pDNA across the nuclear envelope and gene expression (Figure 1).

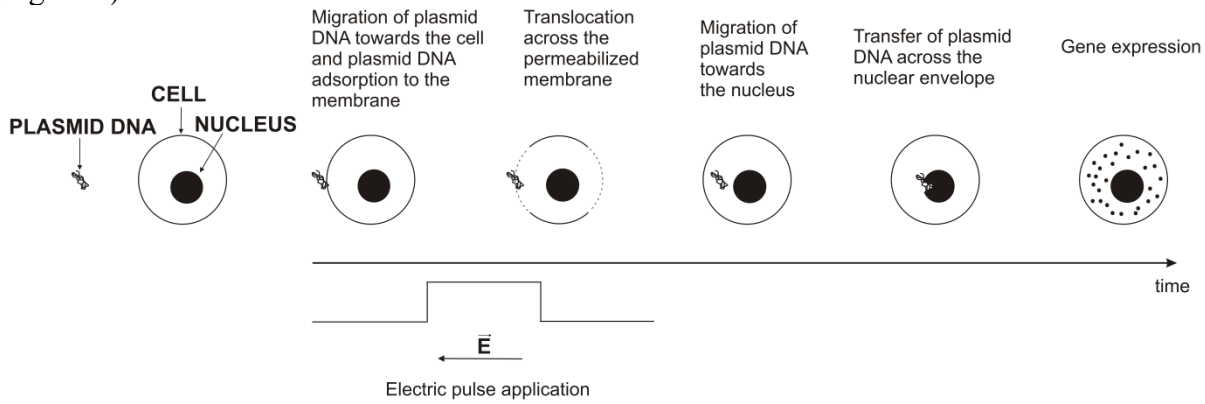

Figure 1. Steps involved in gene electrotransfer.

Many parameters have been described to influence the efficiency of gene electrotransfer. A few published reports have shown that the concentration of Mg^{2+} ions in electroporation media have important impact on forming a complex between DNA and the cell membrane during application of electrical pulses. Namely, DNA is negatively charged polyelectrolyte and Mg^{2+} ions can bridge the DNA with negatively charged cell membrane. But it was shown that Mg^{2+} ions at higher concentrations may bind DNA to the cell membrane strongly enough to prevent translocation of DNA into the cell during electroporation thus gene electrotransfer efficiency is decreased.

The aim of this laboratory practice is to demonstrate how different Mg^{2+} concentrations in electroporation media affect the efficiency of gene electrotransfer and cell viability.

EXPERIMENT

We will transfect Chinese hamster ovary cells (CHO-K1) with plasmid DNA (pEGFP-N_1) that codes for GFP (green fluorescent protein) using two different electroporation media (see Protocol section). To generate electric pulses Jouan GHT 1287 electroporator (Jouan, St. Herblain, France) will be used. Pulses will be monitored on osciloscope (LeCroy 9310C).

We will determine gene electrotransfer efficiency and cell viability for both electroporation media.

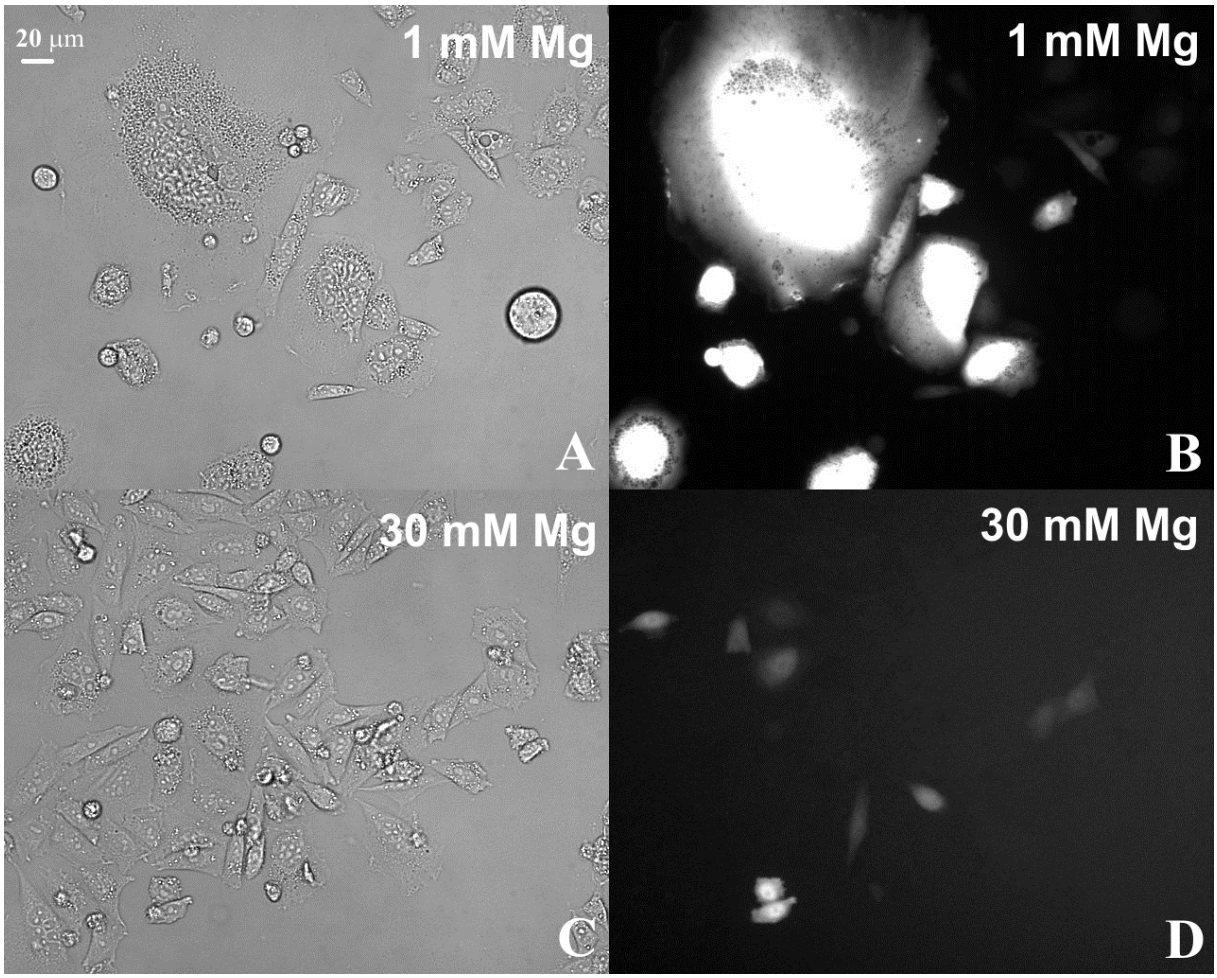

Figure 2. Gene electrotransfer of plated CHO cell 24 h after pulse application in 1 mM Mg or 30 mM Mg media. 8 x 1 ms (stainless steel wire electrodes with inter-electrode distance d = 2 mm; applied voltage U = 140 V resulting in electric field strength E = 0.7 kV/cm) pulses were applied with repetition frequency of 1 Hz to deliver pEGFP-N_1 (concentration of DNA in electroporation media was 10 μg/ml) into the cells. Phase contrast images of treated cells for (A) 1 mM Mg and (C) 30 mM Mg media and fluorescence images of treated cells for (B) 1 mM Mg and (D) 30 mM Mg media are presented. To visualize transfection 20x objective magnification was used.

Protocol 1/2 (Gene electrotransfer with different electroporation media): CHO cells will be grown in multiwells as a monolayer culture in Ham's tissue culture medium for mammalian cells with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at 37º C. Cells will be plated 24 h before the experiment in concentration 5 x 10^4 cells per well.
Just before the experiment remove culture media and replace it with 150 μl of electroporation media containing plasmid DNA with concentration 10 μg/ml. Use 1 mM or 30 mM electroporation media. Sucrose molarity has also been changed in order to attain the molarity of the media:

a) **1 mM Mg media** (10 mM phosphate buffer Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose; pH = 7.2)
b) **30 mM Mg media** (10 mM phosphate buffer Na_2HPO_4/NaH_2PO_4, 30 mM $MgCl_2$, 160 mM sucrose; pH = 7.2)

Incubate cells with plasmid DNA for 2-3 minutes at room temperature. Then apply a train of eight rectangular pulses with duration of 1 ms, U = 140 V resulting in electric field strength E = 0.7 kV/cm and repetition frequency 1 Hz to deliver plasmid DNA into the cells. Use stainless steel wire electrodes with inter-electrode distance d = 2 mm.
Cells in the control are not exposed to electric pulses.
Immediately after exposure of cells to electric pulses add 37.5 µl of fetal calf serum (FCS-Sigma, USA). Incubate treated cells for 5 minutes at 37ºC and then add 1 ml of culture media.

Protocol 2/2 (Determining gene electrotransfer efficiency and cell viability): After 24 h incubation at 37ºC determine the difference in gene electrotransfer efficiency and cell viability for both electroporation media by fluorescent microscopy (Leica, Wetzlar, Germany) at 20x magnification using GFP filter with excitation at 488 nm.

You will determine gene electrotransfer efficiency from the ratio between the number of green fluorescent cells (successfully transfected) and the total number of viable cells counted under the phase contrast. You will obtain cell survival from phase contrast images as the ratio between the number of viable cells in the treated sample and the number of viable cells in the control sample.

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NOTES & RESULTS

Electroporation media	Treated viable cells	Viable cells in control	Green fluorescent cells	Gene electrotransfer efficiency [%]	Viability [%]
1 mM Mg media					
30 mM Mg media					

NOTES & RESULTS

Visualization of local ablation zone distribution between two needle electrodes L3

Tjaša Potočnik, Alenka Maček Lebar
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 60 min
Max. number of participants: 4
Location: Cell Culture Laboratory 3
Level: basic

PREREQUISITES

Participants should be familiar with Laboratory safety (S1) and Electroporation hardware safety (S2). No other specific knowledge is required for this laboratory practice.

THEORETICAL BACKGROUND

Electroporation is the method in which by applying external electric field of sufficient amplitude and duration membrane of exposed cells becomes permeabilized for molecules that otherwise cannot pass cell membrane. After reversible electroporation cell membrane reseals. With increasing amplitude of electric field the level of cell membrane permeabilization and the number of cells that are permeabilized increases. When pulses with sufficient magnitude and duration are applied, cell death is achieved and the process is defined as irreversible electroporation (IRE). IRE is an emerging ablation technique inducing apoptosis in successfully treated cells or tissues. Usually there is a sharp border between treated and untreated tissue regions because only the cells that are exposed to high enough electric field are ablated. Effective prediction of electric field can be obtained by numerical modeling, which includes the shape and position of the electrodes and parameters of electric pulses (amplitude, duration, number, frequency) used as well as electrical properties of the tissue. Using treatment planning, IRE offers benefits over other cancer therapies because it can be performed near large blood vessels, nerves, and ducts without causing damage to these structures, sparing extracellular matrix.

