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Welcome note

Dear Colleagues, dear Students,

The Workshop and Postgraduate Course on Electroporation-Based Technologies and Treatments (EBTT) at the University of Ljubljana was organised for the first time in 2003. In more than twenty years, over 1000 participants from 45 different countries have attended the school. And also this year we can say with great pleasure: "with the participation of many of the world's leading experts in this field". However, the aims and objectives of the school remain unchanged: to provide participants with sufficient theoretical background knowledge and practical skills to enable them to use electroporation effectively in their working environment.

It is a great pleasure to welcome you to the EBTT organised by the University of Ljubljana and the Institute of Oncology Ljubljana, which takes place at the Faculty of Electrical Engineering as an integral part of the Interdisciplinary doctoral programme Biomedicine of the University of Ljubljana. From the very beginning, we were aiming to prepare lab work for the participants that would complement the lectures. Since the preparation of lab work takes more time than the preparation and organisation of lectures, we introduced lab work at the second workshop in 2005. The lab work covers different aspects of research: biological experiments in the cell culture labs, microbiology lab, tissue lab; numerical and molecular dynamics modelling, e-learning using computer classrooms, development of pulse generators and electrical measurements using the electronic lab workshop.

After the experience of 2020, when we organized the school completely online due to the pandemic, we decided to continue organizing the course as a hybrid course to enable participation even for those who still have difficulties with travel or suffer from a lack of time. The team here in Ljubljana will therefore offer both practical lab work on site and live webinars on the lab work so that you can benefit the most even if you are not in the lab.

The biological experiments were pre-recorded and will be organized in the Infrastructural Centre "Cellular Electrical Engineering", part of the University of Ljubljana's network of research and infrastructure centres, in the Laboratory of Biocybernetics. Lab works would not be possible without the extensive participation and commitment of numerous members of the Laboratory of Biocybernetics, for which I would like to thank them all cordially.

It also needs to be emphasized that all written contributions included in the proceedings were thoroughly reviewed and subsequently edited. We would like to thank all authors and reviewers for their diligent work. I would also like to express our sincere gratitude to the faculty members and invited lecturers for their lectures delivered at the course. Finally, I would like to thank our sponsors as well as the Bioelectrochemical Society and the International Society for Electroporation-Based Technologies and Treatments for supporting us and making our EBTT possible.

I sincerely hope you will enjoy the experience, benefit from being with us and expand your professional network.

Sincerely Yours,

Damijan Miklavčič

INVITED LECTURERS AND NEW FACULTY MEMBERS

The role of PEF in enabling future food production systems

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INTRODUCTION

Nanosecond pulsed electric field (nsPEF) technology is an innovative approach in bioprocessing that enhances cell growth and biomass yield across various organisms, including yeast. By applying ultra-short, high-intensity electric pulses, nsPEF induces intracellular effects that stimulate cellular processes without compromising cell viability. This technique has shown significant potential in increasing cell density, accelerating growth rates, and boosting the production of valuable biomolecules. Its precise, non-invasive nature makes nsPEF a proven solution that will significantly contribute to future food production systems, supporting the stable supply of affordable, nutritious, and sustainable food to meet the growing global demand [1].

MATERIAL & METHODS

The Stellar platform (Buhler AG, Switzerland), utilizing nanosecond pulsed electric field (nsPEF) technology is a first of its kind technology platform. The platform delivers ultra-short, high-intensity electric pulses designed to induce specific intracellular effects in a reliable and controlled manner. Key technical specifications include the application of electric pulses ranging from 5 to 5000 nanoseconds, with electric field strengths between 0.5 kV/cm to 50 kV/cm. Voltage increases between 10% to 90% of the target voltage occur within 0.1 to 100 nanoseconds. The precise control ensures non-invasive application, with the capability to scale from laboratory to industrial levels with the largest scale industrial installation to date at 300 m^3/h.

RESULTS

The application of nsPEF technology significantly enhances the metabolic activity of various biological cells, which is closely linked to the principle of electropermeabilization. In experiments involving Escherichia coli and Pichia pastoris, nsPEF treatment, using pulse lengths of approximately 100 nanoseconds and electric field strengths between 5 to 10 kV/cm, resulted in a marked increase in metabolic activity. This stimulation is primarily due to the increased mass transfer of molecules and ions across the cell membrane, facilitated by application of nsPEF. The rapid voltage changes induce temporary pores in the cell membrane, allowing enhanced uptake of nutrients and other molecules essential for metabolic processes. Consequently, treated cells demonstrated up to a 30% increase in metabolic activity, as measured by fluorescein diacetate (FDA) cleavage assays.

The underlying principle of electropermeabilization also explains the observed increase in biomass yield. The nsPEF treatment optimizes intracellular conditions, promoting faster cell growth and division. This effect is particularly notable in single-cell cultures, where the biomass yield increased by up to 50% compared to untreated controls. The enhanced mass transfer not only boosts metabolic activity but also accelerates the overall growth rate of the cells, leading to higher cell densities and biomass production.

Additionally, the improvement in product titer yields is directly related to the optimized metabolic activity and growth conditions induced by nsPEF. As cells become more metabolically active and proliferate more rapidly, the production rates of targeted bioproducts, such as lipids and recombinant proteins, also increase, resulting in higher product titres per cell. In treated cultures, lipid content increased by up to 55%, demonstrating the direct impact of nsPEF on bioprocess efficiency. This enhancement is attributed to the increased intracellular energy availability and the improved synthesis pathways facilitated by the nsPEF-induced permeabilization.

The Stellar platform's flexibility further amplifies these benefits by allowing the application of nsPEF technology across a wide range of organisms and strains, from laboratory to industrial scales. This system's adaptability makes it effective in both batch and continuous recirculation processes, processing diverse biological cells, including bacterial, yeast, fungal, microalgae, plant and mammalian cells. The ability to scale nsPEF technology from small to large operations enhances its integration into various bioprocessing systems, ultimately improving the overall efficiency and scalability of these operations. The system's capability to adapt to different scales and biological contexts underscores its potential to revolutionize future food production systems, making it a versatile and powerful tool for optimizing metabolic activity, growth, and product yields in a variety of applications.

CONCLUSION

The nsPEF technology excels in flexibility and adaptability, making it applicable across diverse organisms and bioprocess scales. Effective in both laboratory and industrial settings, this technology enhances metabolic activity, biomass yield, and product titre across various biological cells. Its seamless integration into bioprocessing systems positions nsPEF as a revolutionary tool in the bio-based industry, driving advancements in sustainable and efficient production methods.

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Collateral effects of electroporation: heating, electrical stimulation, and electrochemical reactions

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INTRODUCTION

Applying electrical energy to living tissues for inducing electroporation can cause diverse phenomena in addition to electroporation. Three well-identified collateral phenomena, which in most cases are undesired, are heating, electrical stimulation, and electrochemical reactions.

HEATING

Except for superconductors, all electrical conductors dissipate electrical energy in the form of heat when current flows through them. This phenomenon is known as Joule heating or resistive or Ohmic heating and obeys:

$$P = I^2 R$$

where P is the power of heating, I is the current flowing through the conductor and R is its resistance. This equation, known as Joule's law, can also be expressed for infinitesimal volumes at each point of space:

$$p = \frac{\left|\vec{J}\right|^2}{\sigma} = \left|\vec{E}\right|^2 \sigma$$

where σ is the electrical conductivity and $\vec{J}$ and $\vec{E}$ are the current density and electric field respectively. In electroporation scenarios, with field magnitudes in the order of hundreds of V/cm and conductivities in the order of S/m, Joule heating would cause harmful temperatures if very short exposures, much shorter than a second, were not used. The dependence of heating on the square of the electric field is relevant because it implies that heating is particularly high where the electric field is larger, typically at the periphery of the electrodes due to the edge effect. This is further exacerbated by the increase of σ with the temperature.

The temperature increase due to the Joule effect can be accurately predicted with numerical models, even for complex tissues. However, thermal damage models used to predict the physiological impact of the intense temperature peaks caused during electroporation are intended for longer exposures and, although are used by the electroporation community, their accuracy is still unknown.

ELECTRICAL STIMULATION

The nervous system transmits signals in the form of action potentials. Action potentials are sudden transitions in transmembrane resting voltage that propagate along the cell. Electrical stimulation consists in nonphysiologically triggering action potentials by delivering electric fields.

In clinical applications of electroporation, electrical stimulation is not desired because, by exciting efferent nerves, it causes muscle contractions and, by exiting afferent nerves, it causes pain [1].

Both electroporation and electrical stimulation occur when the transmembrane voltage is artificially increased above a threshold due to the presence of the electric field. This implies that unsought stimulation occurs very frequently when electroporation is intended. Advantageously, high-frequency biphasic fields can cause electroporation whilst minimizing electrical stimulation [2].

ELECTROCHEMICAL REACTIONS

Electrical conduction in metallic electric circuits is provided by the flow of electrons whereas in living tissues (and in suspensions) the moving charge carriers are ions. Conduction across the interface between the electronic conductor and the ionic conductor, that is, across the electrode, can be capacitive or electrochemical. Capacitive conduction is only substantial for very brief or ac currents of small magnitude. In most electroporation scenarios, conduction across the electrode is mediated by electrochemical reactions. In electrochemical reactions, the chemical species in the living tissue and the electrode exchange electrons thus altering their chemistry.

Electrochemical reactions are generally deleterious for electroporation applications because they modify pH and cause the release of gases and metallic ions. (However, it is worth noting that it has been proposed their synergy with electroporation to achieve large ablation volumes [3]).

Electrochemical reactions are a surface phenomenon (occur at the electrode) and, because of that, are of particular concern when the electrode area is large compared to the treated volume, as is typically the case in microfluidic setups.

Electrochemical reactions are difficult to model, particularly at the anode (positive electrode) because multiple reactions can occur concurrently.

Interestingly, high-frequency biphasic currents minimize electrochemical reactions. This effect, combined with lower stimulation, explains the emergence of high-frequency biphasic fields for electroporation-based ablation.

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Challenges and limitations in high power pulse forming for electroporation

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INTRODUCTION

Electroporation is a polarization-based phenomenon requiring precise generation of electric pulses and thus dependent on the electronics and the pulsed power generators. A pulsed power generator is a device capable of delivering short (e.g., microsecond range, but not limited to) and high-power pulses to the load, which can range from planar microfluidic electrode systems to high volume and constant flow treatment chambers. In the biomedical context typically standard electroporation cuvettes are used for in vitro research, while applied and translational research is performed using an array of electrodes (numerous various structures depending on the application) in direct contact with tissue [1]. The technological and engineering challenges in the development of the pulse forming circuits and electroporators vary depending on the application, the load and the parametric range.

THE CHALLENGES & LIMITATIONS

An electroporator can be simplified to a three-element scheme: 1) power supply; 2) pulse forming circuit and 3) the load [2]. However, the devil is in the details. If one works in the lab employing reversible electroporation and microsecond range (50+ µs) pulses with standard 1 mm cuvettes, a 300–500 V / 50 A generator would cover most of the research needs and will take less than 6 hours to be assembled from scratch by an experienced electronics engineer. However, the higher the voltage is required and the shorter the pulses become (e.g., sub-microsecond) the more the complexity of electronics increases and eventually sky-rockets to systems requiring a team of engineers, years of development accompanied by lots of coffee and despair. Even a basic resistor, which could be a wire-wound one in a price range of a single bubble gum is no longer applicable in the nano-pulse forming circuit [3]. The requirement to use state-of-art ceramic low-inductance high voltage resistors arises, which of course cost a fortune and even have problems in the supply chain. Some electronics components become so crucial and so special, that in specific cases half-a-year factory lead time and governmental authorization is required to acquire them. To simplify: a capacitor is no longer a simple capacitor and even wire is no longer a wire, but a complex signal transmission system with matched impedance [4]. Otherwise, the pulse will be altered with oscillations and reflections, the duration and form will not be the ones expected and, as usual, in a worst-case scenario irreversible damage of the components will be inflicted accompanied by smoke, sparks and potential danger towards the operator.

Finally, the lab-scale devices and prototypes are just one family of devices [5], which features lots of flexibility and compromises, while medical grade devices require additional safety solutions and certification [6], which influences their complexity.

THE LECTURE

In this lecture the review of basic concepts of pulse generation will be reviewed and problems of pulse forming will be highlighted. The importance of impedance, the influence of transients' processes on the pulse and their cause, risks of voltage breakdown and basic characteristics of semiconductors will be reviewed. It is expected that the attendees of the lecture will definitely plan to buy an oscilloscope and learn the basics of pulse metrology after the lecture (if they haven't done so already). Finally, some understanding of why the state-of-art electroporation systems cost a fortune will be acquired.

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Advances in irreversible electroporation technology and novel clinical applications

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INTRODUCTION

Irreversible Electroporation (IRE) is a minimally invasive tissue ablation technique that involves applying low-energy pulses to create defects in the lipid bilayer, inducing cell death. [1]. IRE is unique among ablation techniques in affecting only the cell membrane while tissue molecules such as collagen structures and proteins; remain intact, thereby making treatment near critical structures possible. We developed an advanced form of the technology, high frequency irreversible electroporation (H-FIRE) [2], that is being used for the treatment of cardiac arrhythmias, cancer and other malignancies. This new therapy mitigates the need for a neuromuscular paralytic during treatment.

Different waveforms and fields elicit different cell death mechanisms, including necrosis, apoptosis, pyroptosis, and necroptosis [3]. Pulsed electric fields have been shown to increase antigen activity with the release of damage associated molecular pathways (DAMPs) and proteins [4]. By modifying the pulse parameters and field strength, specific modes of cell death can be triggered [5]. We will discuss such mechanisms and modeling techniques to avoid thermal damage. We will also discuss recent advances in pulsed electric field therapies to target other aspects of the tumor microenvironment and potential clinical applications.

METHODS

A human glioblastoma cell line, U251, was cultured in a 2D monolayer cell culture platform and covered in low conductivity buffer for electroporation treatments. Ablation areas (n=5) were measured using fluorescent imaging to determine electric field thresholds for each waveform. To normalize the level of cell death, energy matching was used across all chosen waveforms. Cell samples were collected at 6h for a colorimetric Caspase 3/7 assay to quantify apoptotic activity. A fluorometric Caspase 1 assay was used on samples collected 30 min after treatment to quantify pyroptotic activity.

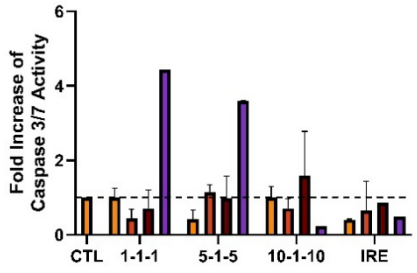

Figure 1: Apoptotic caspase 3/7 cleavage. (n=2)

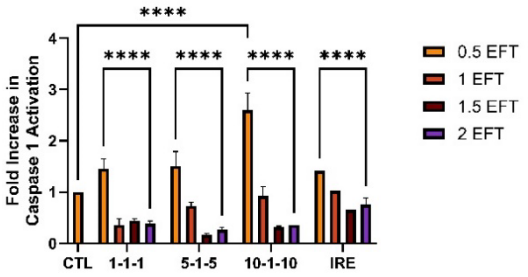

Figure 2: Pyroptotic Caspase 1 cleavage. (n=3)

RESULTS

We found that Caspase 3/7 activation (Figure 1) was significantly higher for shorter pulse widths (1 and 5μs) and stronger electric fields. Minimal caspase 3/7 activation was observed with higher pulse widths (10μs and IRE). Caspase 1 activation (Figure 2) displayed opposite trends from that of Caspase 3/7 as it was significantly higher at sublethal electric fields. Our results indicate that higher levels of apoptotic cell death occur at shorter pulse widths and increasing in electric field magnitude, and higher levels of pyroptotic cell death occur at longer pulse widths and lower field strengths.

CONCLUSION

We determined that we can activate specific cell death mechanisms by altering the applied pulse parameters. With this information, we can develop computational models for clinical treatment planning to mitigate thermal damage and optimize patient recovery times.

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Nucleic acid sensing

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INTRODUCTION

Germline-encoded pattern recognition receptors (PRRs) are ubiquitously expressed in both immune and nonimmune mammalian cells. PRRs detect two classes of overlapping ligand categories. Initially described ligands, pathogen associated molecular patterns (PAMPs), include microbial components such as lipopolysaccharides, lipoproteins, flagellin and nucleic acids [1]. Subsequently, the concept of damage associated molecular patterns (DAMPs) including proteins, glycans, ions, metabolites and nucleic acids was described [2]. PRR binding and activation lead to inflammatory signalling and cell death.

Intracellular delivery of exogenous DNA and RNA for therapeutic purposes by any method, including electroporation, electropermeabilization, or electrotransfer, should induce PRR activation.

METHODS

Established assays such as cell death quantification, real time RT-PCR and RNA sequencing, protein detection and functional assays, microscopy as well as unique molecular methods were employed in vitro and in animal samples.

RESULTS

Any intracellular delivery of exogenous DNA and RNA for therapeutic purposes by any method, including electroporation, electropermeabilization, or electrotransfer will induce PRR activation. The downstream effects of nucleic acid delivery are observed in tumor and nontumor cells, tumors, skin and muscle. Several PRRs bind intracellular plasmids [3]. Although not specifically assessed, it is probable that these effects modulate nucleic acid-based therapies in companion animals and humans.

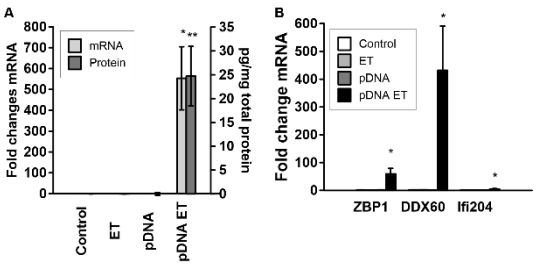

Figure 1: Effects of DNA electrotransfer in C2C12 myoblasts. A. Interferonβ (IFNβ) secretion. B. Regulation of select PRR mRNAs.

Cells respond to DNA electrotransfer with IFNβ secretion (Figure 1a), a common marker of PRR activation. PRR mRNAs are also regulated (Figure 1b).

From 15 minutes to 4 hours after electrotransfer, several PRRs bind plasmid DNA (Figure 2). These observations implicate PRR activation.

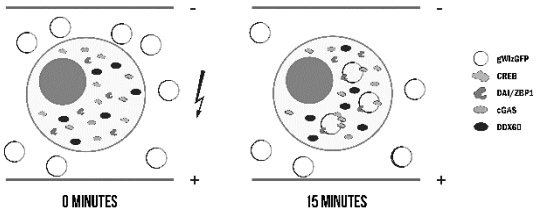

Figure 2: PRRs bind plasmid DNA after electrotransfer.

CONCLUSIONS

Ubiquitous, evolutionary cell and tissue responses must be incorporated into therapeutic design. While PRR effects are associated with exogenous nucleic acid delivery, intense pulse application alone may impact intracellular structures containing nucleic acids such as nuclei, mitochondria and membraneless organelles to produce similar effects.

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Electroporation-based gene editing enables advanced adoptive cancer immuno-cellular therapeutics and next-generation smart-cells

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INTRODUCTION

Leveraging electroporation-mediated gene editing, cell therapy is becoming part of the pharmaceutical arsenal through two major evolutions: (1) it is transitioning from the realm of grafts, essentially defined by a process serving a patient bespoke therapy, to that of robust and reproducible "off-the-shelf" pharmaceutical products, and (2) chosen favorable scenarios are programmed into cells ("smart-cells") that they can execute once infused into patients.

Triple negative breast cancer (TNBC) has limited therapeutic options and the worse prognosis compared to the other subtypes of breast cancer[1]. CAR-T cell therapy could be an invaluable option for TNBC patients. However, the immunosuppressive tumor micro-environment (TME) of solid cancers, challenge CAR T-cells to efficiently mount an anti-tumor response[2]. Some of the key mechanisms of immune evasion are mediated by PDL1/PD1 and TGFB1/TGFBR2 interactions resulting in T-cell exhaustion or impaired proliferation.

Here we describe a pre-clinical investigation of UCARTMUC1[3], a mucin-1 targeting "smart" T-cell leveraging electroporation-mediated TALEN®-based gene editing for robust and safe multiplex reprograming of an allogeneic CAR T-cells to circumvent TME barriers.

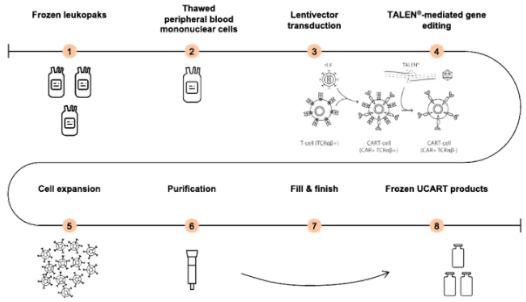

Figure 1: UCARTMUC1 manufacturing process leveraging electroporation-mediated TALEN®-based gene editing.

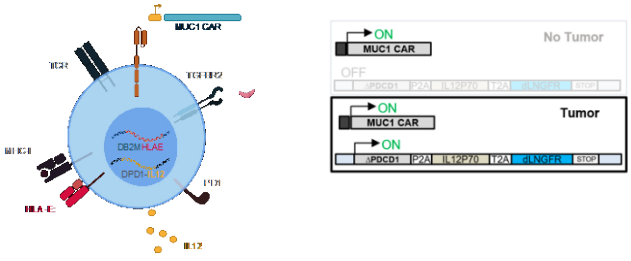

Figure 2: UCARTMUC1 combinable attributes: MUC1-CAR, TRAC^{KO}, ΔPD1-IL12, ΔB2M-HLAE-E and TGFBR2^{KO}, and activation-induced PD1-targeted IL12 cassette.