Electroporation can be detected by measuring increased transport of molecules across the membrane. Cell uptake of dyes, either fluorescent molecules (lucifer yellow, yo-pro-1, propidium iodide) or colour stains (such as trypan blue), is most often used for *in vitro* electroporation detection. Trypan blue can be used as an indicator of plasma membrane integrity and of cell viability. Trypan blue is normally impermeant to healthy cells. When cell membrane integrity is compromised, the dye is able to enter the cell and stains cellular structures blue, especially nuclei, making the cell appear blue. Cells that take up this dye several hours after exposure to electrical pulses are usually considered dead or dying.

The aim of this laboratory practice is to visualize local ablation zone distribution between two needle electrodes with increasing pulse amplitude using trypan blue.

EXPERIMENT

We will visualize local ablation zone distribution between two needle electrodes using trypan blue. The effect of the pulse amplitude on the local ablation zone distribution between two needle electrodes will be determined for a train of eight 100 μs rectangular pulses delivered with the repetition frequency 1 Hz. The area of blue cells that is a consequence of efficient ablation increases with increasing pulse amplitude is presented in Figure 1.

Protocol: You will use Chinese hamster ovary cells (CHO), plated 48 h before experiment in concentration 2.5 x 10^5 cells per tissue culture dish. Cells are attached to the culture dish surface. Immediately before electric pulses are applied replace the growth medium with electroporation buffer. As electroporation buffer you will use isotonic 10 mM K_2HPO_4/KH_2PO_4 containing 1 mM $MgCl_2$ and 250 mM sucrose with pH 7.4. You will use needle electrodes 1 mm apart. For pulse delivery Gemini X2 electroporator (Hardvard apparatus BTX, USA) will be used. It can produce square and exponential pulses. During the experiment current will be monitored with an oscilloscope and a current probe. Electric field in the needle surrounding can be calculated numerically.

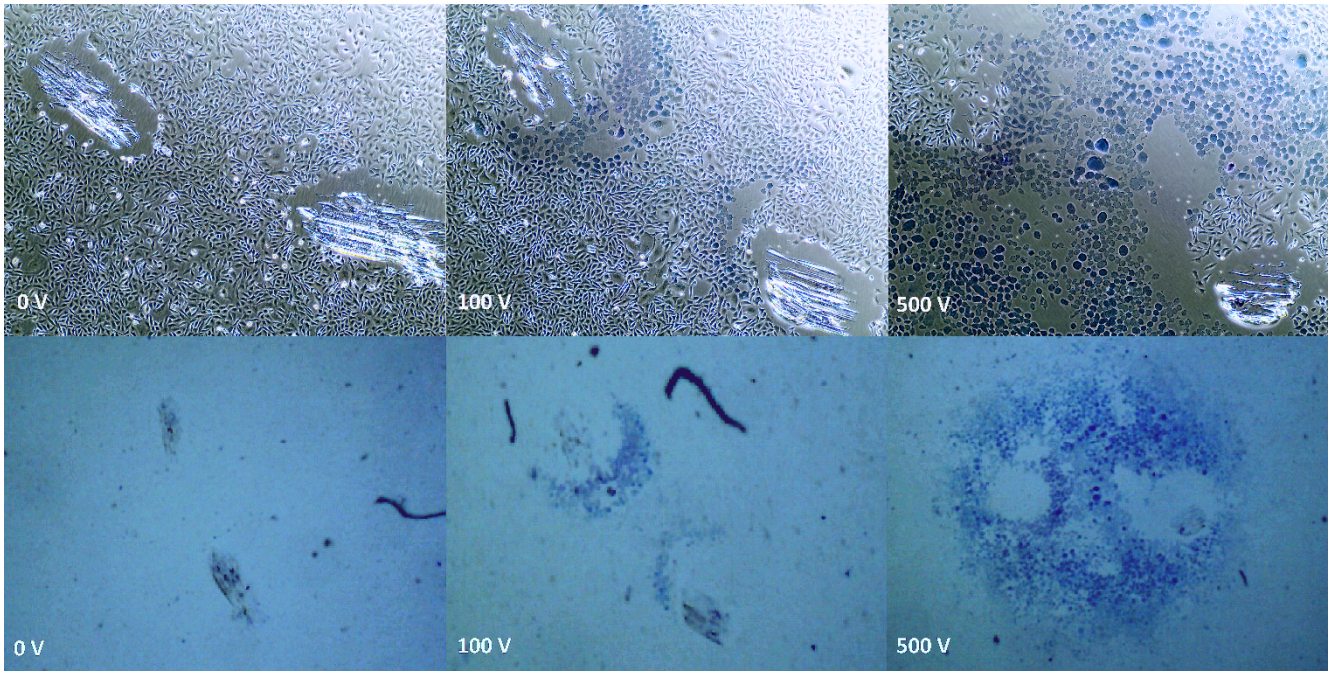

Figure 1: The sequence of the images of local ablation zone after cells were exposed to electric pulses with increasing pulse amplitude. The images were obtained by light microscopy under 10 × objective magnifications (top row) and under 5 × objective magnifications (bottom row).

Remove the tissue culture dish from the incubator and replace the growth medium with 500 µl of electroporation buffer. Carefully place needle electrodes on edge of tissue culture dish and apply electric pulses. Electric pulse parameters used are: 8 pulses, 100 µs duration and pulse repetition frequency 1 Hz, while pulse amplitude increases gradually. Increase the pulse amplitude from 0 V to 100 V, 300 V, 500 V and 700 V. After electroporation leave cells for 10 minutes at room temperature. Remove electroporation buffer and add 500 µl of trypan blue to tissue culture dish. Leave the cells for 5 minutes at room temperature then replace the trypan blue with 500 µl of fresh electroporation buffer. For visualization of local ablation zone, EVOS XL Core Imaging System (Invitrogen™) will be used.

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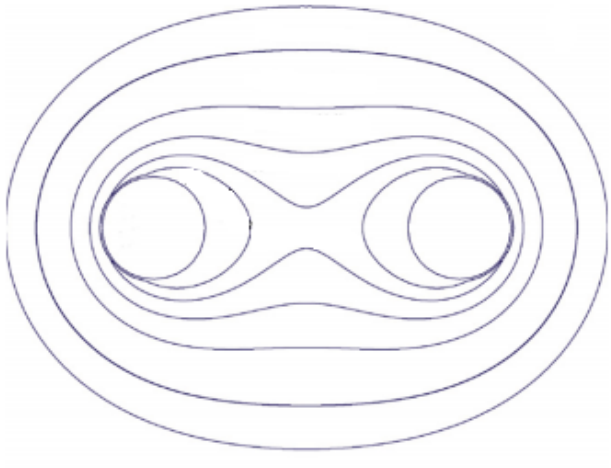
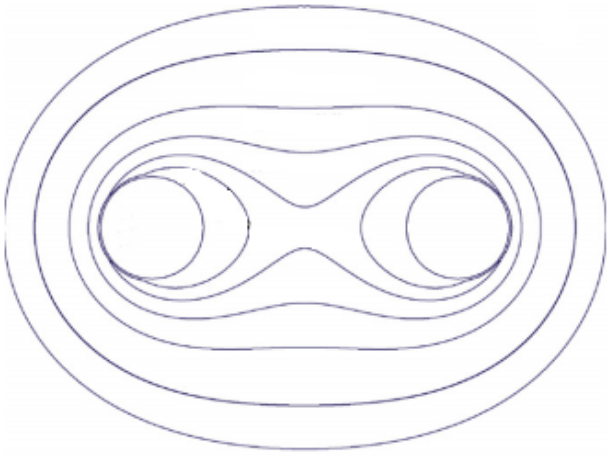
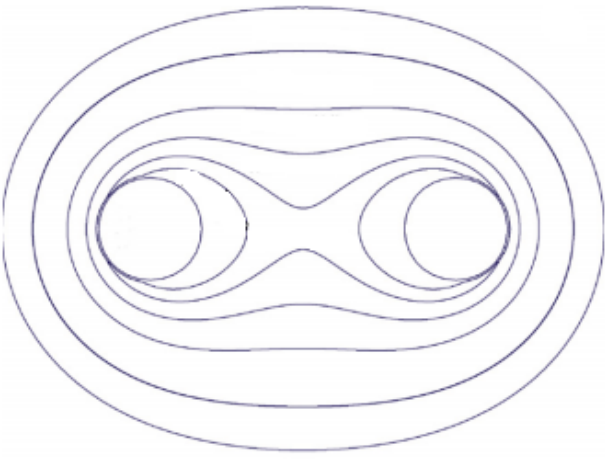
NOTES & RESULT



100 V



500 V



700 V

NOTES & RESULTS

Electroporation of planar lipid bilayers L6

Peter Kramar, Aljaž Velikonja, Alenka Maček Lebar
University of Ljubljana, Faculty of Electrical Engineering, Slovenia

Duration of the experiments: 120 min
Max. number of participants: 4
Location: Laboratory for skin and planar lipid bilayers
Level: Basic

PREREQUISITES

Participants should be familiar with Laboratory safety (S1). No other specific knowledge is required for this laboratory practice.

THEORETICAL BACKGROUND

A planar lipid bilayer can be considered as a small fraction of total cell membrane. As such has often been used to investigate basic aspects of electroporation; especially because of its geometric advantage allowing chemical and electrical access to both sides of the lipid bilayer. Usually a thin bi-molecular film composed of specific phospholipids and organic solvent is formed on a small aperture separating two aqueous compartments. Electrodes immersed in these two aqueous compartments allow to measure current and voltage across the lipid bilayer (Figure 1).

Two different measurement principles of planar lipid bilayer's properties can be used: voltage or current clamp method. Planar lipid bilayer from an electrical point of view can be considered as imperfect capacitor, therefore two electrical properties, capacitance and resistance, mostly determine its behaviour.

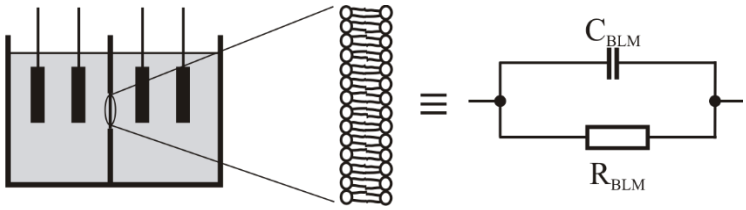


Figure 1: Equivalent circuit of a planar lipid bilayer.

The aim of this laboratory practice is to build a planar lipid bilayer by painting method (Muller - Rudin method) or/and foldig method (Montal – Mueller) and to determine capacitance and resistance of the planar lipid bilayer using LCR meter. Basic aspects of planar lipid bilayer electroporation will be given by observing formation of the pores and determining its breakdown voltage.