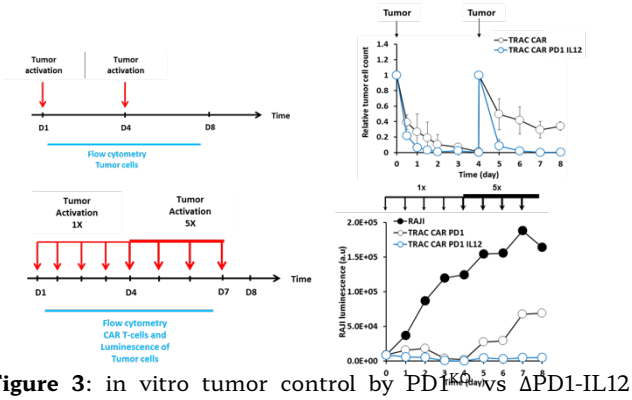

Figure 3: in vitro tumor control by PD1^{wt} vs ΔPD1-IL12 versions of UCARTMUC1.

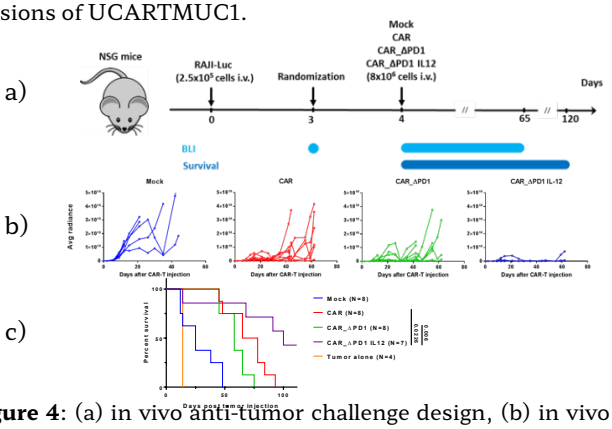

Figure 4: (a) in vivo anti-tumor challenge design, (b) in vivo tumor control by PD1^{wt} vs PD1^{KO} vs ΔPD1-IL12 versions of UCARTMUC1, (c) correlative mouse survival rates.

DISCUSSION

Electroporation-mediated TALEN®-based gene edited UCARTMUC1 "smart" cells were manufactured with various versions of attributes. Our results show superior in vitro and in vivo tumor control with disruptive Il-12 knock in at the PD1gene. UCARTMUC1 illustrates the "smart" cell versioning approach to overcome tumor natural defense mechanisms aiming to change the immunological scenario taking place in in patients.

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Scaling non-viral gene delivery for cell-based therapies

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INTRODUCTION

Immunotherapy is currently at the cutting edge of basic scientific research and pharmaceutically driven clinical application. This trend is in part due to recent strides in targeted gene modification and expanded use of CRISPR/Cas complex editing for therapeutic development.[1] Electro-mechanical transfection involving use of electric fields coupled with high fluid flow rates is a scalable strategy for cell-based therapy development and manufacturing. Unlike purely electric field-based or mechanical-based delivery methods, the combined effects result in delivery of genetic material at high efficiencies and low toxicity. This study focuses on delivery of reporter mRNA to show electro-mechanical transfection successfully used in human T cells.

MATERIALS AND METHODS

Transfections were performed with commercially sourced mRNA encoding GFP (TriLink Biotechnologies). T cells were counted, pelleted (500 x g, 5 min), and resuspended in proprietary transfection buffer at densities of 10-50e6/mL for the 100 µL samples. In a 50-fold scale up demonstration (5 mL) we transfected 50M T cells at a density of 10e6/mL with 1 mg of GFP mRNA. Payload was added at a fixed maximum of 10% volume and the cell:payload solution was mixed via pipetting. Final density in recovery solution for both platforms was 1e6/mL, containing 10% transfection buffer and 90% cell culture media. Cells were cultured at 37°C with 5% CO_2 in a standard cell incubator.

RESULTS AND DISCUSSION

Non-viral transfection is an attractive method of engineering cells. This work presents a new transfection technology utilizing electrical energy with continuous flow that demonstrates several advantages over other non-viral transfection methodologies. It has generally been accepted that electroporation requires additional optimization in the process of scaling up from research to manufacturing volumes, due to changing geometries of both electrodes and cuvettes [2]. To address this issue, the field of electroporation-based transfection has seen the advent of workarounds including microfluidics, batch-based automation, and nanostructures [3]. However, these solutions have been unable to meet the need for high-throughput development and large volume manufacturing requirements in the evolving cell and gene therapy industry. Electro-mechanical transfection scales with time, therefore processing larger volumes only requires operating for a proportional length of time. To this end electro-mechanical transfection can process up to 100 mL of fluid (10-100B cells, depending on cell concentration) in roughly 3 minutes, from input to output bag, via a peristaltic pump (Masterflex® L/S). The transfection parameters identified during optimization on the small-scale system directly apply to the larger volume system because these systems utilize the exact same electro-mechanical transfection apparatus (Figure 1A). The results show no significant loss in cell viability 24-hours after electro-mechanical transfection, with viabilities of 73.5% and 71.0% in small and large volume platforms respectively (Figure 1B). The observed efficiency was also similar 24-hours after electro-mechanical transfection, 94.3% and 92.2% in small and large platforms respectively (Figure 1C). Thus, electro-mechanical transfection can easily scale up for clinically relevant processing volumes. This study demonstrates that non-viral electro-mechanical transfection is an efficient and scalable method for cell and gene therapy engineering and development.

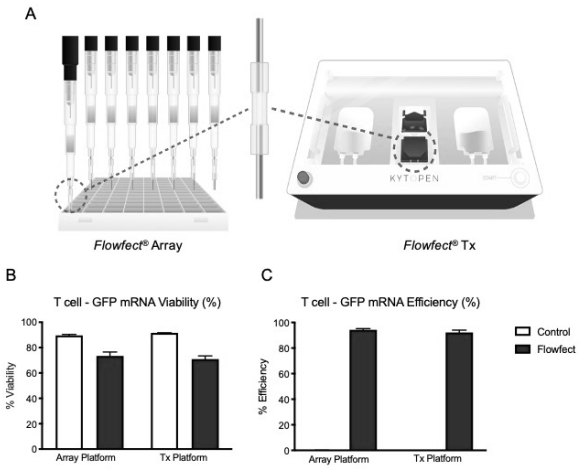

Figure 1: Non-viral gene delivery technology directly translates from small-scale research transfections to large-scale cell manufacturing procedures. A) Schematic showing how the electro-mechanical flowcell allows for direct translation from one platform to the other. Expanded human T cells were assessed for B) cell viability (7AAD negative) and C) GFP mRNA transfection efficiency at 24 hours. Bar graphs are Mean±SD Array n=6 Tx n=2.

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Effects of electroporation on the vasculature

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INTRODUCTION

Electroporation/electropermeabilization (EP), i.e. the application of electric pulses to cells or tissue, leads under suitable conditions to a reversible permeabilization of the cell membranes and thus facilitates the entry of exogenous molecules into the cells. Reversible EP of tissue is feasible, efficient and tolerable in humans. The most advanced routine clinical application is electrochemotherapy (ECT), in which cytotoxic drugs are delivered to cells to treat tumors. More recently, this approach has also been used in the treatment of vascular malformations, where it has been termed bleomycin electrosclerotherapy (BEST). If excess current is applied, this leads to irreversible electroporation (IRE). The use of IRE to treat various types of cancer is intensively researched and several clinical trials are ongoing. In addition, IRE has recently been successfully adapted for the treatment of cardiac arrhythmias, where it is referred to as pulsed field ablation (PFA). Since the early experiments with EP and ECT in mouse tumors, it became clear that the EP-based treatments have an effect on the normal and tumor vasculature in addition to the direct cytotoxic effect of the therapy on the tumor cells. This is now directly exploited in BEST and is an important factor for the success of PFA in the treatment of cardiac arrhythmias.

ENDOTHELIAL CELLS RESPONSE TO ELECTROPORATION

Endothelial cells, the inner lining of blood vessels, show varying sensitivity to EP depending on the electric field intensity, pulse duration and tissue type. In vitro studies on human endothelial cells have shown that reversible EP induces cytoskeletal changes, most notably disruption of actin and tubulin filaments and the disruption of cell-to-cell junctions, which are responsible for controlling vascular permeability [1]. Moreover, in vitro studies demonstrated that EP increases the permeability of endothelial monolayers in a dose-dependent manner.

DIFFERENTIAL EFFECTS OF EP AND ECT ON NORMAL AND TUMOR BLOOD VESSELS

The application of EP pulses to normal blood vessels increases the permeability of the affected blood vessels, causes transient vascular lock, i.e. a decrease in perfusion, and modulates the diameter of the affected blood vessels. Similarly, the application of EP or ECT to tumors increases the permeability of the affected blood vessels and causes vascular lock [2]. In normal blood vessels, these effects are short-lived, whereas in tumor vessels they are long-lasting and disappear more than 72 hours after ECT.

In tumors, EP leads to an immediate abrogation of blood flow, i.e. a vascular lock that lasts for more than 60 min. Interestingly, the tumor-supplying arterioles react to EP in the same way as normal vessels, namely with rapid vasoconstriction and increased permeability [3]. This suggests that this is the main cause of the immediate vascular lock observed after EP. EP also leads to increased permeability of tumor blood vessels to macromolecules and a partial, long-lasting decrease in perfusion. Thus, EP has a differential effect on normal and tumor blood vessels. Moreover, ECT has a direct cytotoxic effect on tumor endothelial cells, i.e. it has a vascular disrupting effect, which is now also exploited in BEST.

EFFECTS OF IRE ON BLOOD VESSELS

The effects of IRE on blood vessels were first confirmed with magnetic resonance imaging (MRI) of perfusion in the rat brain after EP, showing that IRE increases the permeability of blood vessels to macromolecules [4]. Importantly, further studies conducted on various normal blood vessels showed that while IRE can destroy the endothelial cells, the large blood vessels retain their functionality and are repopulated with endothelial cells after therapy.

CONCLUSIONS

In summary, EP-based treatments have effects on the endothelial cells and thus on the normal and tumor vasculature. This can be exploited to destroy endothelial cells, as is the case with ECT and BEST, or one can rely on their function-preserving property when it comes to normal blood vessels, as is the case with IRE and PFA.

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Real-time imaging and conductance tracking of individual electropores in live cells

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INTRODUCTION

With over 50 years of electroporation research, mechanisms responsible for long-lasting membrane permeabilization remain elusive. The formation and persistence of hydrophilic pores in cell membrane has never been clearly shown. In artificial lipid bilayers and molecular models, the lifetime of membrane pores after an electric shock is limited to nano- or microseconds. In contrast, permeabilized state of electroporated cells can last minutes. The lifespan of individual electropores in cells could not be measured directly. The controversy between experimental cell data and modeling raised questions if electropores are indeed responsible for the prolonged membrane permeabilization. An alternative explanation to permeabilization was a diffuse impairment of the membrane barrier function, possibly due to lipid oxidation.

APPROACH

Discerning individual membrane lesions in electroporated cells has been a challenge due to their size, which falls below the resolution limit of optical microscopy. Nonetheless, we have succeeded in tracking individual electropores by imaging Ca^{2+} entry with total internal reflection fluorescence (TIRF) microscopy. TIRF imaging is restricted to an extremely shallow (~100-nm) subplasmalemmal layer, which facilitates the observation of Ca^{2+} fluxes through individual pores instead of seeing a diffuse fluorescence cloud.

A whole-cell voltage clamp configuration was established in human embryonic kidney cells placed on glass coverslips with an indium tin oxide (ITO) layer as electrical ground. Voltage steps, 1 to 25 ms, applied between the pipette and the ITO, induced lesions in the membrane portion adjacent to the ITO. Loading cells with the Ca^{2+} sensitive dye CAL-520 enabled dynamic visualization of Ca^{2+} fluxes through these lesions. Pore conductance was tested by small, non-permeabilizing voltage steps applied across the membrane by the patch clamp amplifier.

RESULTS

Applying 1-ms voltage steps to -400 mV caused a rapid appearance of multiple bright spots in TIRF images (Fig. 1), reflecting multiple points of Ca^{2+} entry through the electroporated membrane. Most of these fluorescence transients disappeared within 10-50 ms but some were present for over a minute. These experiments provided the first evidence of electropore longevity in the absence of an applied membrane potential.

Hyperpolarizations to about -100 mV for 25 ms induced a diffuse zonal electropermeabilization, manifested by momentary and subtle Ca^{2+} fluorescence upticks ("scintillae") over large membrane areas. Charging the membrane to -200 mV and beyond produced one or several focal fluorescence transients. Their brightness decreased sideways from the center, consistent with Ca^{2+} entry through a pore followed by a radial diffusion. These transients disappeared within 10-20 ms after the voltage step and marked the formation of short-lived electropores with 50- to 200-pS conductance. Larger voltage steps increased the brightness, the size, and the number of transients. At -240 mV we observed high-conductance pores (> 1 nS) that persisted for seconds.

Similar effects were observed when cells were electroporated by applying the electric field pulses externally, without patch clamp. However, the scintillae were replaced by a diffuse macro-flash of fluorescence lasted about 10 ms or less. The macro-flash was always present after 300-ns pulses (4 to 16 kV/cm) but only occasionally after 1-ms pulses (0.2-0.4 kV/cm). Pores opened by external pulses could also persist for over a minute.

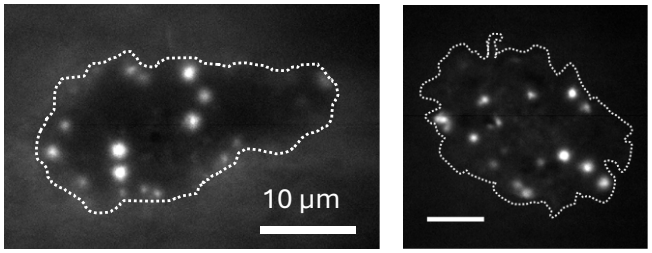

Figure 1: Ca^{2+} fluorescence transients (bright spots) imaged within 6 ms after a 1-ms voltage step to -400 mV, in two different cells. White dotted contours emphasize the cell's footprint. Images taken before the electroporation had no bright spots (not shown).

SUMMARY

We introduced a novel experimental technique for tracking individual electropores by imaging Ca^{2+} entry with TIRF microscopy. The experiments unequivocally demonstrated that focal electroporation lesions (pores) in live cells persist (conduct electric current and Ca^{2+} ions) for tens of seconds after an electroporation shock [1,2].

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Electrophysiological effects of electroporation on myocardium

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INTRODUCTION

Pulsed Field Ablation (PFA) is a promising cardiac ablation modality that offers a potentially safer and shorter procedure times compared to traditional thermal ablation methods [1]. In PFA, electric pulses are commonly delivered using an endoluminal catheter which induces irreversible electroporation (IRE) to the myocardium surrounding the electrode location. While the tissue under IRE results in a durable ablative lesion, reversible electroporation (RE) beyond the that area can induce a transient electrophysiologic signal disruption [2].

Intracardiac electrograms (iEGMs) have traditionally been used to assess the formation of cardiac lesions. However, PFA's stunning effect can lead to overestimation of the treated area, potentially compromising treatment efficacy.

To improve the success rates of PFA treatments, a deeper understanding of the electrophysiological effects of electroporation is essential. This knowledge will support the development of novel intra-procedural measurements to predict lesion durability.

This lecture will provide an overview of the primary electrophysiological effects of electroporation on the myocardium and discuss current approaches for evaluating the irreversibility of PFA-induced lesions.

ELECTROPHYISIOLOGIC EFFECTS

The sudden membrane conductivity increase induced by electroporation triggers initially the depolarization of cardiomyocytes as excitable cells. However, during the resealing process, the interactions between the induced ion leaks, membrane channels, and the altered transmembrane voltage prevent the normal depolarization of the cell.

Although, in-vitro studies [3] and numerical models [4] have been employed to elucidate this complex mechanisms, further studies are still needed to fully understand the electrophysiological response to electroporation.

Despite the current lack of knowledge, several methods have been already proposed to be used as feedback methods to guide the physicians during the procedures.

INTRACARDIAC ELECTROGRAMS

Conventional bipolar iEGMs are obtained by measuring the potential difference between two electrodes placed around the region of interest. While this approach offers high spatial resolution of the cardiac surface, it lacks information about deeper myocardial layers. Unipolar iEGMs, recorded between a single electrode at the area of interest and a dispersive electrode, have been shown to contain information correlated to lesion extension and durability.

TISSUE ELECTRIC PROPERTIES

Impedance measurements have been widely adopted by the electrophysiology community as an indicator of lesion formation in thermal ablations. This passive electrical properties of the tissue, already proposed as a potential tool for real-time monitoring in electroporation procedures [5], could be able of discriminating between reversible and irreversible areas in the myocardium.

TISSUE OPTICAL PROPERTIES

Optical Coherence Reflectometry (OCR) is a non-invasive imaging technique with microscopic resolution. When combined with a Polarization-Sensitive system (PS-OCR), tissue birefringence can be measured. This optical property correlates with tissue microstructural anisotropy which is high in healthy myocardium. When exposed to irreversible damage, the affected tissue loses its ultrastructure, resulting in a reduction in birefringence [6]. Since myocytes with reversible effects preserve their ultrastructural conformation, PS-OCR can be used as a feedback mechanism.

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Pulsed field ablation treatment of cardiac arrythmias

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NOTES

SHORT PRESENTATIONS BY STUDENTS AND PARTICIPANTS

Acoustic pressure analysis due to high-frequency electric field on the biological cell during electroporation

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INTRODUCTION

The biological cells when exposed to an electric field are stretched parallel to the direction of the applied electric field. This stress produces acoustic pressure waves in the extracellular fluid which could be measured by the pressure transducers. The multiphysics investigation on this deformation [1,2] and acoustic pressure produced during reversible electroporation is proposed using different cell contours. The transmembrane potential, total elastic strain energy, Maxwell stress tensor (MST) and Helmholtz wave equation in the cell are compared using multiphysics for action under unipolar and bipolar nano pulse electric fields. The polarization of cervical cells is expressed using Debye expression [3] which occurs as the cells are dispersive at high frequencies. Our model explains the temporal evaluation of electroporation and the acoustic wave produced in the fluid due to the bi-lipid membrane under constant stress which produces electrodeformation. This method can find application in acoustic imaging where the back-projection method is applied.

METHODOLOGY

Finite element analysis is conducted on a 100µm × 100µm computational domain. Copper electrodes are placed above and below the square domain, and an electric field of 10 kV/cm is applied for 100 nanoseconds. Perfectly matched layers (PML) are used to minimize boundary effects. Cells of varying shapes—circular, oblate, and prolate—are positioned at the center of the domain. The Laplace equation, and Debye second-order model are solved throughout the entire domain. Smoluchowski statistical model is solved at the membrane to find out pore density. The cell membrane is modelled as viscoelastic, using. Generalized Maxwell model in COMSOL for simulation. The Navier-Stokes and Helmholtz equations are solved to generate acoustic waves, which are captured by 100 pressure sensors placed around the biological cell model within the domain. The Delay and Sum (DAS) algorithm is implemented in MATLAB and using the pressure signals image reconstruction is done. Figure 2 shows the final outcome.

RESULTS AND FIGURES

Figure 1 shows the pressure wave captured for different shapes and their temporal plot. The sensors show a characteristic N shaped acoustic pressure wave [4]. Figure 2 shows the reconstructed image of the oblate (1:2) using the pressure waves captured by 100 sensors placed in the computational domain. Delay and sum (DAS) back projection algorithm has been used for image reconstruction.

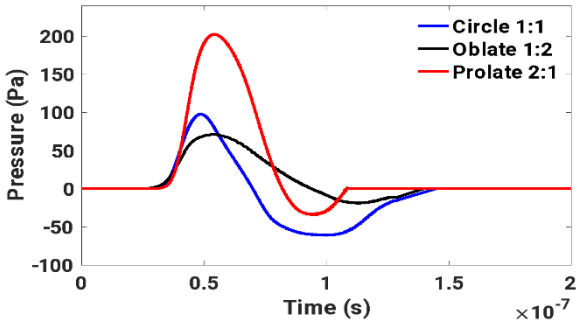

Figure 1: Acoustic pressure wave temporal plot generated during electroporation captured by point pressure sensor in computational domain.

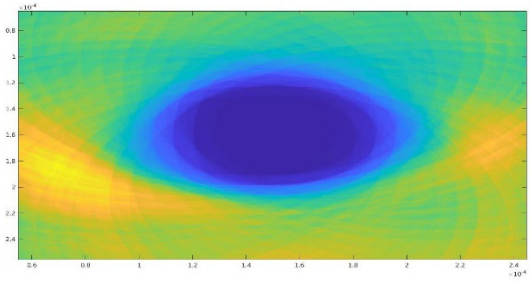

Figure 2: Reconstructed image of oblate (1:2) after applying delay and sum (DAS) algorithm.

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Effect of electroporation in combination with inorganic particles used in tattoo ink

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INTRODUCTION

Electroporation is a technique that exploits electrical pulses to increase cell membrane permeability. In order to kill tumor cells, electroporation is combined with low systemic or local concentrations of anticancer agents, which can thus massively penetrate into permeabilized tumor cells. This modality is also known as electrochemotherapy (ECT). ECT can be used to treat several types of cancers, including skin cancers, such as melanoma [1]. In parallel, an increasing number of individuals are opting to wear one or more tattoos for aesthetic or reparative purposes. Tattoo inks contain micro- and nano- particles (NPs), composed of titanium dioxide and iron oxides, which are used as pigments. In case of ECT treatment, the presence of these particles in the skin could alter or amplify the effects of electroporation [2], if a tattoo is present in the zone that is being electroporated.

METHODS

Characterization of the particles contained in a sample of tattoo ink was carried out by transmission electron microscopy, to determine NPs morphology and nanoparticle tracking analysis-NTA (Nanosight), to determine NP number. The iron content was quantified by inductively coupled plasma spectrometry.