EXPERIMENT

Protocol:

Muller-Rudin method

Form a planar lipid bilayer by covering the surface of the aperture in a barrier separating two compartments of a measuring vessel with a lecithin solution (20 mg/ml of hexane). After evaporation of hexane, fill compartments with solution consisting of 0.1 mol KCl, 0.01 mol of HEPES, at pH=7.4. Connect the electrodes and apply a drop of lecithin dissolved in decane (20 mg/ml) to the aperture by

the micropipette or paint it by a teflon brush. Measure capacitance and determine if the formation planar lipid bilayer is appropriate.

Montal – Mueller method
Cover the surface of the aperture in a barrier separating two compartments of a measuring vessel with 1 µl lecithin solution (10 mg/ml of hexane and ethanol absolute in ratio 9:1). After evaporation of hexane and ethanol, add on the aperture 1,5 µl solution of pentan and hexadecane in ratio 7:3. Fill compartments with solution consisting of 0.1 mol NaCl, 0.01 mol of HEPES, at pH=7.4. On the solution surface apply 2 µl of lecithin solution in each compartment. Wait approximately 15 minutes that lipid molecules are equally spread on the solution surface. Then rise the solution surface above aperture synchronously in both compartments by pumps. Measure capacitance and determine if the formation planar lipid bilayer is appropriate.

Measuring methods: When the planar lipid layer is formed, we apply the current or voltage to the planar lipid bilayer. In the current clamp method the current is applied to the planar lipid bilayer and we measure voltage across the bilayer. Apply a linearly increasing current and record a voltage across the bilayer. During the experiment you will obtain the time course of the voltage across the bilayer and the plot of the programmed current flowing between two current electrodes. In the voltage clamp method the voltage across the planar lipid bilayer is applied and current, which flows through planar lipid bilayer, is measured. To the planar lipid bilayer apply a linearly increasing voltage and record a flowing current. Like at the current clamp method you will obtain the time course of the flowing current and the plot of the programmed voltage across the planar lipid bilayer.

From collected data determine the breakdown voltage (U_{br}) and the lifetime (t_{br}) of planar lipid bilayer.

FURTHER READING:

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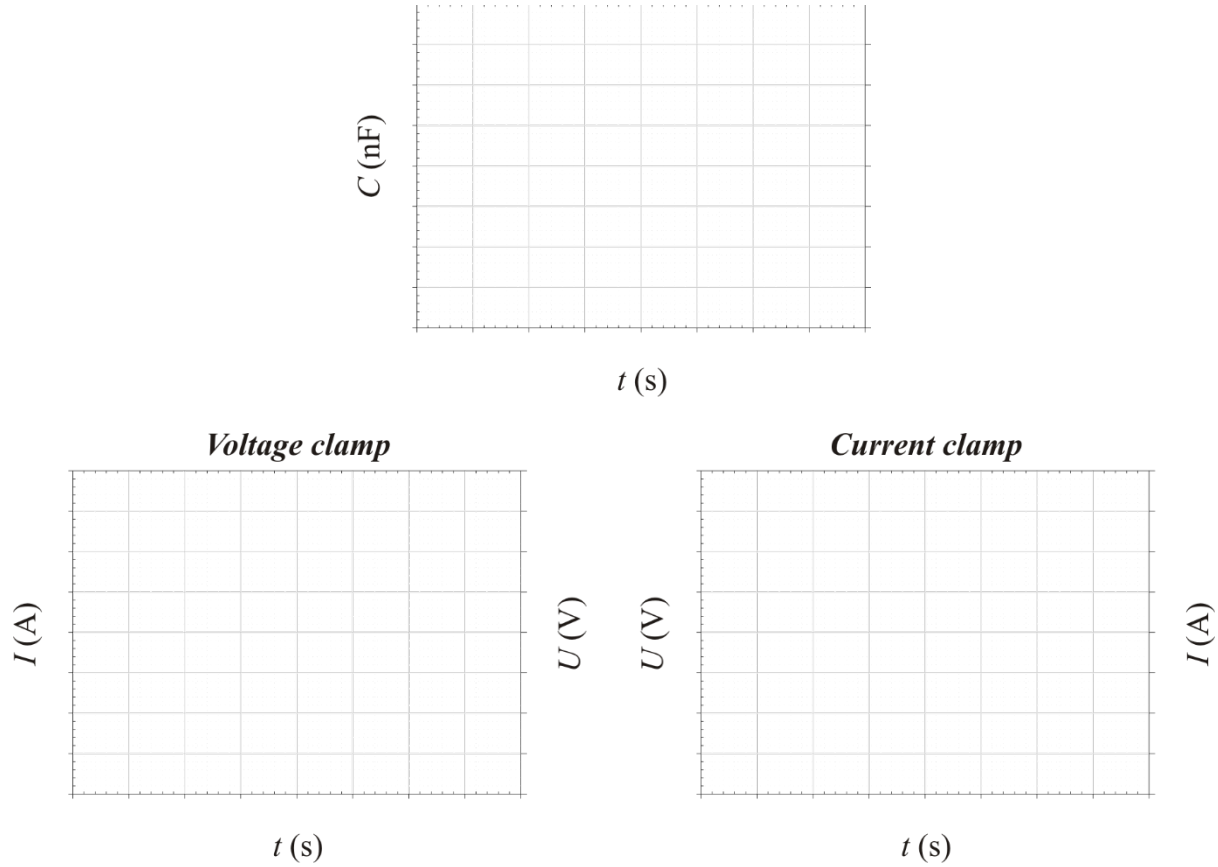
NOTES & RESULTS

NOTES & RESULTS



NOTES & RESULTS

E. coli inactivation by pulsed electric fields in a continuous flow system L7

Saša Haberl Meglič, Karel Flisar
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiment: day 1: 90 min; day 2: 60 min
Max. number of participants: 4
Location: Microbiological laboratory
Level: Basic

PREREQUISITES

Participants should be familiar with the Safety rules and Rules for sterile work in cell culture laboratory. No other specific knowledge is required for this laboratory practice.

THEORETICAL BACKGROUND

The first description of the profound effect of electrical pulses on the viability of a biological cell was given in 1958. If a cell is exposed to a sufficiently high electric field, its membrane becomes permanently permeable, resulting in leakage of cellular components, which leads to cell death. The method gained ground as a tool for microbial inactivation and the influence of different pulsed electric fields (PEF) on microbial viability was extensively studied on various microorganisms.

Since PEF microbial inactivation in controlled laboratory conditions showed promise, the idea arose of also removing pathogenic microorganisms from various water sources, hospital wastewaters and liquid food, without destroying vitamins or affecting the food's flavour, colour or texture. In order to facilitate PEF application on a large scale, the development of flow processes has been pursued. A standard PEF treatment system therefore consists of a pulse generator that enables continuous pulse treatment, flow chambers with electrodes and a fluid-handling system.

Several parameters have been described, which can influence inactivation of microbial cells. Specifically in a continuous flow system the flow rate of a liquid must be adjusted in order for each bacterial cell to be exposed to appropriate pulse conditions.

The aim of this laboratory practice is to demonstrate how different pulse parameters in a continuous flow system affect bacterial inactivation.

EXPERIMENT

We will inactivate *Escherichia coli* K12 TOP10 cells carrying plasmid pEGFP-N1, which encodes kanamycin resistance (Clontech Laboratories Inc., Mountain View, CA, USA) in a continuous flow system (see Figure 1) using different electric pulse parameters. To generate electric pulses square wave prototype pulse generator will be used. Pulses will be monitored on osciloscope (LeCroy 9310C). The inactivation level will be determined by plate count method.

Bacterial cells will be grown prior experiment for 17 hours at 37°C in Luria Broth (LB) medium (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) with shaking.

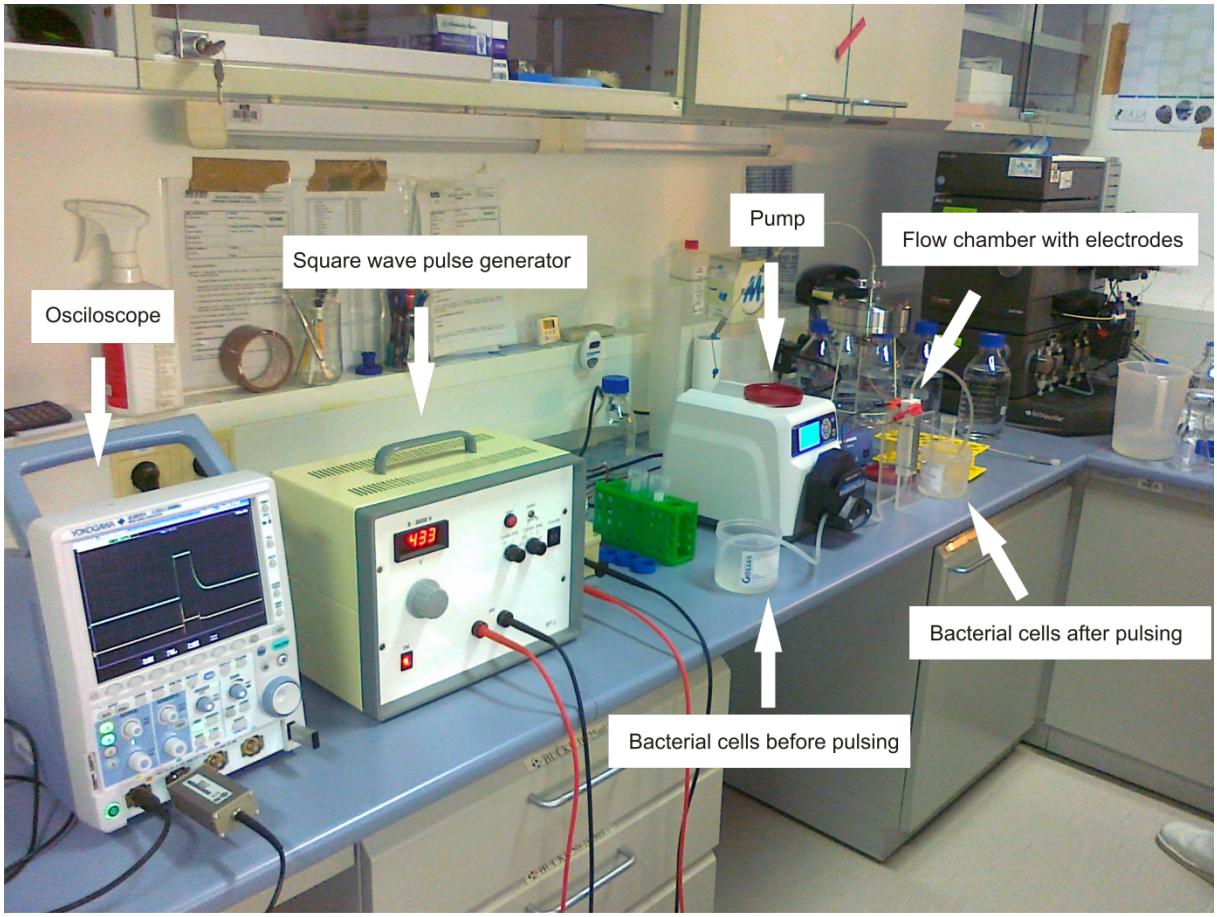


Figure 1. Continuous flow electroporation system. The circuit system includes a flow chamber with electrodes and a prototype square wave pulse generator. Voltage and current are both monitored throughout the experiment.

Protocol 1/2 (Electroporation of bacteria in a continuous flow system): On the first day of experiment *E. coli* cells will be centrifuged (4248 g, 30 min, 4°C) and the pellet will be re-suspended in 10 ml of distilled water and 100x diluted. The total volume of prepared bacterial cells for the treatment will be 0.3 L.

In order to determine the number of bacterial cells in our sample, you will prepare serial dilutions of bacterial sample ranging from 10^{-1} to 10^{-6} (in 900 µl of sterile distilled water you will dilute 100 µl of bacterial sample). You will pipette 100 µl of dilutions 10^{-5} and 10^{-6} on LB agar containing kanamycin antibiotic and evenly spread the liquid with sterile plastic rod.

The exposure of cells to electric pulses in flow through chamber in a continuous flow system depends on the geometry of the chamber, the frequency of pulses at which electroporator operates. The number of pulses is given by equation 1. At that flow, the desired number of pulses are applied to the liquid and thus to the cells in the flow-through chamber. Because the volume of our cross-field chamber between the electrodes (Q = 0.0005 L) and the frequency (10 Hz in our case) are constant, the flow through the chamber can be determined:

$$q = \frac{f}{n} \cdot Q \qquad (1)$$

where q (L/min) is the flow rate, Q (L) the volume between the two electrodes and n is the number of pulses received by the fluid in the chamber in residence time. For a frequency of 10 Hz, you will calculate the flow rate (q) at which the whole liquid will be subjected to at least one pulse. For PEF flow through

treatment you will use 0.3 L (10^{-2} dilution) of prepared bacterial cells. Bacterial cells will be pumped through the system at the calculated flow rate and pulses will be applied by prototype pulse generator.

After PEF treatment take 100 µl of treated sample and prepare dilutions ranging from 10^{-1} to 10^{-6}. You will pipette 100 µl of dilutions 10^{-4}, 10^{-5} and 10^{-6} on LB agar with kanamycin antibiotic and evenly spread the liquid with sterile plastic rod.

Protocol 2/2 (Determining bacterial viability): After 24 h incubation at 37ºC count colony forming units. Express the viability as log (N/N_0), where N represents the number of colony forming units per ml in a treated sample (bacterial cells exposed to electric pulses) and N_0 the number of colony forming units per ml in an untreated sample (bacterial cells not exposed to electric pulses).

Example of determining bacterial viability:
You counted 70 CFU in a control sample (dilution 10^{-7}) and 30 CFU in a treated sample (dilution 10^{-5}).

Number of bacterial cells per ml (control sample) = 70 x 10^7 (dilution factor of sample) x 10 (dilution factor of plating) = 7 x 10^9 bacterial cells/ml

Number of bacterial cells per ml (treated sample) = 30 x 10^5 (dilution factor of sample) x 10 (dilution factor of plating) = 3 x 10^7 bacterial cells/ml

log N/N_0 = log (3 x 10^7/ 7 x 10^9) = ***-2.368***

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NOTES & RESULTS

NOTES & RESULTS

Analysis of electric field orientations on gene electrotransfer efficiency L8

Saša Haberl Meglič, Matej Reberšek
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiment: day 1: 90 min; day 2: 60 min
Max. number of participants: 4
Location: Cell Culture Laboratory 1 (1st day) and 3 (2nd day)
Level: Advanced

PREREQUISITES

Participants should be familiar with the Safety rules and Rules for sterile work in cell culture laboratory. The basic knowledge of handling with cells is required for this laboratory practice.

THEORETICAL BACKGROUND

Gene electrotransfer is a non-viral method used to transfer genes into living cells by means of high-voltage electric pulses. An exposure of a cell to an adequate amplitude and duration of electric pulses leads to transient increase of cell membrane permeability for molecules which are otherwise deprived of membrane transport mechanisms. This allows various nonpermeant molecules, including DNA, to be transported across the membrane and enter the cell. Although mechanisms of the process are not yet fully elucidated, it was shown that several steps are crucial for gene electrotransfer: interaction of plasmid DNA (pDNA) with the cell membrane, translocation of pDNA across the membrane, migration of pDNA towards the nucleus, transfer of pDNA across the nuclear envelope and gene expression.

Many parameters (such as electric pulse protocol) can influence the first step (interaction of DNA with the cell membrane) and by that gene electrotransfer efficiency. Therefore different electric pulse protocols are used in order to achieve maximum gene transfection, one of them is changing the electric field orientation during the pulse delivery. Since DNA is a negatively charged molecule and it is dragged towards the cell with the electrophoretic force in the opposite direction of the electric field, changing electric field orientation increases the membrane area competent for DNA entry into the cell.

The aim of this laboratory practice is to demonstrate how different pulse polarity affects the efficiency of gene electrotransfer and cell viability.

EXPERIMENT

For the experiment we will use Chinese hamster ovary cells (CHO-K1) and plasmid DNA (pEGFP-N_1) that codes for GFP (green fluorescent protein). To generate and deliver electric pulses a high-voltage prototype generator and electrodes with four cylindrical rods, which were developed at a Laboratory of Biocybernetics will be used. Pulses will be monitored on osciloscope (LeCroy 9310C).

Pulse protocols (see also Figure 1):

a) SP (single polarity): the direction of electric field is the same for all pulses
b) OBP (orthogonal both polarities): the direction of the electric field is changed between the pulses

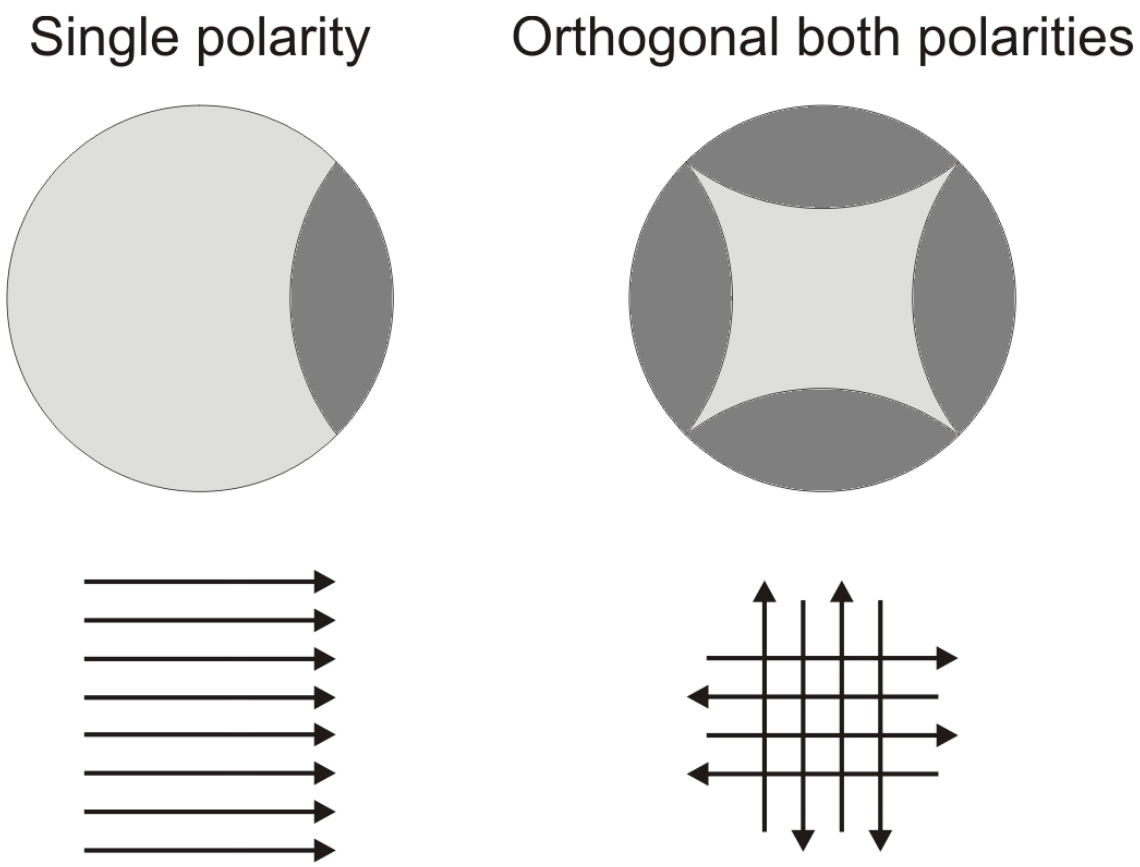

Figure 1: Two different pulse protocols will be used: single polarity (SP) and orthogonal both polarities (OBP)

Protocol 1/2 (Gene electrotransfer with different pulse parameters): CHO cells will be grown in multiwells as a monolayer culture in Ham's tissue culture medium for mammalian cells with 10% fetal bovine serum at 37º C. Cells will be plated 24h before the experiment in concentration 5 x 10^5 cells per well.
Just before the experiment remove culture medium and replace it with 150 µl of electroporation buffer containing plasmid DNA with concentration 10 µg/ml. Incubate cells with plasmid for 2-3 minutes at room temperature. Then apply a train of eight pulses with amplitude of 225 V, duration of 1 ms and repetition frequency 1 Hz using single polarity and orthogonal both polarities (see Pulse protocols) to deliver plasmid DNA into the cells.
Cells in the control are not exposed to electric pulses.
Immediately after exposure of cells to electric pulses add 37 µl of fetal calf serum (FCS-Sigma, USA). Incubate treated cells for 5 minutes at 37º C and then add 1 ml of culture medium.

Protocol 2/2 (Determining gene electrotransfer efficiency and cell viability): After 24 h incubation at 37º C determine the difference in gene electrotransfer efficiency and cell viability for both pulse protocols by fluorescent microscopy (Leica, Wetzlar, Germany) at 20x magnification using GFP filter with excitation at 488 nm.

You will determine gene electrotransfer efficiency from the ratio between the number of green fluorescent cells (successfully transfected) and the total number of cells counted under the phase contrast. You will obtain cell survival from phase contrast images as the ratio between the number of viable cells in the treated sample and the number of viable cells in the control sample.

FURTHER READING:

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NOTES & RESULTS

Pulse parameters	Gene electrotransfer efficiency [%]	Cell viability [%]
Single polarity		
Orthogonal both polarities		

NOTES & RESULTS

Monitoring of electric field distribution in biological tissue by means of magnetic resonance electrical impedance tomography L10

***Matej Kranjc*[1], *Igor Serša*[2]**
[1]*University of Ljubljana, Faculty of Electrical Engineering*
[2]*Jozef Stefan Institute*

Duration of the experiment: day 1: 90 min
Max. number of participants: 4
Location: MRI Laboratory (Jožef Stefan Institute)
Level: Basic

PREREQUISITES

Participants should be familiar with Laboratory safety (S1). No other specific knowledge is required for this laboratory practice.