The effect of co-exposure of tattoo ink and electroporation (pulse duration 100 µs, N=8, pulse repetition rate = 1 Hz, intensity 0 to 600 V/cm) on cell permeability was studied using normal dermal fibroblasts cultured in 2D isolated from a healthy skin biopsy. After uptake of 50 µM propidium iodide (PI), cell permeability was monitored by fluorescence microscopy.

RESULTS

Tattoo ink contains electron dense nanoparticles exhibiting different morphologies, including elongated structures (**Figure 1**), which could locally amplify the electric field. The average size of these nanoparticles determined by NTA is 256 nm and the average number of particles is 2,81 x 10^{14} per 1 mL of tattoo ink suspension.

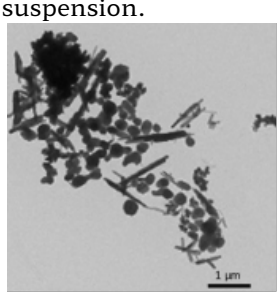

Figure 1: Characterization of nanoparticles in a diluted tattoo ink suspension.

After exposure to tattoo ink or its supernatant at different dilutions and electroporation at different intensities on normal dermal fibroblast cells cultured in 2D, there does not appear to be a significant difference in results between ink and supernatant (**Figure 2**). Except for the supernatant, diluted to 0,01%, which reduced viability in the absence or presence of pulses. At 0 and 200 V/cm, no difference was observed between cells exposed to pulse buffer without and with tattoo ink or supernatant. With pulses at 400 and 600 V/cm, a higher percentage of permeabilized cells was observed when cells were exposed to tattoo ink or supernatant compared with control cells.

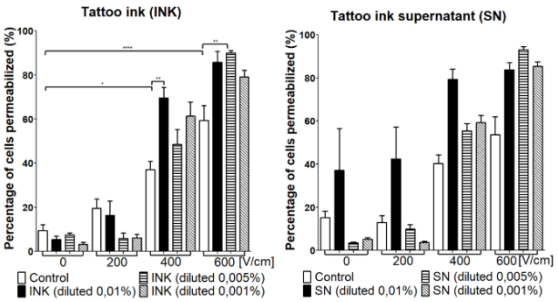

Figure 2: Histogram showing the percentage of permeabilized cells under different conditions.

CONCLUSION

The shape of the particles contained in tattoo ink is highly heterogeneous, and includes elongated structures, which could, *via* the lighting rod effect, locally amplify the applied electric field.

The pulsation of cells at 400 or 600 V/cm in combination with tattoo ink at different dilutions results in greater permeabilization with respect to the control. This effect might be either due to the particulate matter or due to the dispersion agents/degradation products, present in the liquid, which is used to disperse tattoo pigments.

At the highest concentration, the supernatant of tattoo ink increases the fraction of PI-positive cells in the absence or presence of electric pulses. At lower supernatant concentrations, the membrane permeability is not altered at 0 and 200 V/cm and the viability of cells is not affected, but pulses of 400 and 600 V/cm result in higher fraction of PI-positive cells in comparison to controls. Taken together, these results suggest that tattoo ink supernatant might be toxic, probably because it contains metallic ions or surfactants, that might, in combination with intense electric pulses, result in reduced cell viability.

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Investigating the stimulation of fungal growth and metabolism by pulsed electric field treatment

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INTRODUCTION

Microscopic and macroscopic fungi play an important role in food and biotechnology. Yeasts and moulds are employed to produce bulk chemicals, e.g. ethanol and citric acid from sustainable substrates, but also as recombinant expression hosts for biotechnological products. Yeast biomass itself is used as a source of vitamins, trace elements and proteins for applications in food and feed. The fruiting bodies of basidomycota (typically referred to as mushrooms) have been deliberately grown for human consumption for centuries. Recently, their mycelium has also experienced interest as a novel source of food constituents such as proteins, pigments, and nutritional fibre.

Pulsed electric fields (PEF) have been reported as means of both inactivation and growth stimulation and enhanced uptake of nutrients in yeast and filamentous fungi. The parameters for inactivation (irreversible PEF-treatment) and stimulation (reversible PEF-treatment) depend on the physical characteristics and morphology of the treated organisms (or tissues) and the suspending matrix, thus treatment conditions are rarely applicable for different organisms and must be investigated on a case-by-case basis.

OBJECTIVES

The aim of this presentation is to review the available research on the stimulation of fungi by PEF-treatment, contrast them with own preliminary experimental results, and demonstrate the gaps of knowledge for further own research. The potential of PEF-assisted growth will be emphasized for both macroscopic and microscopic fungi.

MATERIALS AND METHODS

Results from different studies succeeding in the stimulation of fungal growth via PEF treatment will be compiled. The species, extent of stimulation, parameters of PEF treatment, and experimental setups will be displayed and discussed. Own research will be conducted in liquid cultures in shake flasks, fermentation times 3-21 d, parameters will be 0.1-10 kV/cm, 0.5-50 kJ/kg, 5 µs pulses.

RESULTS AND DISCUSSION

The mechanisms of fungal stimulation by PEFs remain unclear, the most prominent hypotheses include the physicochemical electropermeabilisation of the cell membrane and stress induced transcriptional changes to the cell membrane permeability [2,4].

Since, the effects of sublethal PEFs on mycelial growth in liquid culture have yet to be investigated, own experimental work will focus on this topic.

Table 1 shows selected results for the electrostimulation of yeasts, moulds, and fungi in comparison to an untreated control.

Table 1: Selected results for successful stimulation of fungal growth using PEF.

Organism	PEF parameters	Results
Saccharomyces cerevisiae [1]	0.1 and 6 kV/cm	faster carbohydrate consumption, reduced process time
Hanseniospora sp. [2]	72-285 V/cm	faster glucose consumption & growth
Kluyveromyces marxianus [3]	2.4 kV/cm, single pulse	1.4-fold increase in ethanol after 20 h
Aspergillus niger [4]	5-15 kV/cm, 200-2000 pulses, 5 µs	2.17-fold increase of spore germination
Pleurotus ostreatus [5]	1.5 and 6.7 kV/cm, 62.3 kJ/kg	34-45.5% increased fruiting body yield
Lentinula edodes [6]	0.6-1.4 kV/cm, 1-50 pulses of 5.4 J and 0.1 µs	1.3-2.0-fold increased fruiting body yield

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Chemical synthesis of niobium penta-oxide nanomaterials for the antimicrobial applications

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INTRODUCTION

Niobium pentoxide (Nb_2O_5) is a transition metal oxide with a wide range of properties that make it a potentially valuable and highly adaptable material in many different sectors [1,2] This study investigates the chemical synthesis of niobium pentoxide (Nb_2O_5) nanoparticles using hydrofluoric acid (HF) as a catalyst to facilitate the dissolution and reprecipitation of niobium precursors (**Figure 1**). By controlling the molar ratio of HF, temperature, and reaction time, highly crystalline Nb_2O_5 nanoparticles were successfully synthesized. The synthesized nanoparticles were characterized using scanning electron microscopy (SEM), (**Figure 2**) and transmission electron microscopy (TEM) (**Figure 3**) to determine their structural and morphological properties. Notably, the use of hydrofluoric acid led to the formation of well-defined orthorhombic crystalline phases with enhanced surface area, which are critical for catalytic and biomedical applications. In addition, this study will explore the application of these Nb_2O_5 nanoparticles in inhibiting biofilm formation of *Escherichia coli* and *Pseudomonas aeruginosa*. Antibiofilm preliminary results showed a potential that Nb_2O_5 effectively reduces biofilm metabolic activity (~65% and 95%, respectively, (**Figure 4**). These results show the possibility to use the Niobium nanoparticles to overcome the biofilms of its microorganisms, so that more experiments will be performed.

METHODS

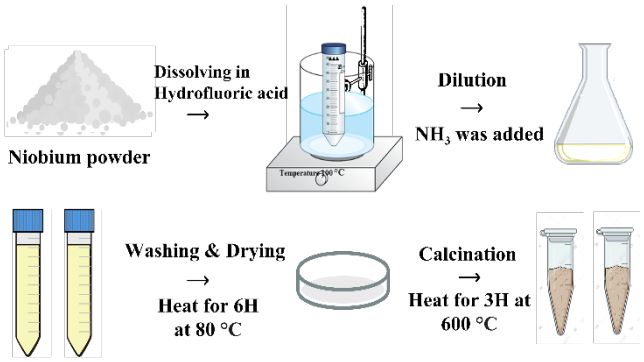

Figure 1: Chemical synthesis of Niobium-pentaoxide

RESULTS AND FIGURES

The results indicate that as the concentration of the niobium precursor increased, the Nb_2O_5 nanoparticles exhibited higher crystallinity and enhanced porosity. SEM and TEM analyses confirmed the formation of well-defined orthorhombic crystalline structures with increased surface area, which is beneficial for applications requiring high surface reactivity. Additionally, the nanoparticles effectively reduced the metabolic activity of *E. coli* and *P. aeruginosa* biofilms, suggesting their potential use in antibacterial applications.

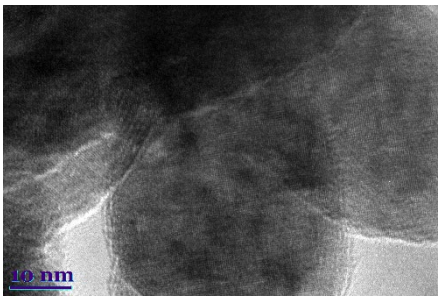

Figure 2: TEM Image of Highly Crystalline NP's

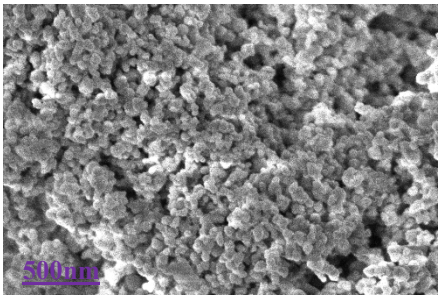

Figure 3: SEM Image of NP's

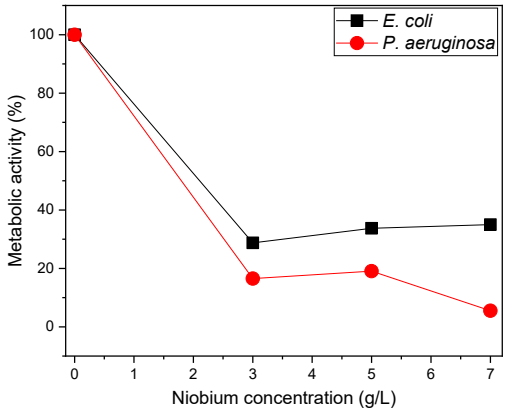

Figure 4: Graphical representation of reduction of metabolic acvtivity.

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Influence of faradaic processes on yeast electroporation: comparative study using conventional and dielectric-coated electrodes

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INTRODUCTION

While conventional understanding attributes electroporation mainly to the action of high electric fields, recent evidence suggests the involvement of electrochemical reactions, particularly the generation of reactive oxygen species (ROS), in the process [1]. This study aims to investigate the influence of faradaic ROS generation on yeast electroporation by comparing the outcomes using conventional metal electrodes with those using electrodes coated with thin films of dielectric material, specifically anodized electrodes.

METHODOLOGY

Various types of electrodes were fabricated from commercially-available metal plates, *i.e.* aluminium, titanium, anodized aluminium (Al/AlO_x), anodized titanium (Ti/TiO_x), and titanium coated with PEDOT:PSS. Hydrogen peroxide (H_2O_2) concentration (as a proxy of ROS), after electroporation was determined using the TMB/HRP assay [2]. *Saccharomyces cerevisiae* (BY4741, MATa) cells were placed in a custom electroporation setup at a concentration of 5×10^8 cells/ml and exposed to 8 pulses of 5 kV/cm intensity, 100 μs duration and 2 Hz frequency. Cell membrane permeabilization was assessed using propidium iodide (PI) uptake and impedance measurements.

RESULTS AND FIGURES

The presence of electrochemical processes during electroporation was established, indicating their potential influence on electroporation efficiency. By removing oxygen from the solution, we were able to significantly reduce the production of H_2O_2, therefore it can be concluded that the main mechanism of H_2O_2 generation is the oxygen reduction reaction, with a smaller contribution of the water splitting reaction. Anodization led to a decrease in H_2O_2 production, with aluminium showing a complete reduction and titanium a partial reduction. This is believed to be due to the varying amounts of charge delivered during electroporation, with titanium delivering approximately twice as much charge as aluminium. On the other hand, coating titanium with PEDOT:PSS resulted in a significant increase in H_2O_2 production.

Following the H_2O_2 mapping experiments, we investigated yeast electroporation. We compared PI uptake measurements with impedance measurements, and also correlated both methods with the total charge delivered during electroporation. The results indicated that cell membrane permeability, as measured by PI uptake, correlated with the charge delivered during the protocol. Impedance measurements showed similar trends, except in the case of anodized aluminium. Here, high permeability to PI was observed along with minimal changes to the conductivity of the sample, indicating minimal loss of intracellular molecules.

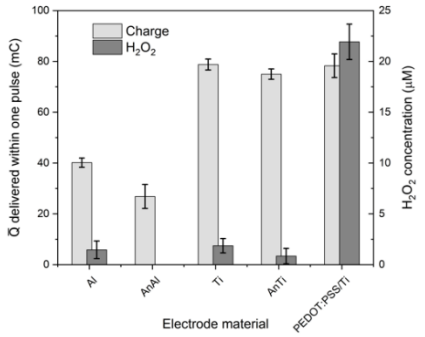

Figure 1: Comparison of H_2O_2 production for different electrode materials after electroporation. 200 pulses, buffer.

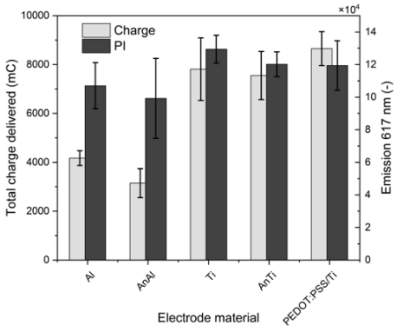

Figure 2: Correlation between total charge delivered during electroporation and PI fluorescence intensity. 8 pulses, yeast.

CONCLUSIONS

Our results demonstrate that the use of anodized electrodes leads to a reduction in the production of H_2O_2 compared to conventional metal electrodes. However, yeast cell membrane permeability after electroporation does not correlate with H_2O_2 production, but rather with the charge delivered by the electrodes. Nevertheless, the effect of ROS on cell viability cannot be excluded.

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Designing and in vitro evaluation of a method for peripheral nerve tissue decellularization based on non-thermal irreversible electroporation

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INTRODUCTION

Electroporation is a method in which pores are created in the cell membrane by using square pulses of the electric field that are applied within a few nanoseconds to milliseconds [1]. During this process, the permeability of the cell membrane increases. This research seeks to find an effective method to destroy rat sciatic nerve tissue cells using non-thermal electroporation.

METHODS

First, using COMSOL Multiphysics, the most effective protocol that does not increase the temperature during the electroporation process was extracted. The effect of myelin sheath on the share of Schwann cells from the intensity of the electric field was also investigated by COMSOL Multiphysics. Then, in in vitro experiments, nerve tissues were exposed to electric pulses with different parameters (number, frequency, pulse width and electric field intensity). Also, the effect of different media for pulse application (such as DMEM, PBS and normal saline) on electroporation efficiency was investigated. Moreover, the effect of giving the cells a chance for programmed cell death in different time intervals was studied. In vivo experiments, the effective protocols identified in the in vitro experiments (field intensity 2100 V/cm, frequency 4 Hz, pulse width 50 μs and 400 pulses) were applied to the sciatic nerve of living rats, and the nerve tissues at different times after the pulses were checked. In addition, an experiment was performed on the SH-SY5Y cell line with the optimal protocol obtained from the previous experiments.

RESULTS

According to the results of COMSOL Multiphysics, the temperature of the tissue increases by about 0.5 degrees Kelvin, and this value is reduced by half in about 250 milliseconds. Also, in the presence of the myelin sheath, the intensity of the electric field is one third of the state in which there is no myelin sheath. According to the results, the period of time after the application of electric field pulses to carry out the processes that lead to cell death (such as apoptosis) was considered and the tissues should not be immediately fixed by formalin and subjected to histological examinations. It seems that increasing the intensity of the electric field and subsequently reducing the pulse width creates more pores in the cell membrane, which results in more cells dying. Using an environment with higher electrical conductivity while applying pulses on the tissue causes a higher rate of cell death [2]. Also, the test results on SH-SY5Y cells obtained with a fluorescence microscope and after staining with ethidium bromide and acridine orange, showed that the cells die in less than three hours with a high rate of apoptosis.

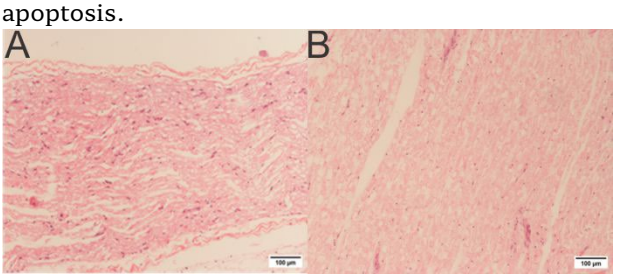

Figure 1: H&E staining results for optimal protocol. A: control group. B: treatment group with field intensity 2100 V/cm, frequency 4 Hz, pulse width 50 μs and 400 pulses (Pulses were applied while the tissue was located in Ringer)

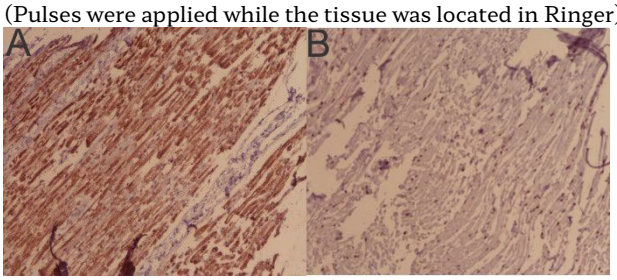

Figure 2: IHC results for the in vivo study. A: MBP antibodies which indicate myelin sheath. B: SOX10 antibodies which stain Schwan cells.

CONCLUSION

The difference in the destruction of cells in the state in which they are located in the tissue and in the state in which they are in the form of cell suspension in the culture medium, is due to the myelination of the peripheral nerve tissue. According to the results, electroporation with the used protocols cannot destroy all peripheral nerve tissue cells, but with the improvement of environmental conditions, this method can be used as an effective method to remove a significant number (70%) of nerve tissue cells.

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Electrochemistry of platinum-iridium (PtIr) electrostimulation electrodes

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INTRODUCTION

Platinum-iridium (PtIr) electrodes are a cornerstone in biomedical applications due to their excellent conductivity, corrosion resistance, and biocompatibility. These electrodes are extensively used in neurostimulation and electroporation-based therapies, both of which rely on electrical stimulation to modulate cellular processes. Despite the known stability of PtIr during chronic use, the electrochemical reactions occurring at the electrode-tissue interface remain poorly understood, particularly in high-voltage applications such as electroporation. Neurostimulation and electroporation apply electric fields to tissues, eliciting different biological responses—ranging from the initiation of action potentials to the creation of transient pores in cell membranes. This study aims to elucidate the key electrochemical reactions occurring on PtIr electrodes during neurostimulation and electroporation, focusing on their effects on the local biological environment and the potential implications for therapeutic efficacy and safety.

METHODOLOGY

We employed a combination of electrochemical and spectroscopic techniques to investigate the behavior of PtIr electrodes during electrical stimulation. Cyclic voltammetry (CV) was used to characterize the electrode response across a range of applied potentials, while amperometric microsensors measured local oxygen, hydrogen peroxide concentrations, pH changes, and hydrogen evolution. Spectrophotometric assays quantified hydrogen peroxide (H_2O_2) and hypochlorite formation, while inductively coupled plasma mass spectrometry (ICP-MS) was used to detect electrode dissolution by measuring Pt and Ir ions in the solution. These experiments were conducted in both standard phosphate-buffered saline (PBS) and more complex cell culture media (DMEM) supplemented with fetal bovine serum (FBS) to mimic physiological conditions.

To investigate the differences between neurostimulation and electroporation, we applied low-voltage bi-phasic stimulation pulses typical of neurostimulation and high-voltage mono- and bi-phasic electroporation pulses.

RESULTS

Our results reveal distinct electrochemical profiles for PtIr electrodes under different stimulation protocols. During low-voltage stimulation, oxygen reduction was the primary reaction, leading to the depletion of dissolved oxygen near the electrode and the formation of hydrogen peroxide [1]. Under higher-voltage conditions, significant water electrolysis occurred, producing hydrogen and oxygen gases and causing shifts in pH near the electrode surface. Electric fields above certain thresholds also promoted the oxidation of chloride ions to hypochlorite, which could pose risks of tissue damage during prolonged applications. Furthermore, we observed electrode dissolution, as evidenced by increased concentrations of Pt and Ir ions in the surrounding solution.

CONCLUSIONS

The electrochemical behavior of PtIr electrodes is strongly influenced by the type of electrical stimulation applied, with neurostimulation and electroporation each creating unique reaction environments at the electrode interface. This research provides critical insights into the electrochemical mechanisms underpinning PtIr electrodes, underscoring the importance of optimizing both neurostimulation and electroporation protocols. A thorough understanding of these reactions paves the way for the development of safer, more effective biomedical devices that leverage electrical stimulation for a variety of therapeutic applications.

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Studying plant tissue electroporation using the indicator triphenyl tetrazolium chloride (TTC)

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INTRODUCTION

Irreversible electroporation (IRE) is an ablation method utilising short, high-intensity electric pulses causing cell death by irreversibly permeabilizing the cell membrane of cells in tissue. IRE ablation causes a very sharp edge of the ablation area. Triphenyl tetrazolium chloride (TTC) is a redox indicator commonly used to differentiate between dead and viable cells in tissue, staining cells with active mitochondrial metabolism, red [1].

The aim of this study was to replicate previous findings suggesting that ablation area is bigger when a sequence of square-wave electrical pulses is followed by an exponentially decaying electrical pulse compared to using only an exponentially decaying pulse [2]. Additionally, the study aimed to investigate whether TTC hyper-staining around the ablated area is due to a loss of turgor pressure or changes in metabolic activity.

METHODS

Experiments were performed on 7-8 mm thick slices of two highly turgid tissues (potato and black radish), and two less turgid tissues (zucchini and melon). Three types of electrical pulses were tested at 500 V, 1000 V and 1500 V: (1) eight monopolar square-wave pulses of 10 µs duration at a frequency of 100 Hz, (2) a single exponential decay pulse with the time constant of approximately 3200 µs, and (3) a sequence combining (1) and (2).

We analysed the size of the electroporated area using TTC staining. The concentration of the TTC solution varied depending on tissue type, with some solutions containing citric acid (CA) to slow down natural melanin formation. Consequently, the submersion times also differed for each tissue type. Concentrations and submersion times for each tissue are given in Table 1.

Table 1: Parameters of TTC staining process.

	TTC concentration	CA concentration	Time in solution
Potato	0.5 %	0.75 %	22 hours
Black radish	0.5 %	0.75 %	22 hours
Zucchini	1 %	None	4 hours
Melon	1 %	None	3 hours

RESULTS

At 1000 V and 1500 V, the sizes of ablated areas in all tissues were statistically similar when using either the exponential decay pulse alone or the combination of eight monopolar square-wave pulses and the exponential decay pulse. The only exception was observed in potato tissue at 1500 V, where the ablation areas differed significantly between the two pulse types ($p = 0.0375$, two-tailed pared Student's t-test). In all other cases the p-values were above 0.05, indicating no significant difference. Due to high variability in results at 500 V, these data were excluded from further statistical analysis.

All 45 slices of potatoes had hyper-stained rings around the ablated area, which darkened over time, while the surrounding tissue did not show increased staining intensity. This suggests that the hyper-staining is mainly a result of turgor pressure loss and consequent increased tissue porosity. This conclusion is supported by results in zucchini, a non-turgid tissue, where no hyper-staining was observed.

After eight hours, 17 out of 45 black radish slices showed hyper-staining. After 22 hours, only 6 slices still showed hyper-staining. This was due to the surrounding area becoming more intensely stained, suggesting that intensified metabolic activity could also contribute to hyper-staining. Additionally, irregularly shaped hyper-stained regions were noted, likely due to tissue inhomogeneities.

The experiments on melon showed similar results as the results on black radish. After three hours, 25 out of 45 slices were hyper-stained. In many slices, the hyper-staining appeared irregularly shaped or was localized to specific regions instead of surrounding the entire ablated area. This irregular pattern could also be attributed to tissue inhomogeneities.

CONCLUSIONS

The eight monopolar square-wave pulses applied before the exponential decay pulse do not affect the size of the ablated area. At 1000 V and 1500 V, the sizes of the ablated areas using the combination of eight monopolar square-wave pulses and the exponential decay pulse vs. only the exponential decay pulse show no statistically significant difference for most tissues.

The cause of hyper-staining cannot be conclusively identified in this study, as the results from black radish and melon were inconclusive. Thus, further research is needed to determine if hyper-staining is caused by a loss of turgor pressure, accelerated metabolic processes, or other factors.

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Continuous electrochemical H_2O_2 delivery for cancer treatment

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INTRODUCTION

Cancer therapy often requires novel approaches to enhance treatment efficacy while minimizing side effects. Hydrogen peroxide (H_2O_2) has shown promise due to its selective oxidative stress induction in tumor cells [1]. H_2O_2 can cause programmed cell death (apoptosis) in cancer cells, making it a potent agent for targeted therapy [2]. However, the challenge lies in achieving continuous and controlled delivery of H_2O_2 at the tumor site, as traditional methods of administration often result in rapid neutralization and clearance from the body [3]. To address this challenge, our study introduces an innovative electrochemical method for the sustained delivery of H_2O_2 directly to cancer cells. Our approach leverages the principles of electrochemical reduction, where dissolved oxygen is reduced at the cathode to produce H_2O_2, offering a promising solution for targeted cancer therapy.

METHODS

We characterized a range of electrode materials, including gold, titanium, stainless steel, and Poly(3,4-ethylenedioxythiophene) (PEDOT), to evaluate their efficiency in generating H_2O_2 via the oxygen reduction reaction (ORR). The electrochemical generation of H_2O_2 was conducted in both phosphate-buffered saline (PBS) and cell culture medium (Fluorobrite™) under chronoamperometric and galvanostatic conditions. Quantification of H_2O_2 was achieved using a spectrophotometric method involving horseradish peroxidase and 3,3′,5,5′-Tetramethylbenzidine. The performance of these electrodes was assessed based on consistency, efficiency, and reproducibility. Additionally, the cytotoxic effects of the generated H_2O_2 were evaluated using an MTS assay on U87 glioblastoma and A375 melanoma cells, which exhibit different sensitivities to oxidative stress [1]. To confirm the specificity of H_2O_2-induced cell death, catalase, an enzyme that decomposes H_2O_2, was added to the system.

RESULTS

Among the tested electrodes, PEDOT exhibited superior performance in generating consistent and efficient levels of H_2O_2 in both PBS and cell culture medium. The continuous electrochemical delivery demonstrated a dose-dependent reduction in cell viability for both U87 and A375 cancer cell lines, with A375 cells being more sensitive to H_2O_2. The addition of catalase restored cell viability, confirming that the cytotoxic effect was specifically due to H_2O_2.

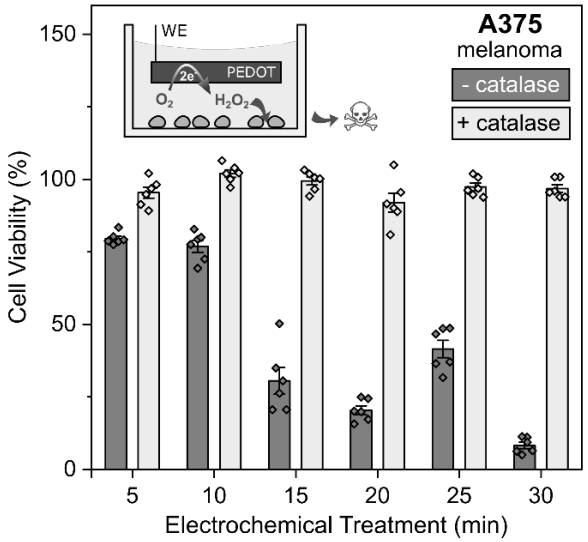

Figure 1: Effect of continuous electrochemical H_2O_2 delivery on the viability of A375 melanoma cells, with and without catalase. The inset illustrates the experimental setup where a PEDOT working electrode (WE) is immersed in the well containing the cells.

CONCLUSIONS

Our results suggest that continuous electrochemical H_2O_2 delivery using PEDOT electrodes is a promising method for targeted cancer therapy. This approach offers controllable, precise, and efficient induction of tumor cell death, presenting a significant advancement in the field of cancer treatment. The technique can be easily integrated with electroporation to achieve a synergistic effect. Future studies will focus on optimizing the electrode design and delivery parameters to further enhance the therapeutic efficacy and explore *in vivo* applications.

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Protocol for a randomized clinical trial investigating the effect of reduced bleomycin in electrochemotherapy on patients with cutaneous malignancies

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INTRODUCTION

Cutaneous tumors across cancer types have long been effectively treated with electrochemotherapy (ECT), involving a standard dose of chemotherapy (15.000 IU/m2 bleomycin) intravenously, followed by brief electric pulses to the tumor. This enhances chemotherapy entry into malignant cells, aiding in their destruction. Studies evaluating reduced bleomycin dosage in electrochemo-therapy have shown positive outcomes [1].

We aim to investigate if halving the chemotherapy dosage during ECT for skin tumors is as effective as standard ECT treatment.

METHODS

We are planning a double-blinded randomized clinical trial where we aim to include 110 biopsy-verified cutaneous metastases of any kind. From previous research we anticipate that one patient will have approximately two tumors [2], why we need to include 55 patients. Patients will be randomly assigned (1:1 ratio) to receive either full or half dose of chemotherapy. The primary endpoint is overall tumor response after three months, assessed by the modified Response Evaluation Criteria in Solid Tumors (RECIST). Additionally, we will collect biological samples during the ECT treatment to measure the distribution of bleomycin in normal tissue, tumors and blood, using liquid chromatography techniques. Furthermore, tumor cell percentage will be correlated with bleomycin concentration. Side effects will be monitored and qualitative interviews with 16 patients pre- and post-treatment will explore their experiences with cutaneous tumors and ECT.

The study will be conducted at Zealand University Hospital, Roskilde and Copenhagen University Hospital, Herlev Gentofte.

DISCUSSION

Advancements in cancer treatment have led to higher survival rates and will likely contribute to a rise in cutaneous malignancies. Moreover, the population we are treating with ECT is getting older [3]. If halving the dose of bleomycin proves to be as effective as standard dosage, it could reduce side effects and thereby benefit elderly, frail and kidney-compromised patients.

Implications of this study will potentially be relevant not only to treatment of cutaneous metastases but also to treatment of deep-seated tumors with use of ECT.

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A universal *trans*-amplifying mRNA system for electrotransfer into tumors

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INTRODUCTION

In the past few years, mRNA molecules have become a promising new therapeutic option in treatment of many disease-types, including cancer. mRNA-based therapeutics are characterised by rapid and high protein expression and the absence of insertional mutagenesis, making them an attractive alternative to conventional gene therapy using plasmid DNA. However, a major drawback of the technology remains its relatively short-lived protein expression, which requires repetitive dosing and slows down the transition to clinical practice.

TRANS-AMPLIFYING mRNA SYSTEM

To overcome these limitations, a bipartite system of *trans*-amplifying mRNA molecules was developed, that aims to increase and prolong the expression of encoded proteins [1,2]. The system consists of two mRNA molecules: one coding for a protein of interest (trRNA-GOI) and the second one for a non-replicating alphaviral replicase (nrRNA-REPL). Upon ribosomal processing, the replication complex matures and amplifies trRNA-GOI molecules. These can again be translated at the ribosomes, resulting in increased cellular expression of protein-of-choice [3].

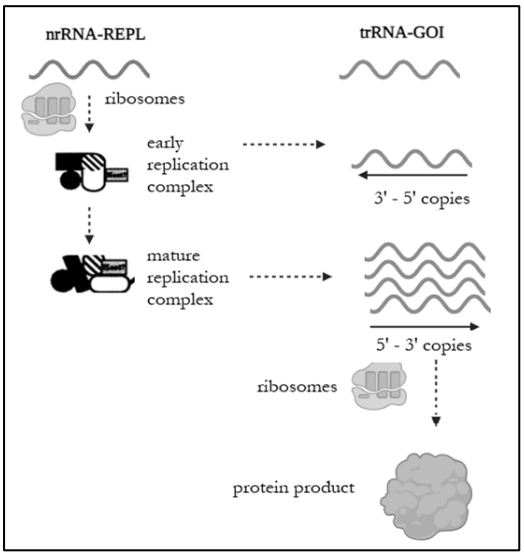

Figure 1: Mechanism of action of trans-amplifying mRNA. nrRNA-REPL – mRNA construct encoding the non-replicating alphaviral replicase, trRNA-GOI – mRNA construct encoding the protein of interest. Image was created in BioRender programme.

AIM OF THE STUDY

Our aim is to design a universal, safe and efficient system of trans-amplifying RNA molecules to increase and prolong the protein expression upon electrotransfer *in vitro* and *in vivo*.

RESULTS

We modified a *trans*-amplifying mRNA system [1,2] and adapted it for two distinct transgenes encoding reporter proteins: trRNA-GFP encoding the green fluorescent protein for *in vitro* experiments with tumor and non-tumor cell lines, and trRNA-LUC encoding firefly luciferase for *in vivo* experiments using mouse melanoma model. The selectivity of the amplification of the trRNA constructs and thus the safety and universality of the system is ensured by designing replicase recognition elements in the untranslated regions of the target mRNAs.

CONCLUSIONS

We designed a universal system of *trans*-amplifying RNA molecules. Its efficacy, specificity and safety will be evaluated after electrotransfer of naked mRNAs into tumor and non-tumor cells and on a mouse melanoma model. We expect this therapeutic approach to be a safe and effective alternative to gene electrotransfer of plasmid DNA.

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Structural effects of pulsed electric fields on collagen secreted by dermal fibroblasts

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INTRODUCTION

Pulsed electric fields can permeabilize the cell membrane. While membrane effects have been studied in detail over the last decades, the effects on the extracellular matrix (ECM), namely the collagen, have been studied to a lesser degree. Increased collagen production can be involved in tumor development, dissemination, and metastasis progression [1]. In addition, collagen can act as a barrier, which prevents the penetration of therapeutics into the tumor. Finding a strategy that would make the collagen more permeable to drugs, could indeed benefit cancer patients. Here we thus evaluated if irreversible electroporation (IRE)- based protocols can remodel the ECM.

METHODS

Dermal sheets produced with primary dermal fibroblasts as described previously [2], were exposed to eighty 100 µs long square wave electric pulses at an electric field intensity of 2000 V/cm, delivered at a pulse repetition rate of 1 Hz. The electric pulses were applied in a low conductivity buffer, once or three times per day (spaced by 3 hours), for one or three days. Structure, amount and spatial distribution of collagen were analyzed in dermal sheets. In addition, potential collagen debris, that might be released into the pulsation medium after IRE protocols were also collected. The collagen within dermal sheets was visualized by second harmonic generation (SHG) microscopy. Collagen residues stemming from dermal sheets into the pulsation buffer were dried, stained with Masson-Trichrome and observed by optical microscopy.

Hyperthermia (1 h exposure of the dermal sheets to 52°C) was used as a positive control, as it is known for its impact on collagen fibers structure [3], while hypothermia (4°C, 1 h exposure) was used as a negative control.

The temperature during IRE protocols was monitored with a temperature probe (Nomad Fiber Optic Thermometer Model NMD 535 A).

RESULTS

The application of tested IRE protocols yields to similar results as hyperthermia exposure with a loss of SHG signal intensity and volume (Figure 1A and 1B). The overall thickness of the dermal sheet did not differ between conditions tested (Figure 1A). The temperature during IRE protocols remained below 32°C suggesting a direct destructurating effect of IRE treatments on collagen structure.

Finally, fragmented collagen was found in the pulsing buffer after pulsed electric fields exposure, indicating collagen fibers alterations by tested IRE protocols.

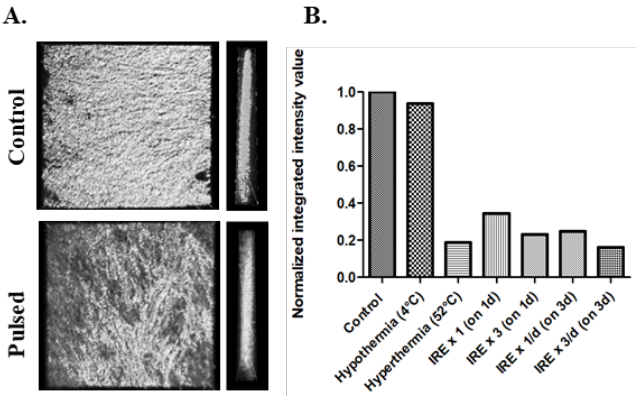

Figure 1: Impact of the treatments on dermal-sheets-containing collagen, determined by SHG microscopy. **A**: SHG microscopy images of control and IRE condition (3/day on 3 days). Collagen appears in light grey. **B**: Quantification of the integrated intensity, normalized to the control, pertaining to the collagen signal in the analyzed volume fraction. Results show the mean of two independent experiments for thermal treatments and IRE (1/d (on 3d)) condition, and one experiment for the other treatments. Each independent experiment contains three biological replicates.

CONCLUSION

This work highlights the potential of pulsed electric fields to alter the collagen structure, indicating that applied IRE treatments could modulate collagen matrix organization. This finding appears promising as it indicates that IRE pulses could potentially be used to prime the extracellular matrix. Targeting the matrix surrounding the tumors might be essential to increase the anti-cancer drug penetration into the tumoral mass. Pulsed electric fields used in irreversible electroporation could thus be a solution to disorganize the collagen and allow better delivery of cancer agents into fibrotic tumors.

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Characterisation of metabolic adaptations induced by electric fields for cancer treatment

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INTRODUCTION

Electroporation is a non-invasive strategy for cancer treatment. Based on the use of the local application electric pulses that induce reversible or irreversible membrane permeabilization, it has fewer side effects than conventional treatments. Electrochemotherapy (ECT) based on the combination of electroporation and chemotherapy is used to favour the cytotoxicity of poorly permeant drugs, as the electrical field facilitates the entry of chemotherapeutic agent into tumour cells. The efficacy of ECT in clinic has been reported for therapeutic agents such as bleomycin, cisplatin, mitomycin c or 5-FU. However, in the absence of ECT, these therapeutic agents are known to induce metabolic adaptations that allow tumour cells to survive. In addition, electroporation disrupts cell homeostasis, of which oxidative stress is a major component. Recently, the combination of metformin, an inhibitor of the mitochondrial respiratory chain, with electrical pulses has been shown to have a synergistic effect to enhance the anticancer effect, suggesting a metabolic adaptation induced by the electrical pulse [1]. Moreover, electrical pulses induce cellular effects such as the release of ATP and HMGB1 (high modility group box 1) [2].

The study of HMGB1 in cancer has shown that HMGB1 treatment of colon cancer cells induces cell death and inhibition of mitochondrial respiration. HMGB1 treatment of cancer cells inhibits PKM2 and alters the metabolism of cancer cells, favouring the use of glucose fermentation and glutaminolysis to meet all energy and metabolic needs [3]. Recently, using an in vivo model of diabetes mellitus, HMGB1 knockout mice showed improved insulin tolerance and lower glycaemia compared to normal mice [4].

As ECT induces HMGB1 release and HMGB1 appears to be an important metabolic regulator, one of the aims of this project is to determine whether HMGB1 release after ECT induces metabolic adaptation in tumours and to characterise this metabolic adaptation in comparison to the change induced by chemotherapy without ECT.

MATERIALS AND METHODS

We are focusing our research on melanoma because ECT is currently used to treat human skin tumours, and because we can easily study the metabolic effects of treatments on normal neighbouring cells.

To this end, we characterise the effects of electroporation and ECT on the oxidative stress and metabolism of melanoma cells using 2D and 3D cell cultures, reconstructed epidermis and reconstructed skin containing melanoma cells [5].

To further characterise the changes in metabolism, exometabolomics and transcriptomics analysis are carried out to determine the changes in cell signalling involved in the metabolic changes after electroporation or ECT treatment.

EXPECTED RESULTS

In this project, we will determine the effects of electroporation and ECT on oxidative stress and metabolic adaptation in cancer cells, in order to find a targetable metabolic vulnerability to increase the efficacy of these strategies.

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Electrolytic electroporation: what we know and where to go

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INTRODUCTION

Electrolysis and electroporation have both been utilized as stand-alone tissue ablation modalities. Electrolysis (or electrochemical treatment) uses a low-magnitude direct electric current to create chemical species at the electrode-tissue interface which then diffuse through the tissue. These electrolytic products result in extreme pH changes and lead to cell death. Electroporation, on the other hand, uses electrical pulses to create permeabilizations in the cell membrane. The permeabilizations produced during irreversible electroporation (IRE) directly lead to cell death by loss of cell homeostasis. When electrolysis and electroporation are combined, a new form of tissue ablation is achieved. Electrolytic electroporation (E2) can be used to increase the ablation volume when compared to irreversible electroporation alone.

PROPOSED MECHANISM

Initial studies have shown that when reversible electroporation (RE) type parameters and low-charge electrolysis parameters were applied across rat liver tissue alone, very minimal tissue ablation was achieved. However, when these two parameters were applied in series, the ablation was seen throughout the entire liver lobe thickness [1]. This drastic increase in ablation volume has been attributed to a combination of the key characteristics of both technologies. It has been proposed that while the low-charge electrolysis alone was not able to produce a strong enough electrochemical effect to target the cells within the liver volume directly, when combined with the permeabilizations induced in the cell membrane by the RE pulses, low levels of electrolytic product were able to penetrate the cell membrane and target the cells from within.

Though additional studies are warranted to understand the E2 effect, this explanation is supported by pathology findings and in vitro studies. For example, Klein et al. [2] developed a set of experiments to isolate the electrolytic and electroporation effects when applying E2 showing that the cell viability dropped drastically when the two effects were allowed to work together. Necroptosis and pyroptosis are cell death pathways attributed to E2 [3] that can both be triggered when cells experience severe acidosis or alkaliptosis, providing additional support for the proposed mechanism of action.

INCREASING ABLATION VOLUME

A variety of E2 treatment parameters have been developed and tested experimentally. Lower voltage pulse sequences have been used to produce electrolytic products while also inducing cell membrane permeabilizations, creating ablation volumes at a significantly lower electric field threshold than required for IRE.

E2 can also be delivered by combining IRE with electrolysis. Here, one of the main advantages is that the E2 effect can be used to expand the ablation volume beyond the electric field threshold needed for IRE and into the surrounding RE zone. A variety of electric parameters have been developed for this application.

CHARACTERISTICS OF E2

Despite a wide range of treatment parameters that have been used experimentally, some key characteristics of E2 have been observed. Histological analysis has shown that, similar to electrolytic ablation alone, E2 results in a markedly different appearance of the treated tissue at the anode compared to the cathode, due to different ablative reactions happening near the different electrodes [4]. The volume of tissue ablation is correlated to the charge delivered, and electrolytic electroporation has been used to create clinically relevant ablation volumes [5]. The extracellular matrix and critical structures within the ablated tissue volume remain undamaged after electrolytic electroporation treatment [2].