THEORETICAL BACKGROUND

A method capable of determining electric field distribution during the pulse delivery has a practical value as it can potentially enable monitoring of the outcome of electroporation which strongly depends on the local electric field. Measurement of electric field distribution enables detection of insufficient electric field coverage before the end of either reversible or irreversible electroporation treatment, thus enabling corrections of field coverage during the treatment and consequently increasing and assuring its effectiveness. As there are no available approaches for measurement of electric field distribution *in situ*, an indirect approach using magnetic resonance techniques was suggested. Magnetic resonance electrical impedance tomography (MREIT) enables reconstruction of electric field distribution by measurement of electric current density distribution, first, and calculation of electrical conductivity of the treated subject during application of electric pulses using MRI data as an input to numerical algorithms, second. This method enables determination of electric field distribution *in situ* also accounting for changes that occur in the tissue due to electroporation.
MREIT is a relatively new medical imaging modality based on numerical reconstruction of electrical conductivity inside a tissue by means of current density distribution measured by current density imaging (CDI) sequence. The MREIT algorithm applied for reconstruction of electrical conductivity of the tissue is based on solving Laplace's equation through iterative calculation. Electrical conductivity is updated after each iteration (k+1):

$$\sigma^{k+1} = \frac{|\mathbf{J}_{\mathrm{CDI}}|}{|\nabla u^k|}.$$

where $\mathbf{J}_{\mathrm{CDI}}$ is current density obtained by CDI and u^{k} is electric potential obtained as a solution of Laplace's equation. When difference between two successive conductivities falls below certain value electric field distribution can be calculated using:

$$\mathbf{E} = \frac{\mathbf{J}_{\mathrm{CDI}}}{\sigma}.$$

The aim of this laboratory practice is to demonstrate monitoring of electric field distribution in a biological tissue using MREIT.

EXPERIMENT

We will monitor current density distribution and electric field distribution in biological tissue exposed to electric pulses by means of MREIT. We will then compare measured current density distribution and reconstructed electric field distribution with simulation results obtained by a numerical model of the tissue.

Protocol

The experiment will be performed on biological tissue (chicken liver) sliced in a disc-like sample measuring 21 mm in diameter and 2 mm in height (Fig. 1a). Electric pulses will be delivered via two cylindrically shaped electrodes inserted into the sample. After the insertion, the electrodes will be connected to an electric pulse generator connected to an MRI spectrometer. The sample will be placed in a 25 mm MR microscopy RF probe (Fig. 1b) inside a horizontal-bore superconducting MRI magnet (Fig. 1c). Electroporation treatment of the sample will be performed by applying two sequences of four high voltage electric pulses with a duration of 100 μs, a pulse repetition frequency of 5 kHz and with an amplitude of 500 V and 1000 V.

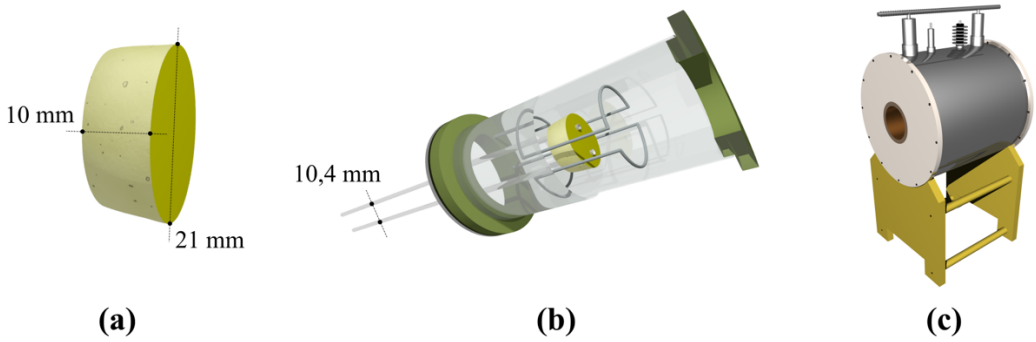

Figure 1: Biological sample (a) placed in a MR microscopy probe (b) inside a horizontal MRI magnet (c).

MR imaging will be performed on a MRI scanner consisting of a 2.35 T (100 MHz proton frequency) horizontal bore superconducting magnet (Oxford Instruments, Abingdon, United Kingdom) equipped with a Bruker micro-imaging system (Bruker, Ettlingen, Germany) for MR microscopy with a maximum imaging gradient of 300 mT/m and a Tecmag Apollo spectrometer (Tecmag, Houston TX, USA). Monitoring of electric field is enabled by CDI, which is an MRI method that enables imaging of current density distribution inside conductive sample. We will apply two-shot RARE version of the CDI sequence (Fig. 2).

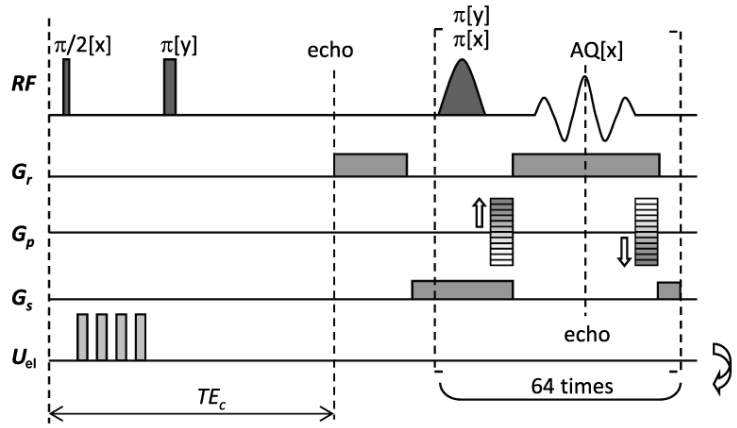

Figure 2: Two-shot RARE pulse sequence used for acquisition of current density distribution. The sequence consists of a current encoding part with a short (100 μs long) high-voltage electroporation pulse (U_{el}) delivered immediately after the nonselective 90° radiofrequency (RF) excitation pulse. In the second part of the sequence signal acquisition is performed using the single-shot RARE signal acquisition scheme that includes standard execution of readout (G_r), phase-encoding (G_p) and slice-selection (G_s) magnetic field

gradients. Due to auxiliary phase encoding induced by the electric pulse, the RARE sequence is repeated twice, each time with a different phase of the refocusing pulses (0°and 90°), and the corresponding signals are co-added.

Electric field distribution in the sample will be reconstructed by iteratively solving Laplace's equation using J-substitution mathematical algorithm and finite element method with the numerical computational environment MATLAB on a desktop PC. We will compare measured current density distribution obtained by means of CDI and reconstructed electric field distribution obtained by means of MREIT in the sample with simulation results obtained by a numerical model of the sample.

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NOTES & RESULTS

NOTES & RESULTS

Measurements of the induced transmembrane voltage with fluorescent dye di-8-ANEPPS

L11

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Duration of the experiments: 60 min
Max. number of participants: 4
Location: Cell Culture Laboratory 1
Level: Advanced

PREREQUISITES

Participants should be familiar with Laboratory safety (S1). The basic knowledge of handling with cells is required for this laboratory practice.

THEORETICAL BACKGROUND

When a biological cell is placed into an external electric field the induced transmembrane voltage (ITV) forms on its membrane. The amplitude of the ITV is proportional to the amplitude of the applied electric field, and with a sufficiently strong field, this leads to an increase in membrane permeability - electroporation. Increased permeability is detected in the regions of the cell membrane where the ITV exceeds a sufficiently high value, in the range of 250 – 1000 mV, depending on the cell type. In order to obtain an efficient cell electroporation it is therefore important to determine the distribution of the ITV on the cell membrane. The ITV varies with the position on the cell membrane, is proportional to the electric field, and is influenced by cell geometry and physiological characteristics of the medium surrounding the cell. For simple geometric shapes the ITV can be calculated analytically (e.g. for a spherical cell, using Schwan's equation). For more complicated cell shapes experimental and numerical methods are the only feasible approach to determine the ITV.

The aim of this laboratory practice is to measure the ITV on a spherical cell by means of a fluorescent potentiometric dye di-8-ANEPPS.

EXPERIMENT

Potentiometric fluorescent dyes allow observing the variations of the ITV on the membrane and measuring its value. Di-8-ANEPPS is a fast potentiometric fluorescent dye, which becomes fluorescent when it binds to the cell membrane, with its fluorescence intensity varying proportionally to the change of the ITV. The dye reacts to the variations in the ITV by changing the intramolecular charge distribution that produce corresponding changes in the spectral profile or intensity of the dye's fluorescence.

Protocol: The experiments are performed on Chinese hamster ovary cells (CHO) grown in Lab-Tek chambers (Nunc, Germany) in culture medium HAM-F12 supplemented with 10% fetal bovine serum, L-glutamine (all three from Sigma-Aldrich) and antibiotics. When cells attach to the cover glass of a Lab-Tek chamber (usually after 2 to 3 hours to obtain attached cells of spherical shape), carefully replace the culture medium with 1 ml of SMEM medium (Spinner's modification of the MEM, Sigma-Aldrich) containing 30 μM of di-8-ANEPPS and 0.05% of Pluronic (both Life Technologies). After staining for 12 min at 4°C, wash the cells thoroughly with pure SMEM to remove the excess dye. After washing leave 1.5 ml of SMEM in the chamber. Place the chamber under a fluorescence microscope (Zeiss AxioVert 200, Germany) and use ×63 oil immersion objective. Position two parallel Pt/Ir wire electrodes, with a 4 mm distance between them, to the bottom of the chamber. Set a single 40 V, 50 ms pulse on the programmable square wave electroporator TSS20 (Intracel). This will result in a voltage-

to-distance ratio of ~100 V/cm. The pulse must be synchronized with the image acquisition. Set the excitation wavelength to 490 nm and use ANEPPS filter to detect fluorescence (emission 605 nm).
Find the cells of interest. Acquire the control fluorescence image and subsequently the image with a pulse, using a cooled CCD camera (VisiCam 1280, Visitron) and MetaFluor 7.7.5 (Molecular Devices). Apply four pulses with a delay of 4 s between two consecutive pulses. For each pulse, acquire a pair of images, one immediately before (control image) and one during the pulse (pulse image) (Figures 1A and B).
Open the images in MetaMorph 7.7.5 (Molecular Devices). To *qualitatively* display the ITV on the cell membrane, convert the acquired 12-bit images to 8-bit images. For each pulse, obtain the difference image by subtracting (on a pixel-by-pixel basis) the control image from the pulse image. Add 127, so that 127, i.e. mid-gray level, corresponds to 0 V, brighter levels to negative voltages, and darker levels to positive ones (Figure 1C). Average the three difference images to increase the signal-to-noise ratio.
To *quantitatively* determine the ITV, open the acquired, unprocessed fluorescence images. Determine the region of interest at the site of the membrane and measure the fluorescence intensities along this region for the control and pulse image. Transform the values to the spreadsheet. Measure the background fluorescence in both images and subtract this value from the measured fluorescence. Calculate the relative changes in fluorescence ($\Delta F/F_C$) by subtracting the fluorescence in the control image F_C from the fluorescence in the pulse image F_P and dividing the subtracted value by the fluorescence in the control F_C; $\Delta F/F_C = (F_P - F_C) / F_C$. Average the relative changes calculated for all four acquired pairs of images. Transform the fluorescence changes to the values of the ITV (ΔF/F = -6% / 100 mV), and plot them on a graph as a function of the arc length (Figure 1D).