MOVING FORWARD

E2 has been demonstrated as a promising method for ablating tissue with lower voltages compared to IRE as well for expanding the ablation volume. Additional studies have shown that many parameters that were traditionally thought to ablate using IRE alone were also producing electrolytic products that influence the treatment outcome. Gaining a deeper understanding of the mechanisms at play is important not only for the further development of E2 but also for increasing our knowledge around IRE. Additional experimental work as well as the development of a model that can account for changes in electrolytic product concentrations and local pH is warranted.

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Localized electroporation of isolated neonatal rat ventricular cardiomyocytes

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INTRODUCTION

Electroporation is a non-viral method for genetic material delivery to the cells which is a crucial step for developing gene therapies. Traditionally, bulk electroporation (EP) has been utilized for most gene electrotransfer (GET) applications *in vitro* [1]. In bulk EP, cells are placed between two electrodes and subjected to an approximately homogenous electric field. The disadvantage of bulk EP is that it can result in quite low cell survival (<20%) in certain types of cells, such as cardiomyocytes, which is thought to be associated with the large cell membrane area that becomes permeabilized [2]. By localizing the electric field over a small membrane area using a nanoporous substrate, it is possible to improve transfer efficiency while maintaining greater cell survival and better control over gene expression [3].

OBJECTIVES

In this study, we investigated the potential of localized EP for transfecting isolated neonatal rat ventricular cardiomyocytes (NRVM).

METHODS

The isolation of NRVM was performed following the protocol from Collesi *et al.* [4]. For experiments, NRVM were grown in a confluent monolayer at a density of 10^5 cells/ml on cell culture inserts on top of PET membranes with 0.4 μm pore diameter (Corning, USA) in DMEM growth medium at 37°C and 5% CO_2. After 5 days the inserts with NRVM were placed onto a 3D printed localized EP device. A conductive ITO-coated glass slide served as the bottom electrode, whereas a Pt-wire was used as the top electrode. The ELECTROcell B10 HVLV (LEROY Biotech, France) pulse generator was used to apply electrical pulses (4´ 5 ms, 10-40 V, 1 Hz). To study electropermeabilization and the concurrent cell uptake of propidium iodide (PI), an 80 μl droplet with concentration of 1 μg/ml was pipetted onto the bottom electrode beneath the insert and the cells were monitored on a Leica Thunder inverted microscope during the pulse delivery (Leica Microsystems, Germany). For evaluation of GET, 80 μl of plasmid pEGFP-N1 at a concentration of 200 μg/ml was used. After 24 h of incubation (37°C, 5% CO_2), EGFP expression was identified under the microscope. The obtained images were analysed using Leica LAS-X and ImageJ software.

RESULTS

Pulse amplitude as low as 10 V resulted in detectable uptake of PI, demonstrating successful membrane electroporation. With increasing the pulse amplitude, the PI uptake progressively increased (Figure 1). EGFP expression was also obtained at pulse amplitude of 20 V.

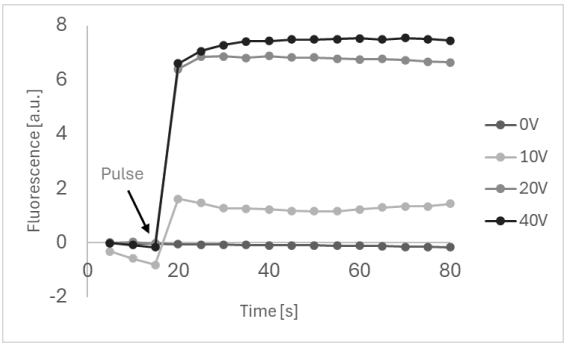

Figure 1: Time course of PI uptake in NRVM.

CONCLUSIONS

Our results present the first step towards developing a localized EP protocol for transfecting cardiomyocytes with nucleic acids that promote cardiomyocyte proliferation. This in turn will help us select nucleic acids to be used for developing cardiac gene therapies for promoting cardiac regeneration using electroporation.

ACKNOWLEDGMENTS

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LABORATORY SAFETY

S1

Laboratory safety

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BIOSAFETY

There are four biosafety levels (BSLs) for working with live organisms; each BSL consists of combinations of laboratory practices and techniques, safety equipment and laboratory facilities. Each combination is specifically appropriate for the operations performed, the suspected routes of transmission of the organisms and the laboratory function or activity.

Biosafety Level 1 represents a basic level of containment. It is suitable for work involving well-characterized agents not known to cause disease in healthy adult humans. The potential hazard to laboratory personnel and the environment is minimal.
Biosafety Level 2 is suitable for work involving agents that can cause human disease and have a moderate potential hazard to personnel and the environment. Precautions must be taken for handling and disposing of contaminated material, especially needles and sharp instruments. The laboratory must have limited access.
Biosafety Level 3 is used in laboratories where work is done with pathogens, indigenous or exotic agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route. Such microorganism can present a serious hazard to workers and a risk of spreading to the community, but there is usually effective prophylaxis or treatment available. BSL 3 requires special facilities with self-closing double doors and sealed windows, decontamination of clothing before laundering and controlled access.
Biosafety level 4 is required for work with pathogens which pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease, for which there is no effective prophylaxis or treatment available. Such organisms present a serious hazard to workers and may present a high risk of spreading to the community. The BSL 4 facility is generally a separate building with specialized ventilation and waste management systems to prevent release of live pathogens to the environment.

GENERAL SAFETY RULES FOR WORKING IN THE LABORATORY

The following basic safety rules should be observed at all times in the laboratory:

1. Wash your hands with liquid soap and dry them with paper towels upon entering and prior to leaving the laboratory.
2. Wear laboratory coat and gloves. Tie back loose hair.
3. Do not carry your personal belongings in the laboratory; place them in specified locations – never on bench tops.
4. Do not smoke, eat, drink, apply cosmetics or insert contact lenses in a laboratory.
5. Keep doors and windows closed during the laboratory session to prevent contamination from air currents.
6. Contaminated spots on clothes or body can be sprayed with disinfectant and washed with water. Contaminated material should be put into special containers.
7. If you had any contact with hazardous chemicals while wearing your gloves, change the gloves before you touch other laboratory equipment, do not touch your face or your clothes with contaminated gloves.
8. Do not allow water or any water-based solution to come into contact with electrical cords or conductors. Make sure your hands are dry when you handle electrical equipment.
9. Report all accidents immediately to the instructor.

RULES FOR HANDLING CHEMICALS

Almost all chemicals can be harmful in some way and prolonged exposure may cause long-term effects as yet unknown. Preparation of hazardous chemicals must be conducted under the fume hood.

When handling chemicals the following rules must be strictly met:

1. **Always read labels before handling any chemical**. Learn hazard warning symbols which are displayed on the labels.
2. Take care to avoid spillage - if this occurs, neutralize any hazard and clean up immediately, including the outside of the container.
3. Some chemicals have a delayed or cumulative effect. Inform the instructor if any feeling of being unwell occurs when using chemicals.
4. Chemicals must not be disposed of by indiscriminate washing down the sink. Carefully read the appropriate material safety data sheet and follow the instructions.

CHEMICAL HAZARD SYMBOLS

Explosive

Mutagenic, carcinogenic, causing respiratory sensitization

Oxidizing

Corrosive

Extremely/Highly flammable

Dangerous for the environment

(Very) Toxic

Pressurized gases

Harmful

PIPETTING TECHNIQUE

Pipetting is one of the most frequent tasks in the laboratory and it directly affects the success and repeatability of the experiments. It is critical to follow good pipetting practice techniques.

ASEPTIC TECHNIQUE

Aseptic technique is a combination of procedures designed to reduce the probability of infection. In spite of the introduction of antibiotics, contamination with microorganisms remains a problem in tissue culture. Bacteria, mycoplasma, yeasts and fungal spores may be introduced by operator, atmosphere, work surfaces, solutions and many other sources. In order to avoid contamination aseptic technique should be used while handling cell cultures.

Correct aseptic technique provides a barrier between microorganisms in the environment and the culture within its flask or dish. Hence, all materials that will come into direct contact with the culture must be sterile and manipulations designed in such manner that exclude direct link between the culture and its nonsterile surroundings.

The elements of aseptic technique are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling.

Rules for aseptic work:

1. Start with completely clear work area and wipe the surface with 70% alcohol and a sterile gauze.
2. Spray and wipe your hands with 70% ethanol.
3. Clean the outside of the containers and other objects with 70% ethanol before placing them in the microbiological safety cabinet.
4. The work surface should be uncluttered and contain only items required for a particular procedure; it should not be used as a storage area.
5. Remove everything that is no longer required and clean with 70% alcohol before the next procedure.
6. Arrange items to have easy access to all of them without having to reach over one item to get to another.
7. Work within your range of vision, e.g., insert a pipette in the pipetting device with the tip of the pipette in your line of sight continuously and not hidden by your arm.
8. Clean up any spillage immediately with absorbent tissues and wipe with 70% alcohol.
9. Remove everything when you finish and wipe the work surface with 70% ethanol.
10. Use ultraviolet light to sterilize the air and exposed work surfaces in the microbiological safety cabinet between uses.

GMO

GMO is an abbreviation for genetically modified organism. GMO is an organism that is created when a recipient (host) organism, with the help of a vector, successfully incorporates the insert in its genetic material and can transfer it to its descendants.
Closed system is a laboratory or some other closed room for GMO work.

Recipient (host) organism = cell/organism which accepts genetic material from the original organism or the environment, replicates and expresses it and can transfer it to its descendants.
Parent organism = recipient organism before the genetic change
Original organism = organism from which the genetic material for transfer in the host is acquired
Vector = DNA tool used in genetic engineering to harbour genes of interest and transfer them to the host
Insert = genetic material that is integrated into a vector

Example: In cell and molecular biology, the GFP (green fluorescent protein) gene **[insert]** is frequently used as a reporter of expression. GFP is a protein that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. It was first isolated from the jellyfish *Aequorea victoria* **[original organism]**, although many marine organisms have similar green fluorescent proteins. It is carried on plasmids **[vector]** to the target cells **[parent organism].** The cells that manage to express the protein are called **host organisms** (GMO).

When working with GMO, traceability is essential. For that it is necessary to keep a good operating and autoclave log book. Operating log is used for writing down essential GMO information, work procedure, solid and liquid waste management and potential work related accidents. Autoclave log is a record of all waste that has been autoclaved.

GMO waste can be deactivated in two different ways – thermic or chemical treatment. Deactivation prevents the GMO`s to migrate out of the closed system. Sterilized liquids can be washed down the sink, dry sterilized solid waste can be thrown in municipal waste.

In case of a GMO accident the biosafety commissioner needs to be informed and his/her directions should be followed. If a spillage occurs there has to be enough absorbent material to absorb all the liquid. Work surfaces should be decontaminated with a disinfectant.

FURTHER READING:

Freshney R. I. Culture of animal cells: a manual of basic technique.3rd ed. Wiley-Liss, Inc. New York, 1994.
http://www.biotechnology-gmo.gov.si/eng/genetically_modified_organisms/index.html

S2

Electroporation hardware safety

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ELECTRIC SHOCK

Possible consequences of the current flow through the human body are ventricular fibrillation, cardiac asystole, respiratory arrest and burns. Voltages greater than 50 V applied across dry unbroken human skin or pulse energies above 50 J can cause ventricular fibrillation, if they produce electric currents above 30 mA in body tissues through the chest area. Frequently, the individual cannot let go of the power source due to involuntary muscle contraction. Side effect are conditioned by path of electric current, its magnitude, tissue characteristics and exposure time. The most sensitive organs to electric properties in human body are the heart and the brain. Human body is much more sensitive to mains-frequency alternating current (50/60 Hz) then to either direct current or high-frequency currents. Pain perception and muscle contraction at a given current level depend strongly on body weight and frequency. For example, 10 mA current at frequency of 50/60 Hz can result in strong muscle contraction, in a person that weights approximately 50 kg, but sensitivity decreases with the frequency increase. The amount of voltage needed to produce same effects depends on the contact resistance between the human and the power source. When dealing with high voltages we always have to keep in mind that air breakdown voltage is about 30 kV/cm, so also a non-direct contact can be dangerous.

GENERAL SAFETY PRECAUTIONS FOR WORKING WITH HIGH VOLTAGES

The following basic safety rules should be observed at all times in the laboratory:

1. Never work alone when dealing with high voltages. Consider having a co-worker with knowledge about equipment and risks.
2. Never leave electrical circuits/devices under high voltages when you are not present.
3. Before working with high voltage devices consider the potential risks. Do not have any contacts with conductive parts of device and keep distance from conductors under high voltage. Keep in mind that air breakdown can occur when dealing with voltages above 30 kV/cm.
4. Before high voltage circuit manipulation, switch OFF the power supply and discharge all high voltage capacitors (preferably through high voltage resistor).
5. Check if all high voltage capacitors are discharge using voltmeter.
6. Use only your right hand to manipulate high voltage electronic circuits, avoid simultaneous touching of two elements and make sure you are not grounded. Wear rubber bottom shoes or sneakers. Set up your work area away from possible grounds that you may accidentally contact.
7. When using electrolytic capacitors:
    b. DO NOT put excessive voltage across them,
    c. DO NOT put alternating current (AC) across them,
    d. DO NOT connect them in reverse polarity.
8. Make sure all high voltage connections, tools and instruments are adequately insulated and rated for the voltage and current used.
9. If someone comes in a contact with a high voltage, immediately shut off the power. Do not attempt to move injured person in contact with a high voltage.
10. In the event of an electrical fire do not use water but special fire extinguishers used for fires caused by electric current.
11. Do not wear any jewellery or other objects that could accidentally come in contact with the conductive parts of electrical circuit.

12. Protect your ears and eyes due to possible discharge sounds and element explosions.

NOTES

LABORATORY EXERCISES

L1

Gene electrotransfer using locally enhanced electric field

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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.

The aim of this laboratory practice is to perform gene electrotransfer on attached cells using classical and localized electroporation

THEORETICAL BACKGROUND
Gene electrotransfer is a safe non-viral method used to transfer genes into living cells with the use of high-voltage electric pulses that cause reversible electroporation [1]. Nucleic acids such as DNA are large molecules that cannot cross an intact plasma membrane. However, if we expose the cells to electric pulses, the DNA can be transported across the electroporated membrane and enters the cell in a process termed gene electrotransfer.

In typical *in vitro* experiments, gene electrotransfer is performed by placing cells in suspension or attached to a surface between two parallel electrodes and exposing them to high-voltage electric pulses. The DNA is in the medium surrounding the cells. While this classical gene electrotransfer can be quite efficient, it can also be damaging to the cells, resulting in a considerable decrease in cell viability. These damaging effects are sometimes attributed to the large surface area of the plasma membranes that becomes electroporated in such experimental configuration [1].
In localized electroporation, nanostructured elements such as nanochannels, nanostraws, or nanopores are used to achieve localization of the electric field over small parts of the plasma membrane, which enhances gene electrotransfer efficiency and improves cell survival [2-5]. Various experimental configurations for localized electroporation have been developed. One of the simplest configurations uses commercially available cell culture inserts containing polyethylene-terephthalate (PET) porous substrate with pore size of 400 nm [2]. The configuration consists of an insert, a multiwell plate, and a pair of platinum/iridium wires as electrodes, one placed above and one below the porous substrate. The cells are grown attached to the upper side of the substrate, whereas the DNA solution is placed below the substrate.

This labwork is conducted by


Tina Batista Napotnik

Tina Cimperman



Klara Bulc Rozman

When applying electric pulses, the pores within the substrate assist in DNA transfer in two ways: 1. they amplify the electric field and enable one to localize electroporation only to small parts of the plasma membrane which are in direct vicinity of the pores; 2. since the electric field within the pores is amplified, the pores act as tubes that electrophoretically pull DNA molecules towards and into the cells. Because the electric field is greatly amplified around the pores of the porous substrate, we can use a much lower pulse voltage for localized electroporation than for the classical one. In fact, application of too high voltage in localized electroporation results in cell damage as well, since electroporation is no longer localized under such conditions.

EXPERIMENT

On day one, we will transfect Chinese hamster ovary cells (CHO) with a plasmid DNA (pEGFP-N_1) encoding green fluorescent protein (GFP) using classical and localized electroporation. On day two, we will determine gene electrotransfer efficiency and cell viability using fluorescence microscopy.

Protocol Day 1: Gene electrotransfer

Classical electroporation: CHO cells will be plated to 24-well plates 24 h before the experiment in concentration of 10^5 cells per well. Just before the experiment, culture medium will be removed and replaced with 150 µl of electroporation medium (10 mM phosphate buffer Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose; pH = 7.2) containing plasmid DNA with concentration 100 µg/ml. Prior to gene electrotransfer, cells will be incubated with plasmid DNA for 2 minutes at room temperature. Afterwards, the Pt-Ir wire electrodes with inter-electrode distance of $d = 2$ mm will be placed into the well. BetaTech Electro cell B10 generator will be used to deliver a train of eight rectangular pulses with duration of 5 ms, U = 140 V (U/d = 700 V/cm), and 1 Hz. Immediately after pulse exposure, 37.5 µl of fetal calf serum will be added to well. The pulse application will be repeated in the next well but with only 20 V (the same as in localized electroporation). After treatment, the cells will be incubated for 5 min at 37°C. Afterwards, 1 ml of culture medium will be added and cells will be incubated for 24 hours in a CO_2 incubator 37°C.

Localized electroporation: CHO cells will be plated into cell culture inserts, adapted for 24-well plates, and containing a PET substrate with pores with a diameter of 400 nm. Two days prior to gene electrotransfer, 0.8×10^4 cells will be plated in 300 µL of culture medium in each insert. The inserts will then be placed into a 24-well plate containing 700 µL of culture medium/well. On the day of experiment, the inserts will be moved to a 24-well Falcon plate with 500 µl of electroporation medium (LCIS) per well containing plasmid DNA at a final concentration of 100 µg/ml. To enable electrophoretic transfer of the negatively charged DNA across the substrate into the cells, the negative electrode should be placed below the substrate and the positive electrode above the substrate, as shown in Fig. 2. Following the experimental setup, a train of eight rectangular pulses with duration of 5 ms, 20 V, 1 Hz will be applied. After 1 min, the insert will be transferred to a new multiwell plate with fresh culture medium and incubated for 24 hours in a CO_2 incubator at 37°C. To test the role of electrophoresis, cells in another insert will be treated in the same way, but with switched polarity of the electrodes (negative electrode above and positive below the substrate).

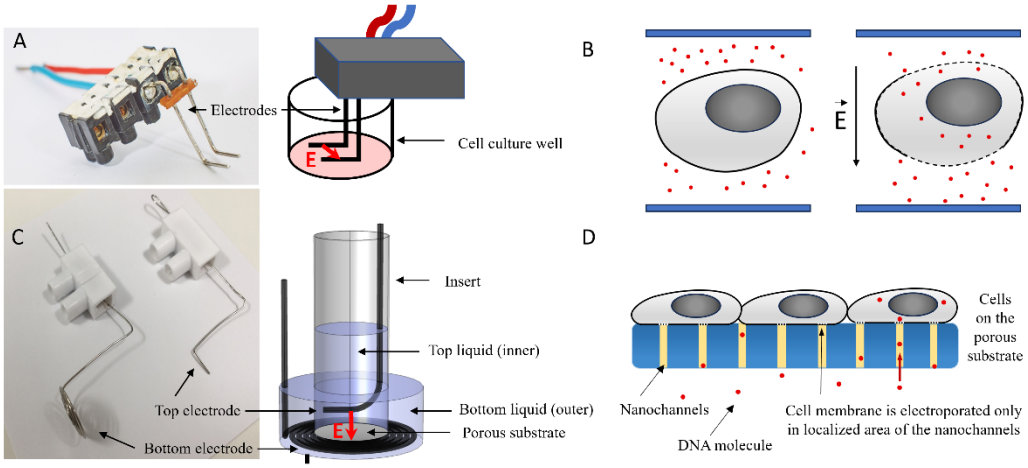

Figure 1. Gene electrotransfer using two methods. (A) Electrodes used for classic electroporation and their position in a well of a cell culture multiwell plate. (B) Bulk electroporation mechanism and pore formation in the presence of electric field. (C) Electrodes used for localized electroporation and their position in a well of a cell culture multiwell plate with porous substrates. (D) Mechanism of localized electroporation on the porous substrate.

Protocol Day 2: Determining cell viability and gene electrotransfer efficiency

After 24 h incubation you will determine the difference in cell viability and gene electrotransfer efficiency for both classical and localized electroporation by fluorescence microscopy (Leica Thunder, Wetzlar, Germany) at 20x magnification using GFP filter with excitation at 488 nm.

For classical gene electrotransfer protocol you will determine cell viability as the ratio between the number of viable cells in the treated sample and in the control sample. Gene electrotransfer efficiency will be determined from the ratio between the number of green fluorescent cells (successfully transfected) and the total number of viable cells counted under the brightfield microscopy. You will determine overall gene electrotransfer efficiency from the ratio between the number of green fluorescent cells in treated sample and the total number of viable cells in control sample. Number of green fluorescent cells will be compared between two presented methods – classical and localized gene electrotransfer.

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NOTES & RESULTS

NOTES & RESULTS

L2

Monitoring cell membrane electroporation with ratiometric fluorescent dye Fura-2AM

Gorazd Pucihar
University of Ljubljana, Faculty of Electrical Engineering

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.

The aim of this laboratory practice is to observe electroporation with fluorescent dye and to determine the effect of cell size, shape and orientation on the efficiency of electroporation.

THEORETICAL BACKGROUND
Exposure of a biological cell to a sufficiently strong external electric field leads to a detectable increase of membrane permeability, a phenomenon termed electropermeabilization. Because it is assumed that an increased permeability is related to the occurrence of hydrophilic pores in the membrane, the phenomenon is often termed electroporation. Provided that the parameters of the electric field (amplitude, duration, number of pulses, frequency) are moderate, the increased permeability is reversible, and cells recover within a few minutes after the exposure. During the state of high permeability the molecules for which the membrane is otherwise impermeable (e.g. drugs, DNA) can be transported across the membrane. Electroporation is nowadays used in biochemistry, molecular biology, and different fields of medicine. It has already become an established method in oncology for electrochemotherapy of tumours, and holds great promises in gene therapy.
The efficiency of electroporation is influenced by the parameters of the electric field, cell size and geometry, and physiological characteristics of the medium surrounding the cell. Different fluorescent dyes (e.g. Propidium Iodide, Lucifer Yellow, Fura-2, Fura-3,...) can be employed to investigate the influence of these parameters on electroporation and the same dyes can be used to monitor electroporation [1, 2, 3, 4].

EXPERIMENT
We will monitor cell membrane electroporation using a fluorescent calcium sensitive indicator Fura-2AM. Calcium ions, present in the extracellular medium, do not readily cross an intact (nonporated) cell membrane and the intracellular Ca^{2+} concentration is low. Once the membrane becomes permeable due to electroporation, Ca^{2+} ions enter the cells, where they bind to the dye and change its excitation and emission spectrum (Figure 1) [5].

This labwork is conducted by

Tina Batista Napotnik

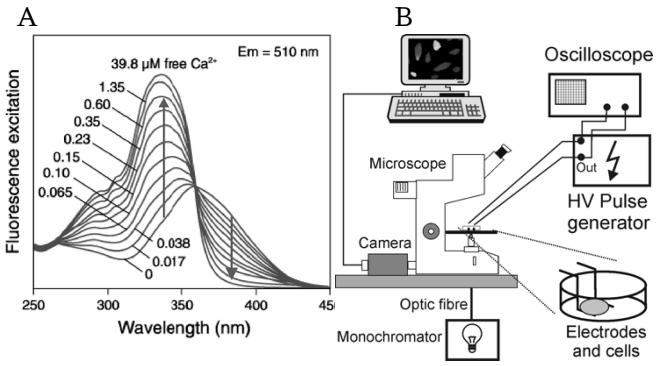

Figure 1: (A) Fluorescence excitation spectra of Fura-2 for different concentrations of Ca^{2+} (image from *http://probes.invitrogen.com/handbook/figures/0554.html*). (B) Schematic of the experiment.

Protocol: The experiments will be performed on H9c2 rat cardiac myoblast cell line grown in Lab-Tek chambers (TermoFisher Scientific) in Dulbecco's Modified Eagle Medium DMEM supplemented with 10% foetal bovine serum, L-glutamine (all three from Sigma-Aldrich) and antibiotics. Plate 8×10^4 cells on cover glass of Lab-Tek chamber and keep them for 24 hours in the incubator. Before experiments, replace the culture medium with fresh medium containing 2 µM Fura-2 AM (TermoFisher Scientific). After 25-30 minutes of incubation at 37°C wash the excess dye and leave 1.5 ml of culture medium in the chamber.
Place the chamber under a fluorescence microscope (Thunder Imager Live Cell system for fluorescence microscopy, Leica Microsystems) and use ×63 objective. Insert two parallel Pt/Ir wire electrodes with a 5 mm distance between them to the bottom of the chamber. Acquire a pair of two images (with 340, 380 nm excitation wavelength) each second for one minute in time-lapse acquisition mode, using sCMOS Leica fast camera and LASX software. Apply the electric pulse at the 5th s of recording: using a BetaTech device, deliver one electric pulse of 100 µs with voltages varying from 150 to 300 V. In LASX software, observe how the ratio image ($R = F_{340}/F_{380}$) changes in time in each cell [3, 4].

Wait for 5 minutes (resealing and recovery) and apply pulse with a higher amplitude. After each pulse, determine which cells are being electroporated (Figure 2). Observe, which cells become electroporated at lower and which at higher pulse amplitudes.

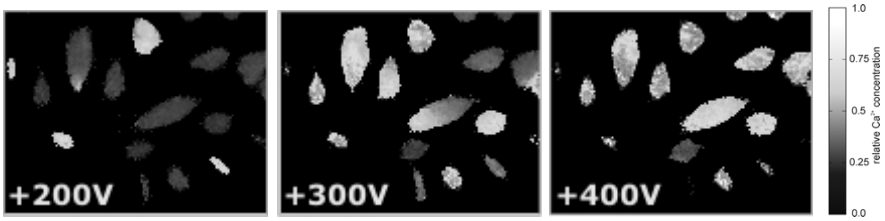

Figure 2: Cells stained with Fura-2AM and exposed to electric pulse with increasing amplitude.

REFERENCES:

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NOTES & RESULTS

NOTES & RESULTS

L3

Visualization of local ablation zone distribution between two needle electrodes

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 2 or 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.

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

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 [1, 2]. 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) [3]. IRE is an emerging ablation technique inducing cell death in successfull 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 [4, 5]. 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 [6, 7]. Trypan blue can be used as an indicator of plasma membrane integrity and cell viability. Trypan blue is normally impermeant to viable 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.

This labwork is conducted by

Alenka Maček Lebar

Jernej Jurič

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.

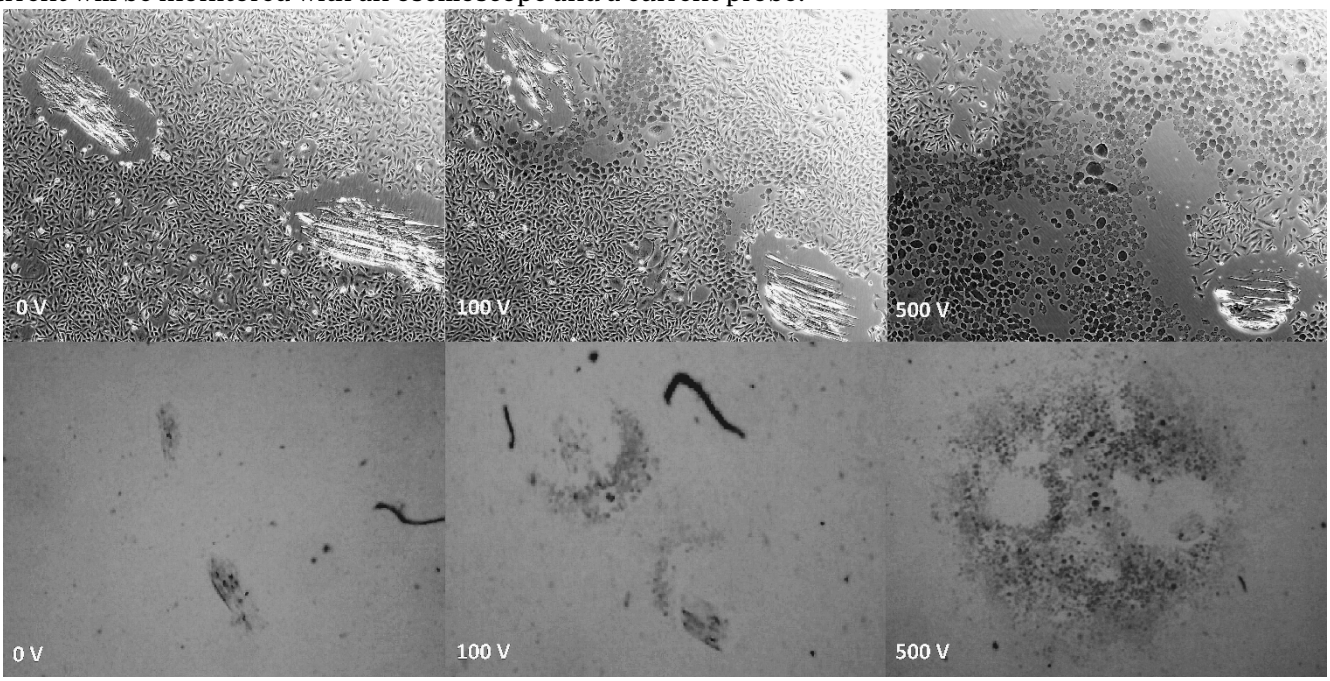

Figure 1: The sequence of the images of local ablation zone after cells were exposed to electric pulses with increasing pulse amplitude. The images were obtained by light microscopy under 10 × objective magnifications (top row) and under 5 × objective magnifications (bottom row).

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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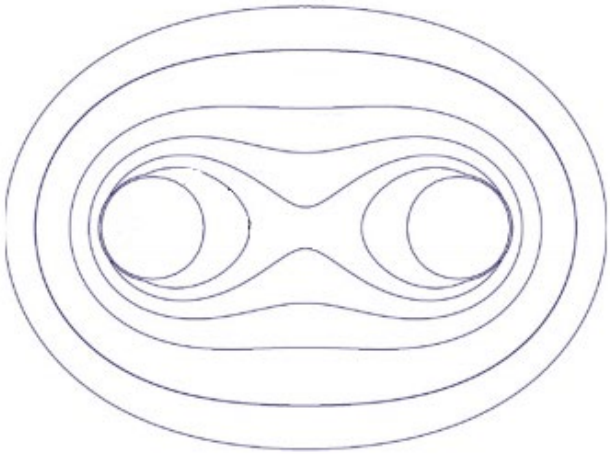
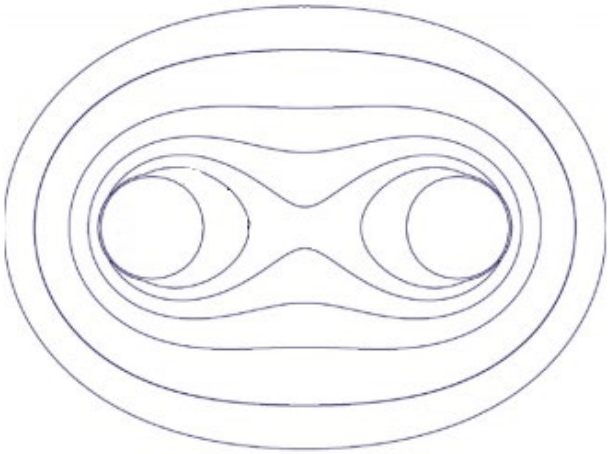
NOTES & RESULTS

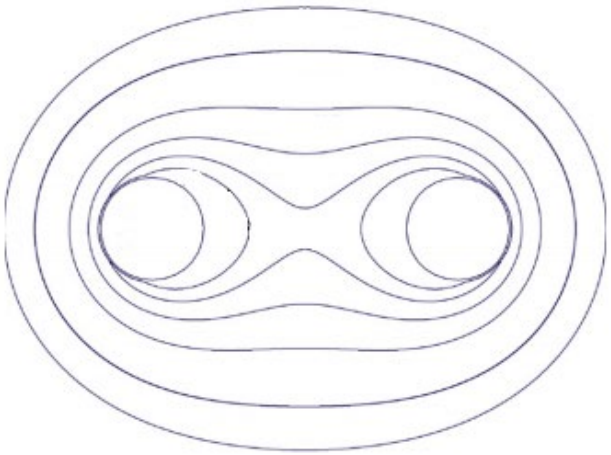
NOTES & RESULTS



100 V



500 V



700 V

L4
Comparison of flow cytometry and spectrofluorometric measurements in cell permeabilization experiments

Janja Dermol-Černe, Lea Vukanović
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 90 min
Max. number of participants: 4
Location: Cell Culture Laboratory 2
Level: advanced

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

The aim of this laboratory practice is the comparison of two different methods of permeabilization detection using fluorescent dye propidium iodide.

THEORETICAL BACKGROUND
When cells are exposed to high electric fields, non-permeant molecules can cross the cell membrane. A commonly used way of detecting cell membrane permeabilization is by using fluorescent dyes such as propidium iodide [1]. When the cell is permeabilized, the propidium ion enters the cell, binds to nucleic acids in the cytosol and nucleus, and upon excitation starts to emit 20-times higher fluorescence than in the unbound state. Cell membrane permeabilization can be determined using different methods, e.g. fluorescent microscopy, spectrofluorometric measurements, flow cytometry, or clonogenic test after electroporation with chemotherapeutics [2-4]. In this lab work, we will compare spectrofluorometric measurements and flow cytometry.
Spectrofluorometric measurements allow for the analysis of a large number of cells at different wavelengths, but the exact number of permeabilized cells cannot be extracted. Namely, as a result, we obtain the sum of the fluorescence intensities of all cells which can conceal subpopulations of differently permeabilized or even non-permeabilized cells [2].
Flow cytometry, on the other hand, gives information on the shape, size, internal structure, and fluorescence of each separate cell, and thus offers possibility to detect subpopulations which differ in any of the measured parameters. Cells (or any other particle) move through a laser beam and refract or scatter light in all directions. Forward scatter (FSC) is the light that is scattered in the forward direction as laser strikes the cell while side scatter (SSC) is the light that is scattered at larger angles. The magnitude of FSC is roughly proportional to the size of the particle and SSC is indicative of the granularity and the internal structural complexity. Fluorescence can be measured at different wavelengths, and the measured signal is proportional to the amount of the emitted fluorescence. After measurements, the analysis is done by gating to separate different cell subpopulations (Figure 1) [2-4].

This labwork is conducted by

Zala Vidic

Vid Jan

EXPERIMENT

We compare the fluorescence detected by flow cytometry (Life Technologies, Attune NxT, USA) and by the spectrofluorometer (Tecan Infinite 200, Tecan, Austria) after one of the two electroporation protocols. First protocol will consist of 50 bipolar pulses consisting of 1 µs long positive and negative pulses with 1 µs delay between them, delivered in 8 bursts at repetition frequency of 1 Hz. The second protocol will consist of monopolar pulses of parameters traditionally used in electrochemotherapy (8×100 µs pulses, 1 Hz repetition frequency) [5]. In both protocols the total duration of pulses will equal 800 µs. To apply the pulses, we use the laboratory prototype pulse HF-IRE pulse generator (University of Ljubljana) based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA), and we monitor the pulses by an oscilloscope and current probe (both LeCroy, USA).

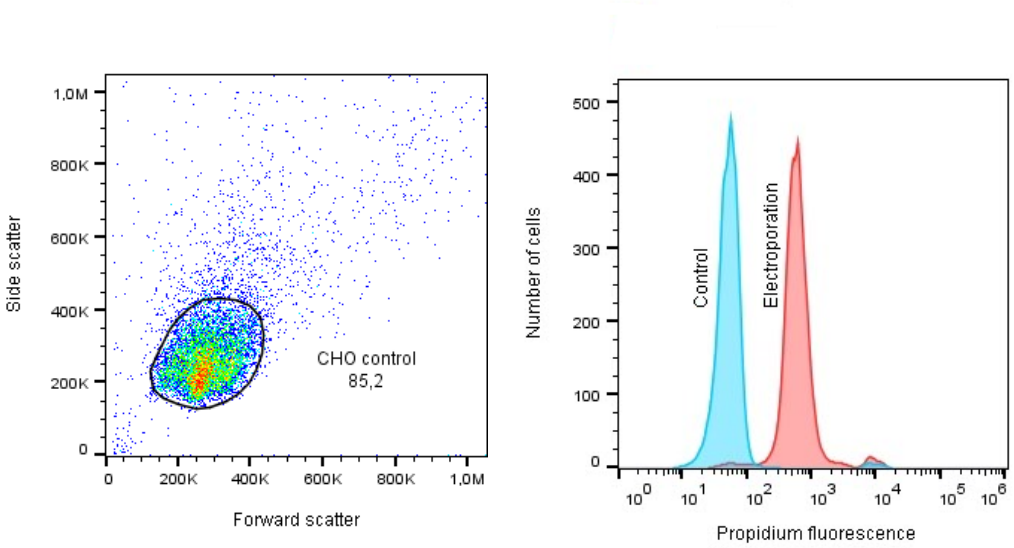


Figure 1: The analysis of the permeabilization data obtained by flow cytometry measurements in the software FlowJo (TreeStar, USA). Left: the viable cells are determined from the FSC-A and SSC-A dot diagram by gating. Right: histogram of measured fluorescence for control and pulsed cells. After electroporation, the cell fluorescence shifts for two decades which allows the discrimination between permeabilized and non-permeabilized cells. The peak at 104 are the dead and/or irreversibly permeabilized cells.

Protocol:

The experiments are performed on Chinese Hamster Ovary (CHO) cells. First, cells are detached by 10x trypsin-EDTA (Sigma-Aldrich, Germany), diluted 1:9 in Hank's basal salt solution (Sigma-Aldrich, Germany). Then, they are centrifuged (180g, 21°C, 5 min), the supernatant is removed and replaced with the low-conductivity KPB buffer (10 mM KH2PO4/K2HPO4, 1 mM MgCl2, 250 mM sucrose) in concentration 107 cells/ml. 100 µl of cell suspension is dispensed in 1.5 ml microcentrifuge tubes (Isolab, Germany). Immediately before pulse application, 10 µl of 1.5 mM propidium iodide (Life Technologies, USA) is added to the tube. Then, 100 µl of cell suspension with propidium iodide is pipetted between 2 mm stainless-steel electrodes. For the application of bipolar and monopolar pulses, we will use a laboratory prototype pulse. We will monitor the delivered voltage and current by an oscilloscope, a differential probe and a current probe. After the pulse application, 80 µl of cell suspension is transferred from between the electrodes to a new 1.5 ml tube. Two minutes after pulse application, the sample is centrifuged (1 min, 2000g, room temperature), the supernatant is removed and replaced by 500 µl of KPB buffer. The change of the buffer stops propidium influx in the cells and allows us to compare different parameters at the same time point. From each tube, 100 µl of the cell suspension is transferred to a 96-well plate in triplicates. 100 µl of the cell suspension is transferred to a 1.5 ml tube. Then, the protocol is repeated for the next sample. When all samples are prepared, we start with the measurements.

First, the fluorescence intensity is determined spectrofluorimetrically. We set the appropriate excitation (535 nm) and emission (617 nm) wavelengths. We measure at an optimal gain which prevents from signal saturation. The optimal gain is automatically determined by the software based on sensor sensitivity and the maximum signal intensity we are measuring. The average fluorescence intensity is calculated for each voltage from the triplicates. We plot the fluorescence intensity in dependence on the applied voltage. Second, we determine the number of fluorescent cells by flow cytometry. On the control cells, we set up the optimal measuring parameters at the lowest flow rate (12.5 µl/min). When optimal parameters are determined, we measure 10,000 events for each voltage with higher flow rate (200 µl/min). By gating, living cells and the percentage of permeabilized cells are determined for each voltage. We plot the cell permeabilization in dependence on the applied voltage for both measurements (spectrofluorometric and flow cytometry) and compare the results.

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NOTES & RESULTS

Bipolar/Monopolar	Bipolar	Bipolar	Bipolar	Bipolar	Bipolar	Bipolar (C)	Monopolar
Voltage (V)							
Fluorescence intensity as measured with spectrofluorometer (a.u.)							
Percentage of fluorescent cells as determined by the flow cytometry (%)							

(C) – combination of negative control and highest voltage

NOTES & RESULTS

L5

Triggering action potential and electroporation in excitable cells exposed to electric pulses

Tina Batista Napotnik, Lea Rems
University of Ljubljana, Faculty of Electrical Engineering, Slovenia

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). No other specific knowledge is required for this laboratory practice.

The aim of this laboratory practice is to observe triggering action potentials and electroporation in genetically engineered tet-on spiking human embryonic kidney (S-HEK) cells with the use of 100 µs electric pulses.

THEORETICAL BACKGROUND
Electric pulses have been used for triggering action potentials in excitable cells (electrostimulation) already for decades. However, when using higher electric fields, the cells' plasma membrane becomes permeabilized and additional ionic currents through pores/defects occurs. These additional ionic currents affect cell excitability in a complex interplay between excitation and electroporation [1, 2].
To better understand the underlying mechanisms of electrostimulation and electroporation in excitable cells (nerves, muscles, cardiac), *in vitro* experimental work is of great importance. Genetically engineered S-HEK cells expressing a minimal complement of sodium and potassium channels ($Na_V1.5$ and $K_{ir}2.1$) needed for excitability are a simple and convenient excitable cell model for studying excitation and electroporation *in vitro* [3, 4].
The use of a fluorescent potentiometric dye ElectroFluor630 and fluorescence microscopy is an effective way to study responses in transmembrane voltage to electric pulses in excitable cells (action potentials and electroporation). Compared to classical electrophysiological methods such as patch clamp, these optical measurements are much easier and time efficient, as they do not require special technical skills. Also, high-voltage electric pulses does not interfere with the measurements (i.e. image acquisition).

EXPERIMENT
We will monitor the changes in transmembrane voltage in excitable S-HEK cells (ATCC CRL-3479) using the fluorescent potentiometric dye ElectroFluor630 (Potentiometric Probes) under a fluorescence microscope and observe the triggering of action potentials and electroporation with 100 µs electric pulses of increasing amplitudes.

This labwork is conducted by

Tina Batista Napotnik

Tina Turk

Protocol: S-HEK cells will be plated to Lab-Tek chambers (Thermo Fisher Scientific) 3 days before the experiment in concentration of 105 cells per well. To prepare the cells for experiment, label the cells with 12 µM ElectroFluor630 in DMEM culture medium for 20 min in a refrigerator at 4°C. Wash the cells three times with a Tyrode solution (2 mM KCl, 125 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 30 mM glucose, pH 7.3) and at the end, add 1.2 ml of low potassium Tyrode solution (0.5mM KCl, 126.5 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 30 mM glucose, pH 7.3) to cells.