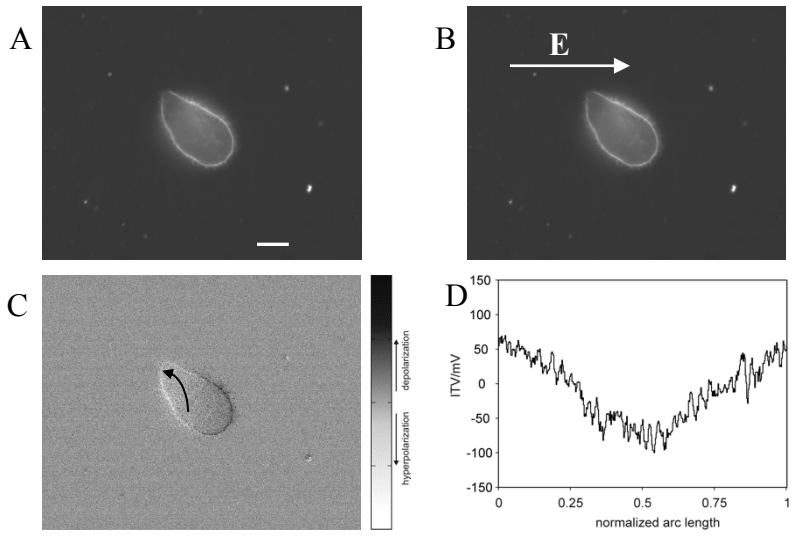

Figure 1: Measurements of the induced transmembrane voltage (ITV) on an irregularly shaped CHO cell. (A) A control fluorescence image of a cell stained with di-8-ANEPPS. Bar represents 10 µm. (B) Fluorescence image acquired during the exposure to a 35 V (~88 V/cm), 50 ms rectangular pulse. (C) Changes in fluorescence of a cell obtained by subtracting the control image A from the image with pulse B and shifting the grayscale range by 50%. The brightness of the image was automatically enhanced. (D) ITV measured along the path shown in C.

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Video Article:

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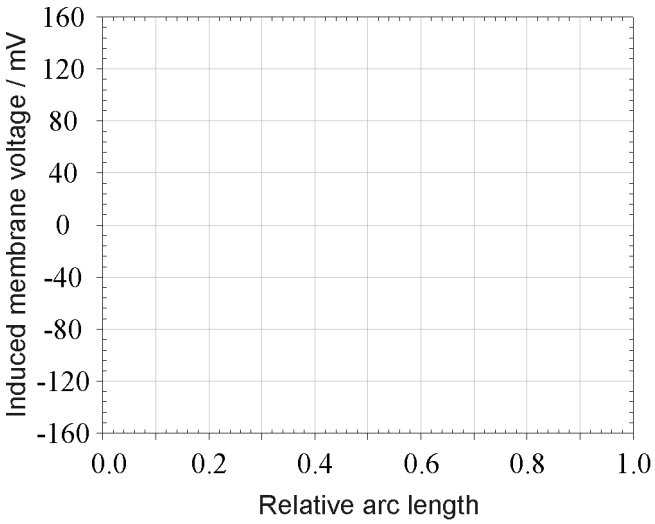
NOTES & RESULTS



NOTES & RESULTS

COMPUTER MODELING

Treatment planning for electrochemotherapy and irreversible electroporation: optimization of voltage and electrode position C1

Anže Županič[1], Bor Kos[2]
[1]Eawag - Swiss federal institute of aquatic science and technology, Switzerland
[2]University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 90 min
Max. number of participants: 6
Location: Laboratory of Biocybernetics
Level: Basic

PREREQUISITES

No specific knowledge is required for this laboratory exercise.

THEORETICAL BACKGROUND

Electrochemotherapy (ECT) is an efficient local treatment of cutaneous and subcutaneous tumors, which combines the delivery of nonpermeant, cytotoxic chemotherapeutics (e.g. bleomycin, cisplatin) and short high-voltage electric pulses. The pulses induce electric fields inside the tissue, thereby increasing cell membrane permeability in tissue (electropermeabilization) to otherwise nonpermeant chemotherapeutics. ECT requires the electric field inside the tumor to be higher than the threshold value needed for reversible electroporation (E_{rev}) while irreversible electroporation (E_{irrev}) in nearby critical structures should be limited. For IRE, the electric field in the entire tumor volume needs to be above the irreversible electroporation threshold. It is not necessary that the whole tumor is electropermeabilized by one pulse or pulse sequence - sometimes a combination of several pulse sequences or a combination of different electrodes is required.

The aim of this laboratory practice is to learn how to use optimization techniques to achieve suitable electric field distribution for electrochemotherapy experimental planning and treatment planning.

EXPERIMENT

A finite element based numerical modeling program package COMSOL Multiphysics version 5.4 (COMSOL AB, Stockholm, Sweden) will be used to optimize voltage between the electrodes and position of the electrodes on a simple 3D model of a spherical subcutaneous tumor and surrounding tissue (Figure 1a). Electrode positions and the applied voltage should be chosen, so that the following objectives are fulfilled:

- For electrochemotherapy: the tumor is permeabilized ($E_{tumor} > E_{rev} = 400\ V/cm$),
- For irreversible electroporation: the tumor is permeabilized above the irreversible threshold ($E_{tumor} > E_{irrev} = 600\ V/cm$),
- the damage to healthy tissue is kept to a minimum.

We will calculate the electric field distribution in the model after each change of the electrode placement or voltage. The final goal of this exercise is to achieve 100 % $E_{tumor} > E_{rev}$ (or 100 % $E_{tumor} > E_{irr}$ when planning for IRE) and minimize E_{irr} in healthy tissue.

Protocol: Build the 3-d model by following the lecturer's instructions and take into account your tissue-specific electric properties. Solve the model and evaluate the initial solution. In case, the initial solution is inappropriate (see e.g., Figure 1b), try to improve on the solution by changing electrode positions and voltage between the electrodes. Calculate the electric field distribution in the model after changing the

electrode positions or voltage and then determine the coverage of tumor tissue with $E_{tumor} > (E_{rev}$ or $E_{irrev})$ and determine damage to healthy tissue due to irreversible electroporation. Repeat the process, until the quality of your solution reaches the set goals. Compare the results with others, who have used different tissue properties. Use a parametric study to find the lowest voltage which achieves the objective for the selected electrode geometry.

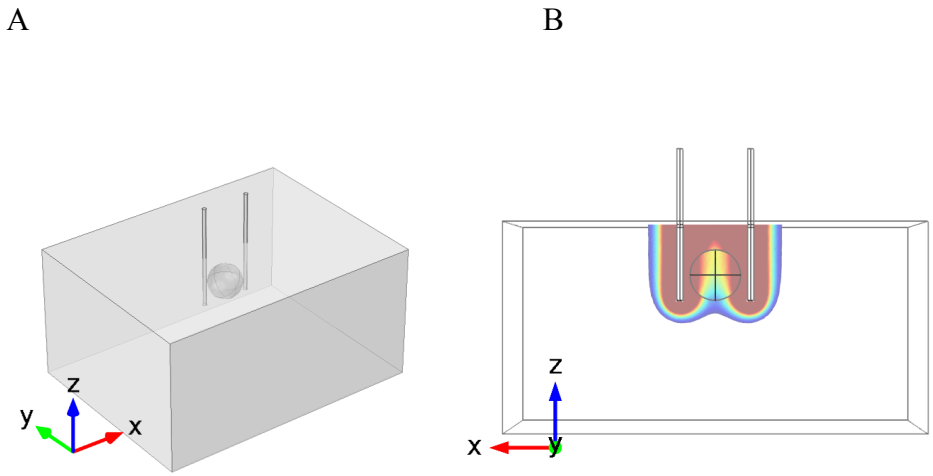


Figure 1: (A) Simple 3D model of tumor and needle electrodes in healthy tissue; (B) electric field over reversible threshold inside the healthy tissue and the tumor.

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NOTES & RESULTS

Numerical Modeling of Thermal Effects during Irreversible Electroporation Treatments

C2

Paulo A. Garcia[1] and Bor Kos[2]
[1]*Virginia Tech – Wake Forest University*
[2]*University of Ljubljana, Faculty of Electrical Engineering*

Duration of the experiment: 90 min
Max. number of participants: 6
Location: Laboratory of Biocybernetics
Level: Advanced

PREREQUISITES

Basic to advanced knowledge of finite element modeling

THEORETICAL BACKGROUND

Irreversible electroporation (IRE) is a new, safe, and effective minimally invasive ablation modality with the potential to treat many currently unresectable and/or untreatable tumors. The non-thermal mode of cell death in IRE is unique in that it does not rely on thermal changes from Joule heating to kill tumor cells thus allowing for successful treatment even in close proximity to critical structures and without being affected by the heat sink effect. Accurate modeling of the electrical and thermal responses in tissue is important to achieve complete coverage of the tumor and ensure that the thermal changes during a procedure do not generate thermal damage, especially in critical structures (e.g. bile ducts, nerves and sensitive blood vessels).

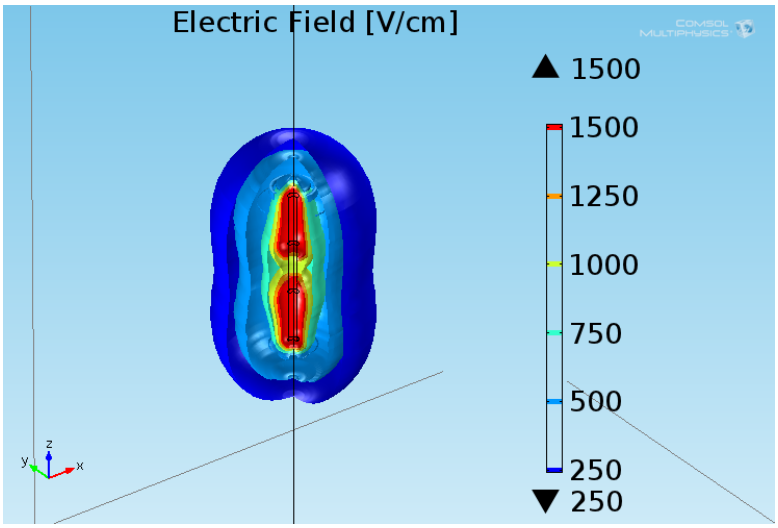

Figure 2: Electric Field distribution resulting from a bipolar electrode with an applied voltage of 1250 V.