Insert two parallel Pt/Ir wire electrodes with a 5 mm distance between them to the bottom of the Lab-Tek chamber. Place the chamber under a fluorescence microscope (Thunder Imager Live Cell system for fluorescence microscopy, Leica Microsystems) and connect the electrodes to the pulse generator. Acquire a set of fluorescence images (635 nm excitation and 700 emission wavelength, ×40 objective) in time-lapse acquisition mode, using sCMOS Leica fast camera and LAS X software: acquire 80 images, one image every 36 ms (around 2.8 s total duration of image acquisition). The first image acquisition represents control without pulse application. Further on, while recording, apply a single electric pulse of 100 µs and 63 V using a TTL signal from the microscope system that triggers the pulse generator and observe the fluorescence signal from the whole field of view.

Every two minutes, record a time-lapse in the same way as before but apply electric pulses of increasing voltage: 63, 75, 88, 100, 125, 150, 175, and 200 V. The electric field to which the cells are exposed is estimated as the applied voltage-to-electrode-distance ratio (E ≈ 126, 150, 176, 200, 250, 300, 350, and 400 V/cm, respectively). At lower electric fields, the pulses will trigger action potentials, at higher electric fields, the pulses will cause electroporation which manifests as a prolonged depolarization.

The fluorescence signal can be further analyzed using a Matlab application: the acquired images are thresholded to extract the signal only from the membranes, the fluorescence signal is corrected for fading and the characteristic parameters of the signal are extracted (number of action potentials, amplitude, recovery to the baseline etc.), as described more in detail in [1].

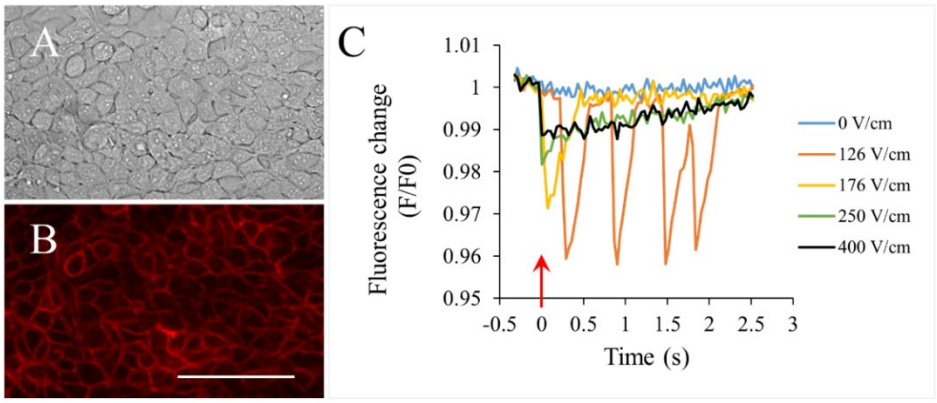

Figure 1. Monitoring triggering excitation and electroporation with 100 µs electric pulses optically with the use of the fluorescent potentiometric probe ElectroFluor630 in excitable S-HEK cells. A brightfield (A) and fluorescence image of S-HEK cells. (C) Time course of ElectroFluor630 fluorescence change in S-HEK cells after applying 100 µs electric pulses of different amplitudes (signal from the whole field of view, thresholded and corrected for fading).

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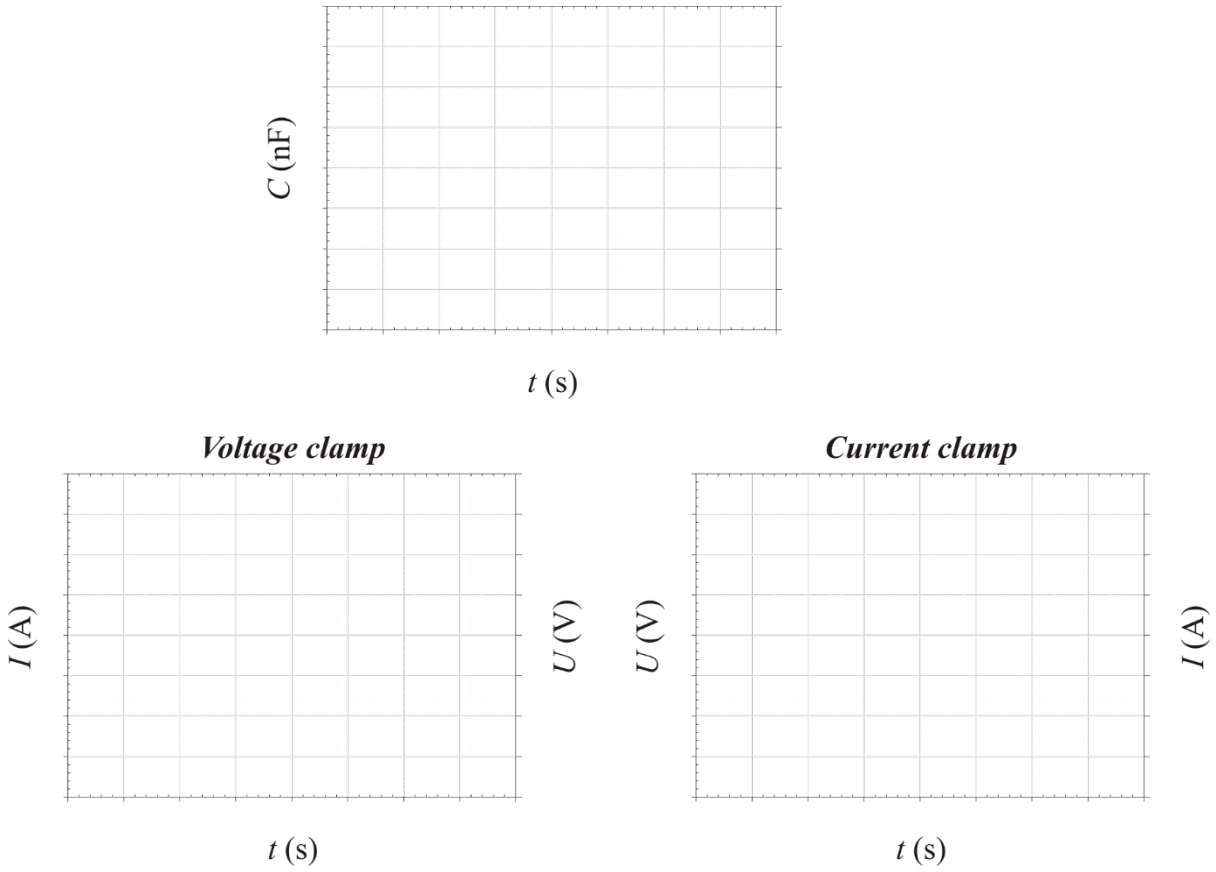
NOTES & RESULTS

NOTES & RESULTS



L6

Eradication of antibiotic-resistant *E. coli* by the combination of antibiotics and electroporation in a continuous mode

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 2
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.

The aim of this laboratory practice is to inactivate antibiotic-resistant bacteria by combining antibiotic and electroporation in a flow-through system.

THEORETICAL BACKGROUND
Antibiotics have long been the mainstay in treatment of bacterial infections, but their overuse and misuse combined with inadequate infection prevention has led to increasing bacterial resistance [1]. Therefore, the development of new approaches for efficient inactivation of drug-resistant bacteria is critical. Electroporation, a process in which electric fields are applied to bacterial cells, has shown promise as an adjunct to conventional antibiotic treatment [2-5].
When a cell is exposed to a sufficiently high electric field, its membrane becomes permanently permeable, leading to the leakage of cellular components and thus to cell death [6]. Electroporation has already been shown to enhance the effect of antibiotics, and various bacteria have been successfully inactivated by combining electroporation and antibiotics [2-5]. In order to enable electroporation on a large scale, the development of flow-through processes has been proposed [7]. Thus, a flow-through treatment system consists of a pulse generator that provides continuous pulse treatment, flow-through chambers with electrodes, and a fluid handling system.
Understanding how antibiotics and electroporation inactivate bacteria is critical not only for developing effective strategies to treat bacteria, but also for preventing antibiotic resistance.

EXPERIMENT
We will inactivate *Escherichia coli* K12 ER2420 cells carrying plasmid pACYC184 encoding tetracycline resistance (New England BioLabs Inc., Ipswich, Massachusetts, USA) in a continuous flow system (see Figure 1) using various tetracycline concentrations and electrical pulse parameters. A prototype square wave pulse generator will be used to generate electrical pulses. The pulses will be monitored using an oscilloscope (LeCroy 9310C). The degree of inactivation will be determined using the plate counting method.

This labwork is conducted by

Saša Haberl Meglič

Karel Flisar

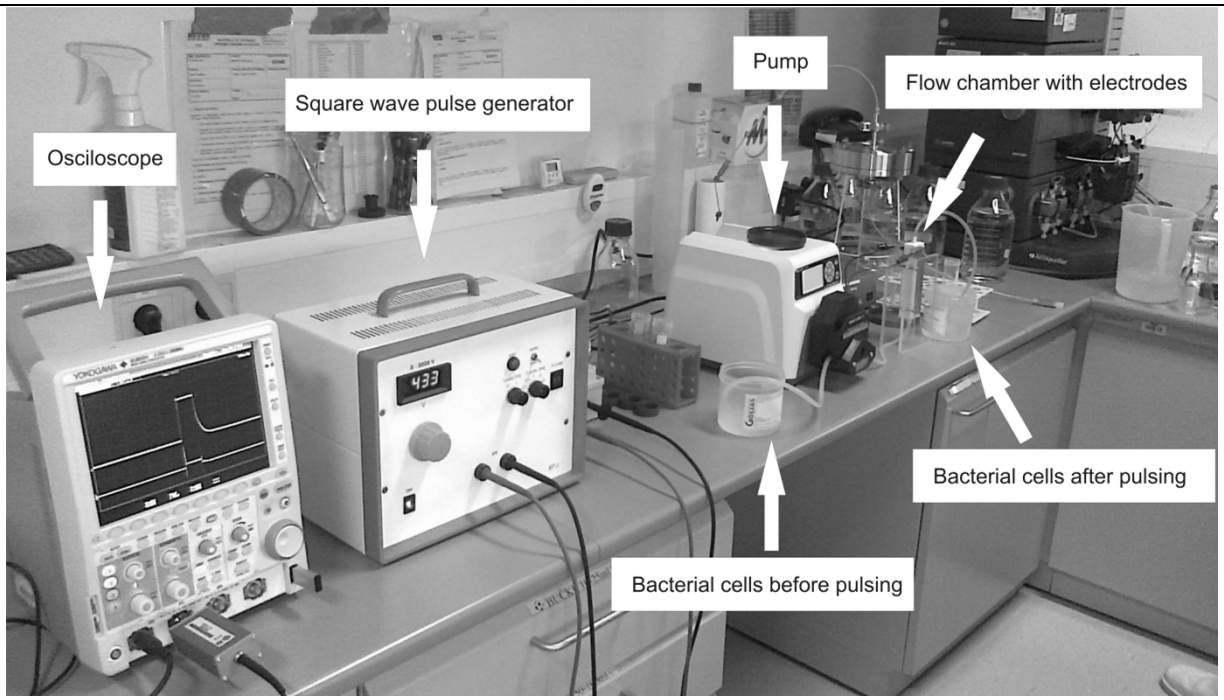


Figure 1. Continuous flow electroporation system. The circuit system includes a flow chamber with electrodes and a prototype square wave pulse generator. Voltage and current are both monitored throughout the experiment.

Protocol 1/2 (Electroporation of bacteria in a continuous flow system): On the first day of the experiment, bacterial cells will be grown for 3-4 hours (until early exponential phase) at 37°C in Luria Broth (LB) medium (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) with shaking. *E. coli* cells will be then centrifuged (4248 g, 30 min, 4°C) and the pellet will be resuspended in 250 mM sucrose.

The exposure of cells to electrical pulses in a flow chamber in a continuous flow system depends on the geometry of the chamber and the frequency of the pulses at which the electroporator operates. The number of pulses is determined by equation 1. At this flow rate, the desired number of pulses is applied to the fluid and thus to the cells in the flow chamber. Since the volume of our cross-field chamber between the electrodes and the frequency 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) is the volume between the two electrodes, and n is the number of pulses received by the fluid in the chamber during the dwell time. For a frequency of 10 Hz, calculate the flow rate (q) at which all the fluid is exposed to at least one pulse. The bacterial cells will be pumped through the system at the calculated flow rate, and pulses will be applied by the prototype pulse generator.

After electroporation treatment, take a small volume of the treated sample and add 40 or 150 µg/ml of tetracycline. Collect 20 µl of the treated sample and prepare dilutions ranging from 10^{-1} to 10^{-6}. Pipette three 10-µl drops of the different dilutions onto LB agar.
To determine the number of bacterial cells in our sample, make serial dilutions of the (untreated) bacterial sample ranging from 10^{-1} to 10^{-7} (dilute 20 µl of the untreated bacterial sample in 180 µl of 0.9% NaCl). Pipette three 10-µl drops of dilutions 10^{-5}, 10^{-6} and 10^{-7} onto LB agar.

Protocol 2/2 (Determining bacterial viability): After 24 hours of incubation at 37°C, count the colony forming units. Viability is expressed as log (N/N_0), where N is the number of colony forming units per ml in a treated sample and N_0 is the number of colony forming units per ml in an untreated sample.

Example of determining bacterial viability:
You counted 20 CFU in a control sample (dilution 10^{-7}) and 10 CFU in a treated sample (dilution 10^{-5}).

Number of bacterial cells per ml (control sample) = 20 x 10^{7} (dilution factor of sample) x 100 (dilution factor of plating) = 2 x 10^{10} bacterial cells/ml

Number of bacterial cells per ml (treated sample) = 10 x 10^{5} (dilution factor of sample) x 100 (dilution factor of plating) = 1 x 10^{8} bacterial cells/ml

log N/N_0 = log (1 x 10^{8} / 2 x 10^{10}) = ***-2.301***

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NOTES & RESULTS

NOTES & RESULTS

L7

Analysis of electric field orientations on gene electrotransfer – visualization at the membrane level

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

Duration of the experiment: 90 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.

The aim of this laboratory practice is to demonstrate how different pulse polarity affects formation of DNA – membrane complex after electric pulse application.

THEORETICAL BACKGROUND
Gene electrotransfer is a non-viral method used to transfer genes into living cells by means of high-voltage electric pulses [1]. 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 [2-4].
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 [5, 6]. 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.

This labwork is conducted by


Tamara Polajžer

Zala Vidic



Klara Bulc Rozman

EXPERIMENT

We will focus on the interaction of DNA with the cell membrane by using TOTO-1 dye. 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 βtech electroporator (Electro cell B10, Betatech, France) and electrodes with 4 mm inter-electrode distance 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) BP (both polarities): the direction of the electric field is changed between the pulses

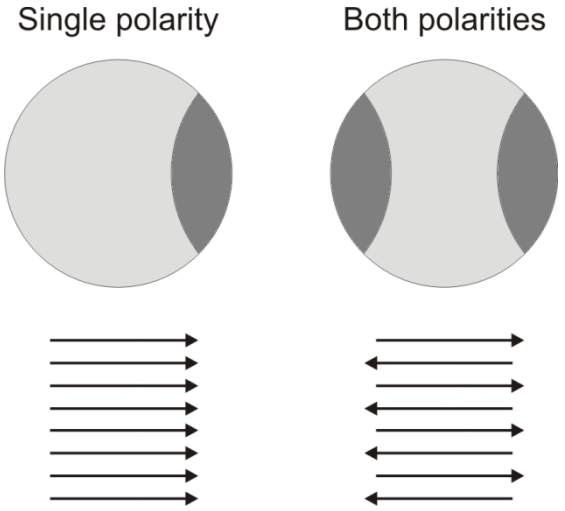

Figure 1: Two different pulse protocols will be used: single polarity (SP) and both polarities (BP).

Protocol:

Interaction of DNA with the cell membrane: CHO cells will be grown in Lab-Tek chambers 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 37° C. Cells will be plated 1 h before the experiment in concentration 1 x 10^5 cells per chamber.

To visualize DNA interaction with cell membrane TOTO-1 nucleic acid stain (Molecular Probes-Invitrogen, Carlsbad, California, USA) will be used. The plasmid pEGFP-N1 will be labelled on ice with 2.3 x 10^{-4} M TOTO-1 DNA intercalating dye 1 h before the experiment. Plasmid concentration will be 1 µg/µl, which yields an average base pair to dye ratio of 5.

Just before the experiment remove culture medium and rinse the cells with 1 ml of electroporation buffer (10 mM phosphate buffer K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose; pH = 7.4). Afterwards add 500 µl of electroporation buffer containing 5 µg of labelled plasmid DNA. Then apply a train of eight pulses with amplitude of 350 V, duration of 1 ms and repetition frequency 1 Hz using single polarity or both polarities (see Pulse protocols).

Immediately after exposure of cells to electric pulses rinse the cells three times with 1 ml of electroporation buffer. Add again 500 µl of electroporation buffer and observe the interaction of DNA with the cell membrane with fluorescent microscopy (Zeiss 200, Axiovert, Germany) using 100x oil immersion objective using TOTO filter with excitation at 514 nm.

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NOTES & RESULTS

NOTES & RESULTS

L8

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

Gorazd Pucihar
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 60 min
Max. number of participants: unlimited
Location: Online ONLY
Level: Basic

PREREQUISITES
No specific knowledge is required for this laboratory practice.

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.

THEORETICAL BACKGROUND
When a biological cell is placed into an external electric field an 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 [1]. 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 and the sensitivity of the detection method [2,3]. 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 strength, and is influenced by cell geometry and physiological characteristics of the medium surrounding the cell [4]. 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 [5].

EXPERIMENT
Potentiometric fluorescent dyes allow observing the variations of the ITV on the membrane and measuring its value [1,2,6,7]. 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 [8]. 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.

This labwork is conducted by

Anja Blažič

Protocol: The experiments follow the protocol in [9] and are performed on Chinese hamster ovary cells (CHO-K1) 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 pulse generator. 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 during a pulse application, 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 ($\Delta F/F$ = –6% / 100 mV), and plot them on a graph as a function of the arc length (Figure 1D).

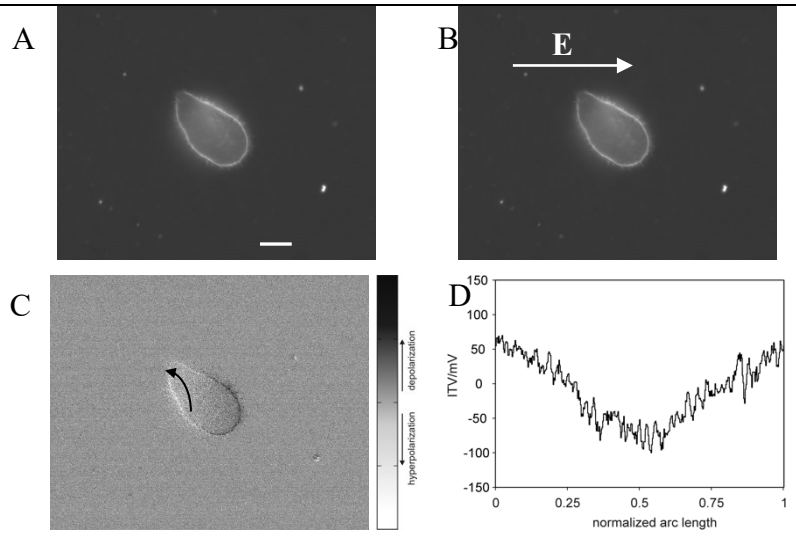

Figure 1: Measurements of the induced transmembrane voltage (ITV) on an irregularly shaped CHO-K1 cell. (A) A control fluorescence image of a cell stained with di-8-ANEPPS. Bar represents 10 μm. (B) Fluorescence image acquired during the exposure to a 35 V (~88 V/cm), 50 ms rectangular pulse. (C) Changes 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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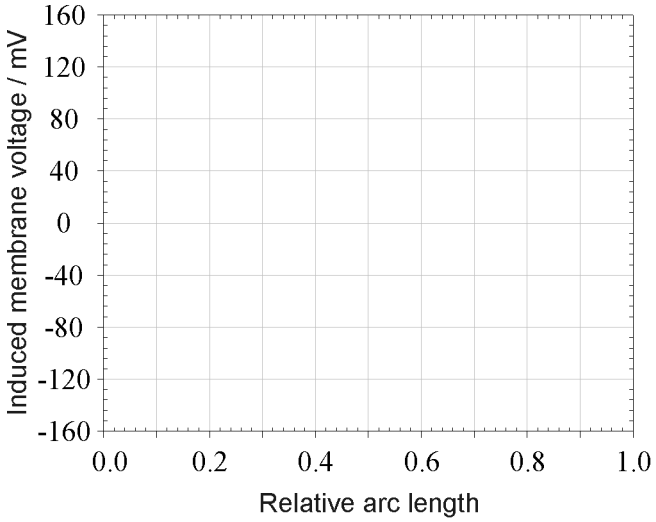
NOTES & RESULTS



L9

Analysis of electric field orientations on gene electrotransfer efficiency

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

Duration of the experiment: 60 min
Max. number of participants: Unlimited
Location: Online ONLY
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.

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

THEORETICAL BACKGROUND
Gene electrotransfer is a non-viral method used to transfer genes into living cells by means of high-voltage electric pulses [1]. 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 [2-4].
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 [5-6]. 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.

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).