The temperature distribution (T) within the tissue will be obtained by transiently solving a modified heat conduction equation with the inclusion of the Joule heating source term $Q = \sigma|\nabla\varphi|^2$

$$\rho C \frac{\partial T}{\partial t} = \nabla \cdot (k\nabla T) + Q \quad (1)$$

where σ is the electrical conductivity, φ the electric potential, k is the thermal conductivity, C is the specific heat capacity, and ρ is the density of the tissue. At each time step, the current density and electric field distribution are determined and updated in the Joule heating term to capture the electrical conductivity changes in liver tissue from electroporation and temperature.

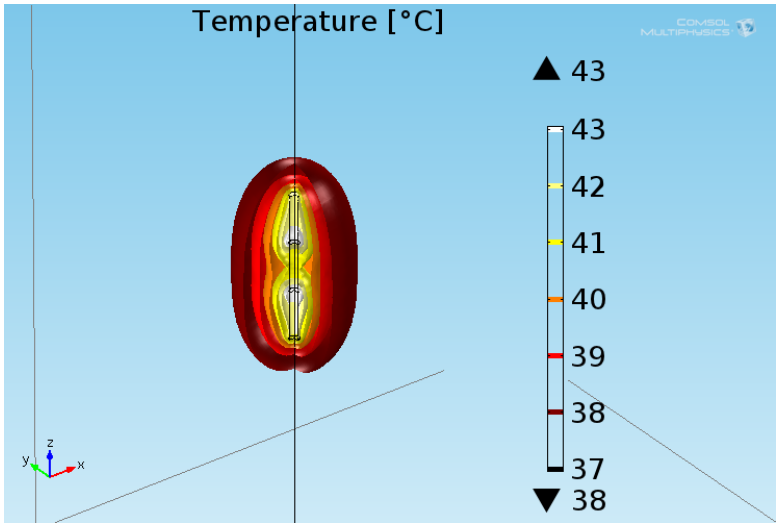

Figure 3: Temperature distribution after a ninety 100-µs pulse IRE treatment in liver tissue at 1 pulse per second.

Thermal damage is a process that depends on temperature and time. If the exposure is long, damage can occur at temperatures as low as 42°C, while 50°C is generally chosen as the target temperature for instantaneous damage. The damage can be calculated based on the temperatures to assess whether a particular set of pulse parameters and electrode configuration will induce thermal damage in superposition with IRE. The thermal damage will be quantified using the Arrhenius rate equation given by:

$$\Omega(t) = \int_{t=0}^{t=\tau} \zeta \cdot e^{\frac{-E_a}{R \cdot T(t)}} dt \qquad (2)$$

where R is the universal gas constant, 8.314 J/(mol·K); ζ is the pre-exponential factor, 7.39×10^{39} s^{-1}, a measure of the effective collision frequency between reacting molecules in bimolecular reactions; E_a the activation energy barrier that molecules overcome to transform from their "native state" to the "damaged state", 2.577×10^5 J/mol for liver tissue. It is important to note that the pre-exponential factor and activation energy are tissue specific parameters that describe different modes of thermal damage such as microvascular blood flow stasis, cell death, and protein coagulation. In terms of finite element modeling of thermal damage, an integral value $\Omega(t) = 1$ corresponds to a 63% probability of cell death and an integral value $\Omega(t) = 4.6$ corresponds to 99% probability of cell death due to thermal effects. In order to convert the damage integral to a probability of cell death, $P(\%)$, we will use:

$$P(\%) = 100 \cdot \left(1 - e^{-\Omega(t)}\right)$$

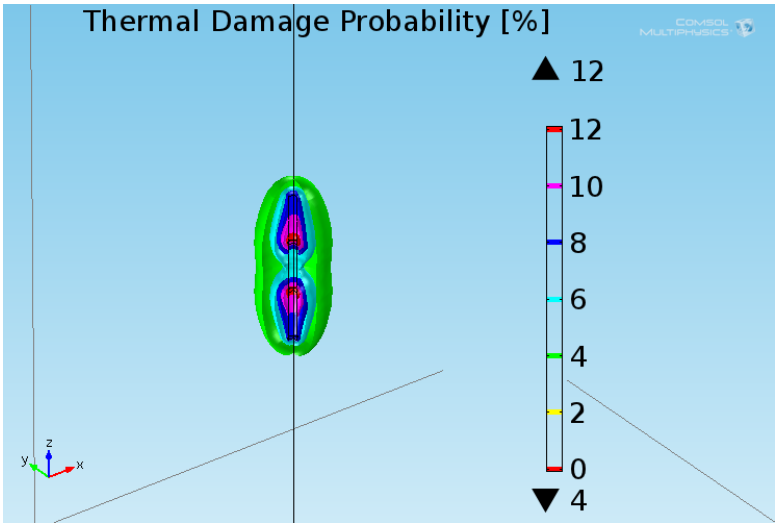

Figure 4: Thermal damage probability of cell death due to excessive thermal effects as a result of Joule heating.

The aim of this laboratory practice is to get familiar with the numerical simulation tools needed for capturing the electrical and thermal responses during a ninety 100-µs pulse IRE. We will accomplish this by coupling the Laplace, Heat Conduction, and Arrhenius equations using COMSOL Multiphysics 5.4 (Comsol AB, Stockholm, Sweden) to determine the IRE zones of ablation and evaluate if the increase in temperature due to Joule heating due to the pulses generates any potential thermal damage.

EXPERIMENT

In this exercise we will compare the effect of a static, σ_0, and dynamic, $\sigma(E)$, electrical conductivity functions in the resulting electrical and thermal effects during an entire IRE protocol in liver tissue. Initially we will determine the volume of tissue affected by IRE from the electric field distributions. We will then evaluate the temperature increase in liver tissue as a result of the Joule heating and determine if there was a probability of cell death due to thermal damage with the given IRE protocols employed. This exercise will provide the participants with accurate predictions of all treatment associated effects which is a necessity toward the development and implementation of optimized treatment protocols.

Specifically:

1) Simulate the electric field distribution using a static conductivity and 1000 V, 1500 V, and 2000 V.
2) Simulate the electric field distribution using a dynamic conductivity and 1000 V and 1500 V.
3) Include the Heat Conduction Equation by coupling with the Laplace Equation via Joule Heating.
4) Explore the resulting temperature distributions as a function of pulse number and frequency.
5) Incorporate the Arrhenius equation to assess potential thermal damage from the Joule Heating.
6) Investigate the effect of pulse frequency (1 Hz, 10 Hz, and 100 Hz) for ninety 100-µs pulses.

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NOTES & RESULTS

Molecular dynamics simulations of membrane electroporation C3

Mounir Tarek
CNRS- Université de Lorrains, Nancy France
Europeen Laboratory EBAM

Duration of the experiments: 90 min
Max. number of participants: 18
Location: Computer room (CIT)
Level: Basic

PREREQUISITES
No specific knowledge is required for this laboratory practice.

THEORETICAL BACKGROUND
The application of high electric fields to cells or tissues permeabilizes the cell membrane and is thought to produce aqueous-filled pores in the lipid bilayer. Electroporation is witnessed when the lipid membrane is subject to transmembrane voltages (TMV) of the order of few hundred millivolts, which results from the application of electrical pulses on a microsecond to millisecond time scale

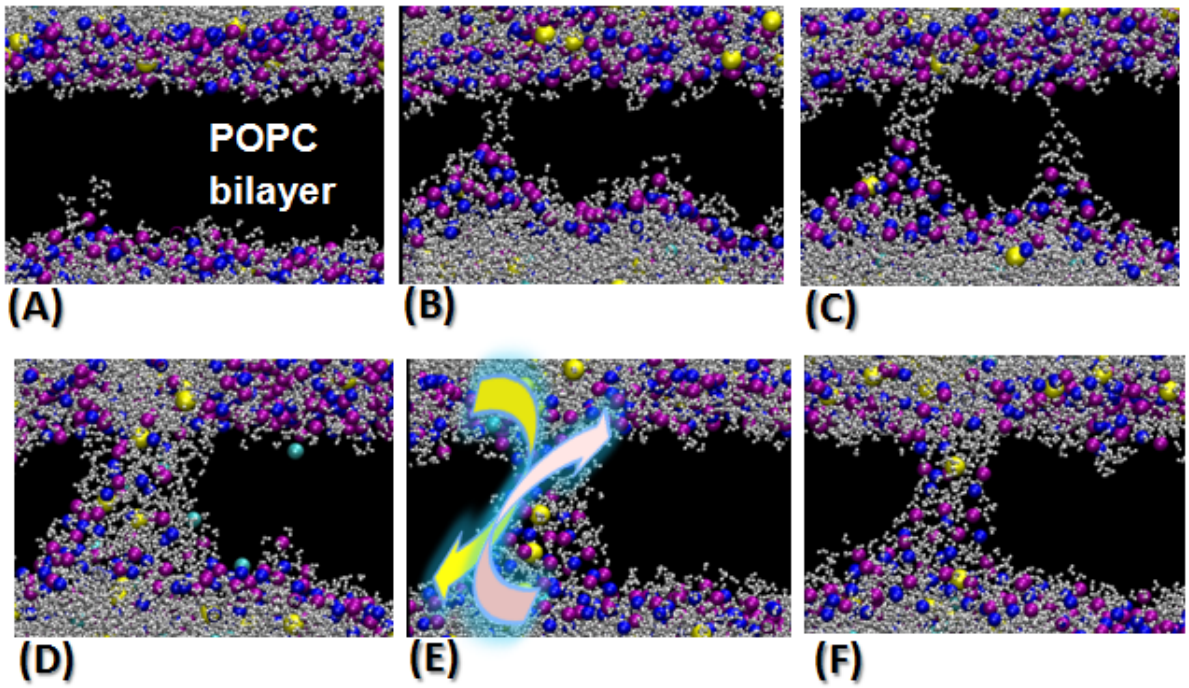

Figure 1: Configurations from the MD simulation for a large POPC subject to a transverse electric field (A) Bilayer at equilibrium. (B-C) Formation of water wires at the initial stage of the electroporation process (D-F) Formation at a later stage of large water pores that conduct ions across the membrane and that are stabilized by lipid head-group (yellow cyan). (Delemotte and Tarek. *J. Membr. Biol.* 2012).

which are sufficient to produce a transient trans-membrane potential and an electrical field across the membrane of the order of ~ 10^8 V/m. This process is believed to involve (1) charging of the membrane due to ion flow, (2) rearrangement of the molecular structure of the membrane, (3) formation of pores, which perforate the membrane and are filled by water molecules (so-called aqueous, or hydrophilic,

pores), (4) an increase in ionic and molecular transport through these pores, and, under appropriate conditions, membrane integrity recovery when the external field stress is removed.

Molecular Dynamics (**MD**) simulations belong to a set of computational methods in which the dynamical behaviour of an ensemble of atoms or molecules, interacting via approximations of physical pair potentials, is determined from the resolution of the equation of motions. MD simulations enable ones to investigate the molecular processes affecting the atomic level organization of membranes when these are submitted to voltage gradient of magnitude similar to those applied during electropulsation. The aim of this practical exercise is to characterize from MD simulations trajectories the electrostatic properties of membranes subject to a transmembrane potential (0 to 2 V).

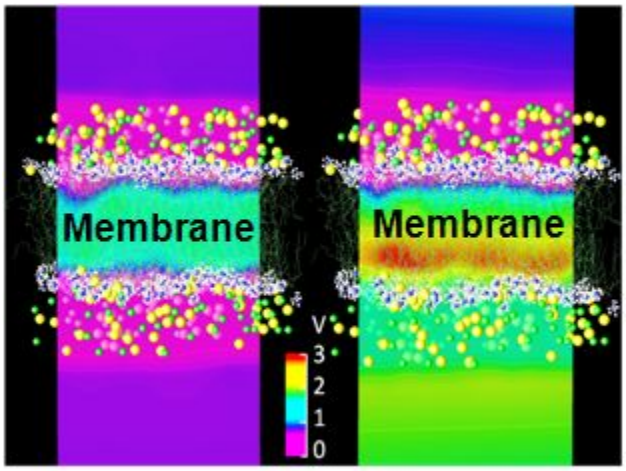

Figure 2: Electrostatic potential maps generated from the MD simulations of a POPC lipid bilayer (acyl chains, green; head groups, white) surrounded by electrolyte baths at 1 M NaCl (Na+ yellow, Cl- green, water not shown) terminated by an air/water interface. Left: net charge imbalance Q = 0 e (TMV=0 mV). Right: Q = 6 e (TMV=2 V).

The aim of this laboratory practice is to get familiar with the tools for molecular dynamics, possibilities to set on models and graphical presentation of atomistic models.

EXPERIMENT

Due to the limited time and large resources needed to generate MD trajectories of membranes, the latter will be provided to the students. The simulations concern pure planar phospholipid bilayers (membrane constituents) and water described at the atomic level. A set of long trajectories spanning few nanoseconds generated with or without a transmembrane voltage induced by unbalanced ionic concentrations in the extracellular and intracellular will be provided. The students will (1) determine the distribution of potential and electric field in model membrane bilayers (2) measure the membrane capacitance, (3) visualize at the molecular level the formation of membrane pores under the influence of a transmembrane voltage, and measure the intrinsic conductance of such pores.

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NOTES & RESULTS

E-LEARNING

Electroporation of cells and tissues - interactive e-learning course E1

Selma Čorović and Samo Mahnič Kalamiza
University of Ljubljana, Faculty of electrical engineering

Duration of the experiment: app. 90 min
Max. number of participants: 18
Location: Computer room (P5)
Level: Basic

PREREQUISITES

No specific knowledge is required for this laboratory practice.

The aim of this laboratory practice is to provide the participants with basic knowledge on local electric field distribution in cells and tissues exposed to high voltage electric pulses (i.e. electroporation pulses) by means of interactive e-learning course content. The e-learning content is based on the available knowledge from the scientific literature.

PROTOCOL OF THE E-LEARNING COURSE

The participants will be gathered in a computer-computer classroom providing each participant with a computer. A short test will be given to establish the baseline knowledge before the e-learning course.
Within the first part of the e-learning course we will bring together the educational material on basic mechanisms underlying electroporation process on the levels of cell membrane, cell and tissues as a composite of cells (Figure 1).

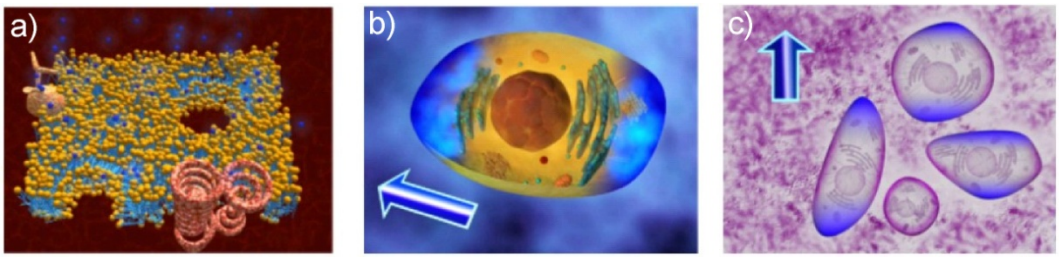

Figure 1: Introduction of small molecules (blue molecules) through a cell membrane (a) into an electroporated cell (b) and into the successfully electroporated cells within an exposed tissue (c) (Čorović et al., 2009).

Within the second part of the course we will provide basic knowledge on important parameters of local electric field needed for efficient cells and tissue electroporation, such as: electrode geometry (needle or plate electrodes as illustrated in Figure 2, electrode position with respect to the target tissue and its surrounding the tissues (Figure 3), the contact surface between the electrode and the tissue, the voltage applied to the electrodes and electroporation threshold values. This part of the e-learning course content will be provided by an interactive module we developed in order to visualize the local electric field distribution in 2D and 3D dimensional tissue models.

The objective of this module is to provide:
- local electric field visualization in cutaneous (protruding tumors) and subcutaneous tumors (tumors more deeply seeded in the tissue);
- guideline on how to overcome a highly resistive skin tissue in order to permeabilize more conductive underlying tissues and

- visualization and calculation of successfully electroporated volume of the target tissue and its surrounding tissue (i.e. the treated tissue volume exposed to the electric field between reversible and irreversible electroporation threshold value $E_{rev} \leq E < E_{irrev}$) with respect to the selected parameters such as: number and position of electrodes, applied voltage on the electrodes.

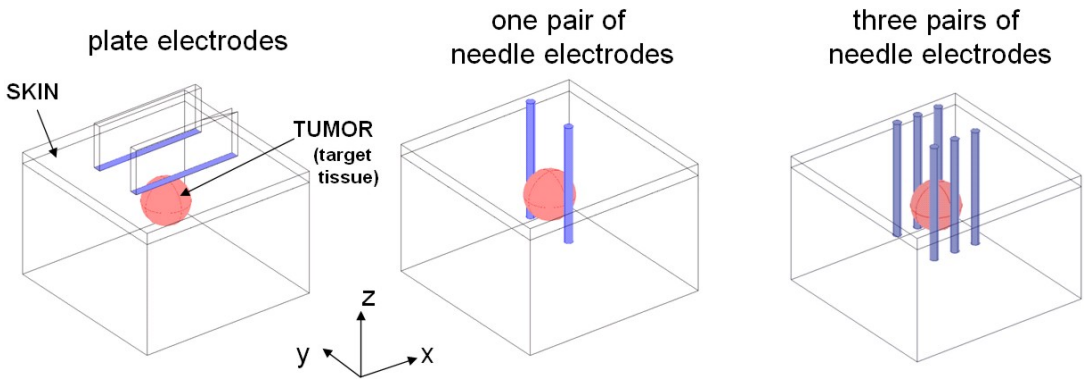

Figure 2: Plate electrodes vs. needle electrodes with respect to the target tissue (e.g. tumor tissue).

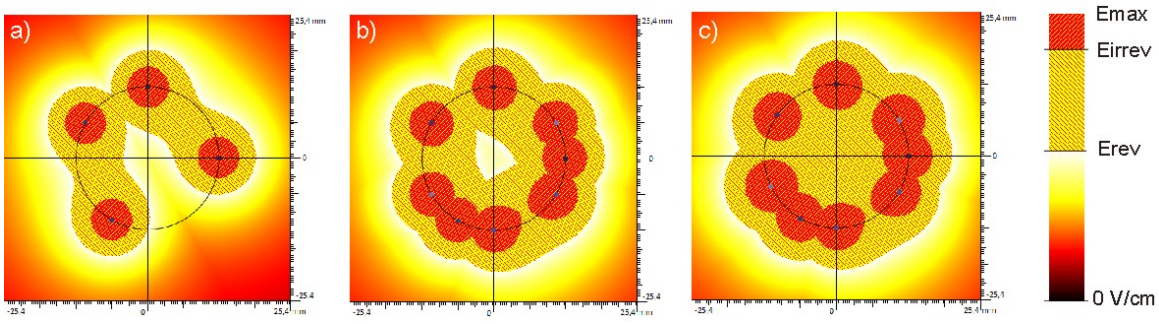

Figure 3: Electric field distribution within the tumor (inside the circle) and within its surrounding tissue (outside the circle) obtained with three different selection of parameters (number and position of electrodes and voltage applied): (a) 4 electrodes, (b) 8 electrodes and (c) 8 electrodes with increased voltage on electrodes so that the entire volume of tumor is exposed to the $E_{rev} \leq E < E_{irrev}$.

After the e-learning course the pedagogical efficiency of presented educational content and the e-learning application usability will be evaluated.

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NOTES & RESULTS

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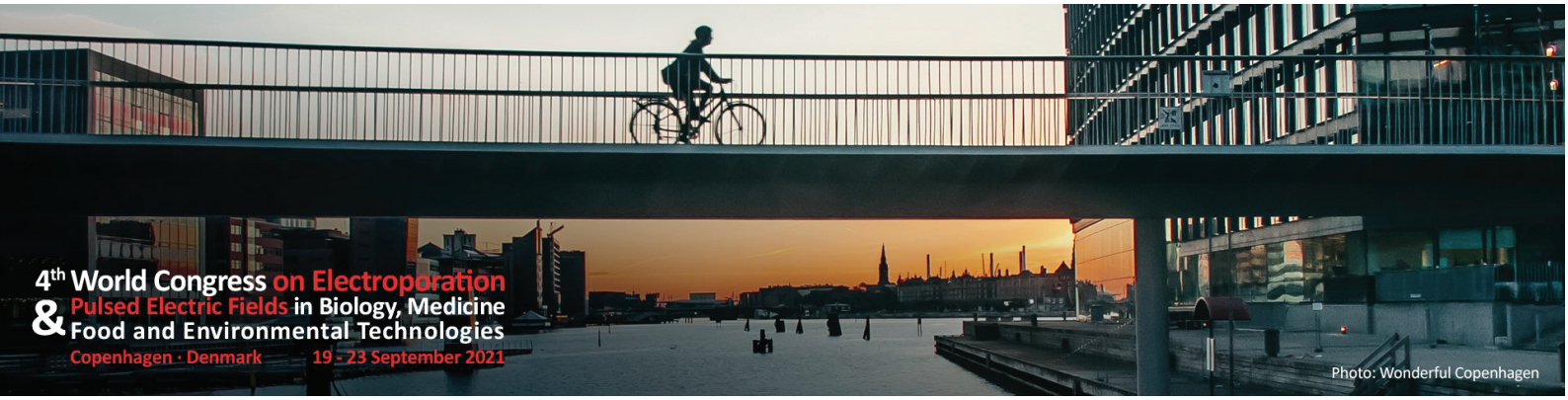

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