This labwork is conducted by

Tamara Polajžer

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

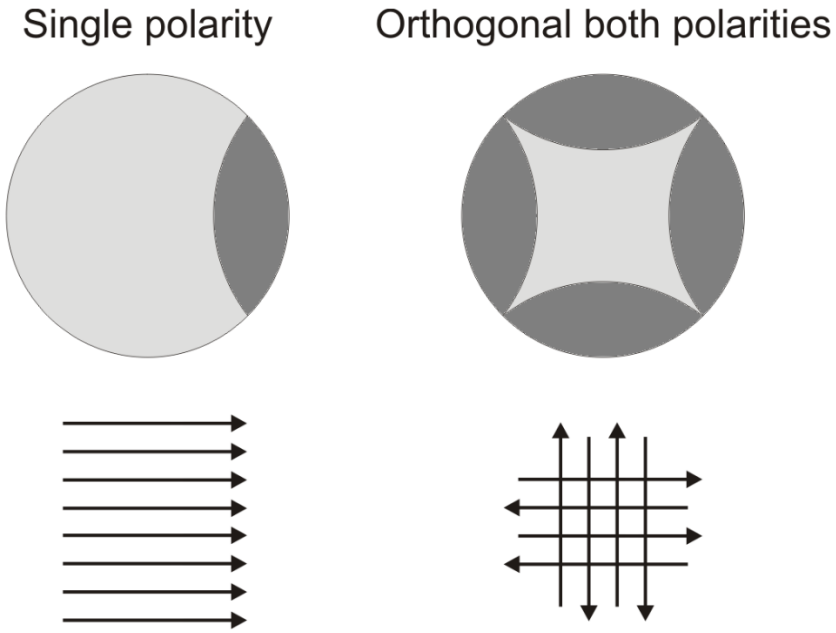


Figure 1: Two different pulse protocols will be used: single polarity (SP) and orthogonal both polarities (OBP)

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 37° 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 37° C and then add 1 ml of culture medium.
Protocol 2/2 (Determining gene electrotransfer efficiency and cell viability): After 24 h incubation at 37° 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.

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NOTES & RESULTS

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

NOTES & RESULTS

L10

Monitoring cell membrane depolarization due to electroporation using fluorescent plasma membrane potential indicator

Anja Blažič, Lea Rems
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 90 min
Max. number of participants: 4
Location: Cell Culture Laboratory 3
Level: Advanced

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

The aim of this laboratory practice is to monitor the time course of transmembrane voltage changes after exposure to electroporation pulses, and to determine the influence of cell-type dependent response, based on the ion channel expression profile.

THEORTICAL BACKGROUND
All cells maintain an electric potential difference across their plasma membranes. This potential difference is called the resting transmembrane voltage (or resting potential) and is maintained by a system of ion channels and pumps. By convention the resting transmembrane voltage is negative, meaning that the cell interior is electrically more negative compared to its exterior [1]. The value of the resting transmembrane voltage changes dynamically with the cell cycle and has an important biological function by controlling the activity of various membrane proteins [2]. When cells are electroporated, their transmembrane voltage changes and the membrane remains depolarized for several minutes after pulse exposure [3-5]. As membrane depolarization acts as a biological signal [2], factors that influence prolonged depolarization upon electroporation can have an important influence on the biological outcome of electroporation.

EXPERIMENT
We will monitor the time course of transmembrane voltage changes using the FLIPR Membrane Potential (FMP) dye [6]. The FMP dye consists of two parts: a fluorescent anionic voltage-sensor dye and a quencher molecule. When the interior of the cell has a relatively negative charge (is negative with respect to its surroundings) the voltage sensor remains on the extracellular side, where the quencher absorbs its fluorescence and consequently minimizes its signal. During membrane depolarization the voltage sensor translocates with positive ions to the intracellular side, which increases the fluorescence intensity inside the cell.

This labwork is conducted by

Anja Blažič

This translocation is reversible, meaning that the voltage sensor can return to the outside of the cell, which makes it possible to monitor membrane depolarization and repolarization as the cell recovers and returns to its resting transmembrane voltage. The fluorescence intensity is linearly proportional to the change in the transmembrane voltage, making this method comparable to patch-clamp measurements upon dye calibration.

Protocol: U-87 MG human glioblastoma cells and Chinese hamster ovary (CHO-K1) cells will be plated in Nunc Lab-Tek II chambered coverglass three to four days before the experiment. On the day of the experiment, we will stain the cells for 30 min at 37°C with the Component A of the FLIPR Membrane Potential Assay Red (Molecular Devices, #R7291), diluted in Live Cell Imaging Solution (Invitrogen, #A14291DJ). Afterwards, the cells will be placed on the microscope stage. Time lapse images of the cells will be acquired before and after the exposure to a single 100 ms pulse of selected amplitude, delivered by a pulse generator B10 HV-LV (Leroy Biotech, France) through a pair of Pt-Ir wire electrodes. We will compare the response of the two cell lines at 37°C, to observe how transmembrane voltage recovery can be influenced by different expression of ion channels (low expression in CHO-K1 cells and high expression in U-87 MG cells). Inverted microscope Leica DMi8 with LED8 illumination source controlled by the LasX software (all from Leica Microsystems) will be used to image cells. The dye will be excited with green LED (555/28 nm), its florescence will pass through DFT51010 filter, and changes in transmembrane voltage will be detected with the Leica DFC9000 Gt camera.

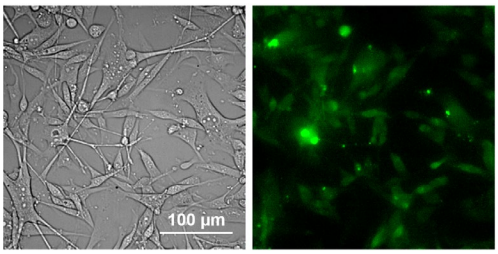

Figure 1: Brightfield image (*left*) and fluorescence (*right*) of U-87 MG cells stained with FMP dye.

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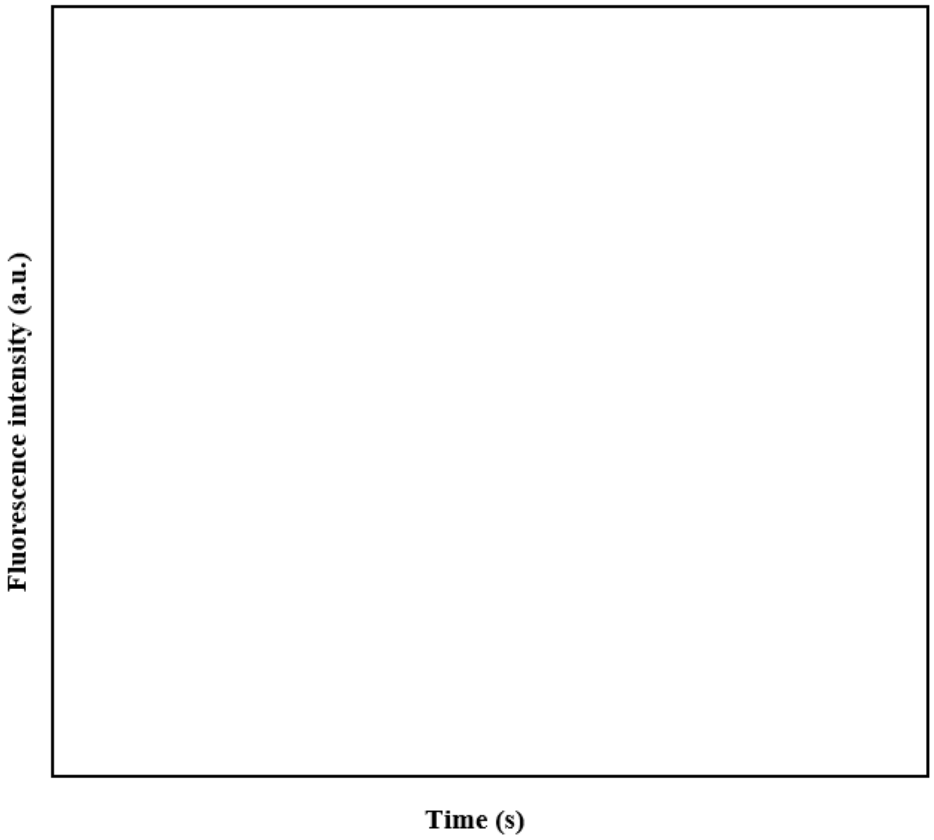
NOTES & RESULS



NOTES & RESULS

L11

Impedance and texture analysis techniques for detecting and characterising electroporation in plant tissues

Samo Mahnič-Kalamiza, Rok Šmerc
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 120 min
Max. number of participants: 4
Location: Tissue Laboratory
Level: Basic

PREREQUISITES
Participants should be familiar with Laboratory safety (S1). No prior knowledge of laboratory work is required. Basic skills of handling electronic instruments such as an oscilloscope and impedance analyser are an advantage, but not prerequisite.

The aim of this laboratory practice is to detect (and quantify) electroporation effects in plant tissues of disparate origin, structure, and water content & solute composition [1], by employing electrical impedance measurements and texture analysis (i.e., tissue's response to mechanic forces). Students will learn of the importance of plant tissue composition and structure, and how these properties impact detection and quantification of electroporation effects in fresh plant matrices.

THEORETICAL BACKGROUND
The application of PEF treatment in food processing is gaining momentum and seeing intensive research and development. New electroporation-based treatments are continuously put to the test and are optimized both at the laboratory and industrial scale processes. PEF treatment offers increasing benefits in terms of low energy use and minimization of food quality deterioration. For successful treatment, an appropriate choice of methods assessing changes due to electroporation occurring in biological matrices of alimentary interest is, however, crucial. Despite a considerable body of literature in the field, detailed information regarding the detection and quantification of the effects of electroporation in complex and highly inhomogeneous multicellular systems, such as real food systems (e.g., plant tissues), is still limited. Moreover, due to the unique characteristics and properties of the biological tissue processed, a case-by-case PEF treatment optimization protocol is often required.
In food-related PEF applications, measurements of the dielectric properties of the tissue are often used to determine the degree of cell membrane disruption by electroporation [2,3]. Electrical impedance spectroscopy (EIS) has been suggested as a reliable method to estimate the extent of tissue damage due to PEF treatment. EIS relies on the theory that, from an electrical point of view, an individual cell can be represented as an insulating membrane exhibiting relatively high resistance to electric current and considerable capacitance, and intra- and extra-cellular media (electrolytes) that behave as a resistive (ohmic) load up to hundreds of MHz.

This labwork is conducted by

Samo Mahnič-Kalamiza

Rok Šmerc

As electroporation affects the permeability (i.e., conductivity) of the cell membrane, multifrequency impedance measurements can be used to assess the degree of membrane permeabilization due to PEF treatment [4,5].

Another way of assessing changes in electroporated plant tissues is offered by texture analysis (texture in the sense of the response of a material to mechanical forces). Plant tissues in structures such as roots, fruits, and tubers, often exhibit considerable turgidity (high turgor pressure) when fresh and hydrated. Increasing membrane permeability of the cells by electroporation can result in release of the intracellular water that is filtered through the extracellular matrix [6]. From the analysis of tissue's response to external force at the exact moment of electroporation and within minutes after, it is possible to evaluate the extent to which the electroporated plant tissue has been affected by the treatment. Texture analysis thus offers an alternative method to evaluating the degree of cell membrane disruption in treatment protocol optimisation where impedance measurements are either unavailable or not practical.

EXPERIMENT

We will perform concurrent sample deformation analysis (at constant loading force) and impedance measurements (pre- and post-pulse delivery) on two plant tissues: an apple fruit sample, and a potato tuber sample. You will familiarize yourself with the principles of the two methods of analysis by experimenting on potato tuber and apple fruit sample, both at a given voltage, after which we will move to a computer and analyse the data that you obtained along with previously gathered data obtained in a voltage escalation study using both types of tissue. You will work with deformation and impedance data recorded for various treatment efficacies (correlating with the extent of changes in tissue caused by electroporation). Together with your data point you will have a total of five different voltages (and thus five different voltage-to-distance ratios) and the control (0 V) to work with. Altogether, you will analyse twelve sets of impedance and texture measurements.

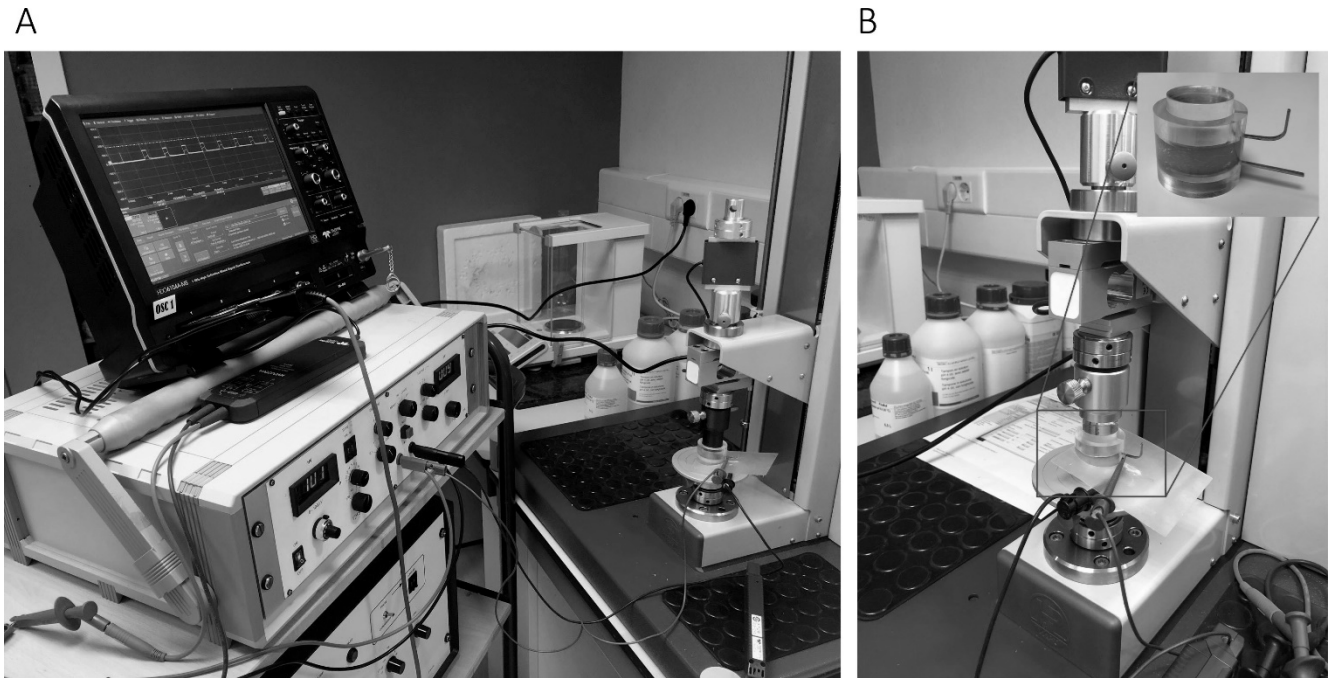


Figure 1: (A) Experimental setup showing the texture analyser, generator, and oscilloscope; and (B) A detailed view of the treatment chamber as set up under the texture analyser piston and of the treatment chamber setup on its own.

Protocol:

You will prepare a sample of apple fruit and of a potato tuber (cultivar depending on availability) cut into a 6 mm thick cylinder of 25 mm in diameter. Sample will then be placed into a cylindrical treatment chamber with plate electrodes at the top and bottom of the sample (see Figure 2), the entire setup will then be placed under the piston of a texture analyser (Hegewald & Peschke Inspect solo 1 kN-M) and subjected to a constant force of 5 N and 10 N for apple fruit and potato tuber, respectively. Electrodes will be connected both to a pulse generator (prototype device), as well as an impedance analyser (an LCR meter, Keysight E4980A), and a switching circuit that will switch between the pulse generator and the impedance analyser to protect the LCR instrument from high-voltage pulses (prototype device).

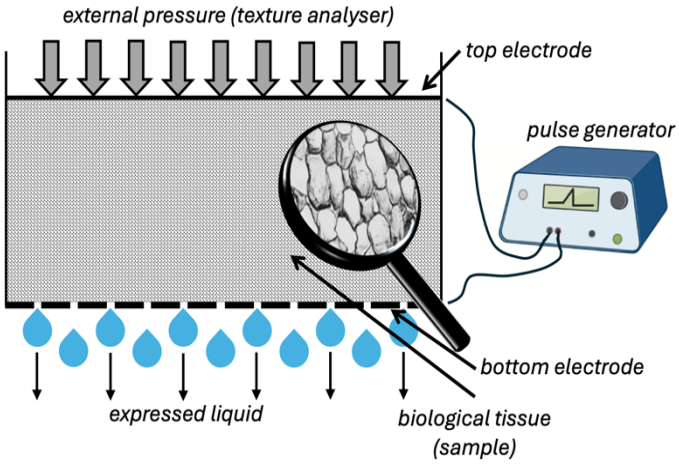


Figure 2: A schematical illustration of a sample placed into a cylindrical treatment chamber with plate electrodes at the top and bottom.

The force will be applied for a total of 3 minutes. After 10 seconds under load, you will measure the pre-pulse impedance, and after 30 seconds under load, you will deliver 8 pulses of 100 µs at 1 s-1 repetition rate, and then immediately measure the post-pulse impedance. The loading of the sample will then continue for another two and a half minutes (until 3 minutes total loading time is reached).

Deformation curves obtained from the texture analyser and impedance measurements will then be imported into MATLAB using scripts prepared in advance for further analysis, during which you will:
Calculate the ratio of post- to pre-pulse electrical impedance of the sample at 5 kHz frequency and plot it versus the applied voltage.
Calculate the total deformation of the sample from the moment of pulse delivery and up to the end of the constant force application and plot this deformation versus the applied voltage.

We will then compare the two functions/plots for both plant tissues and we will discuss the interpretation. The lab work concludes with a printout of graphs that you will paste into your workbook (under NOTES & RESULTS to the right).

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NOTES & RESULS

L12

Modelling, visualising, and tracking pH front formation during pulse delivery to agarose-based tissue phantoms

Samo Mahnič-Kalamiza, Rok Šmerc
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 120 min
Max. number of participants: 3
Location: Tissue Laboratory
Level: Basic

PREREQUISITES
Participants should be familiar with Laboratory safety (S1). No prior knowledge of laboratory work is required. Possession of basic skills of handling chemical/biological laboratory equipment (pipette, analytical scale) is an advantage, but not prerequisite.

The aim of this laboratory practice is to showcase how we can use numerical modelling techniques using advanced finite-element software models (COMSOL Multiphysics) to study complex electrochemical phenomena occurring at the electrode-electrolytic solution interface, and their propagation by electromigration and diffusion in a phantom tissue model. The laboratory exercise will demonstrate, through practical work, how such models can subsequently be validated using practical and comparatively simple lab experiments. The participants will learn just how much electrochemistry is "going on" during a typical electroporation application like gene electrotransfer.

THEORETICAL BACKGROUND
In electroporation applications we inevitably encounter chemical phenomena associated with the direct supply of electrical energy to living organisms by means of metallic electrodes. As these are inserted into an electrolyte solution, (such as is tissue) a special region (layer) forms at the interface between the electrode and the tissue, where dynamic electrochemical processes occur to enable flow of the electric current. The products of these processes can be harmful to the tissue (and electrodes) [1,2]. Assuming monopolar pulse delivery, an acidic pH front forms at the anode and a basic at the cathode, propagating away from the electrodes during, but also after pulse delivery [3,4].

This labwork is conducted by

Samo Mahnič-Kalamiza

Rok Šmerc

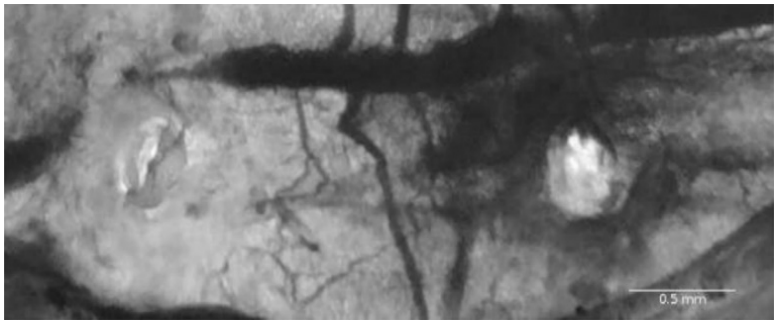

Figure 1: pH changes in the skin due to pulse delivery, surrounding the two needle electrodes (anode-left, cathode-right). Dorsal skin flap of a mouse, observed through a window chamber, in vivo. From [5].

An effective approach to detect and visualize pH changes during the delivery of electrical pulses to model tissues or tissue phantoms involves the use of pH-sensitive dyes or indicators. These dyes exhibit a colour change in response to variations in pH values, allowing for real-time monitoring of pH dynamics within the tissue or tissue phantoms. By incorporating these pH-sensitive dyes into the model tissue, which can be prepared using materials such as collagen or agarose, we can observe and analyse the electrochemical reactions and subsequent pH changes that occur at the interface between the metal electrodes and the tissue [6,7]. This enables a direct visualization of the impact of electrical pulse delivery on the pH environment, providing valuable insights into the underlying processes and helping to optimize the therapeutic applications of electroporation and to validate the models. The pH-sensitive dyes act as visual indicators, offering a convenient and non-invasive means to assess local pH changes induced by the electrical pulses, thus enhancing our understanding of the intricate electrochemical interactions between metal electrodes and biological tissues.

EXPERIMENT

We will first review simulation results demonstrating pH front evolution and advancement in a typical needle-electrode delivery of pulses using a typical gene electrotransfer protocol to i.) an unbuffered medium, and ii.) a buffered medium of comparable buffering capabilities to those of tissue. We will then move to the wet lab portion of the lab work, where we will deliver pulses to agarose phantoms using the same pulse protocol as previously demonstrated through simulation results at various voltages (field strengths). Additionally, we will deliver pulses to the phantoms using an additional protocol employing short, biphasic pulses, to study if these do indeed result in significantly less change in pH as per theory.

We will prepare the agarose phantoms with either unbuffered or buffered saline to demonstrate the natural buffering capabilities of tissue. The two pulse protocols will be chosen to demonstrate the difference between monophasic and biphasic delivery of pulses. The experimental system for observing pH changes consists of a high-speed digital camera with a macro lens, a camera stand with lighting panel, an electroporation pulse generator, and a suitable laptop for capturing the camera's video signal.

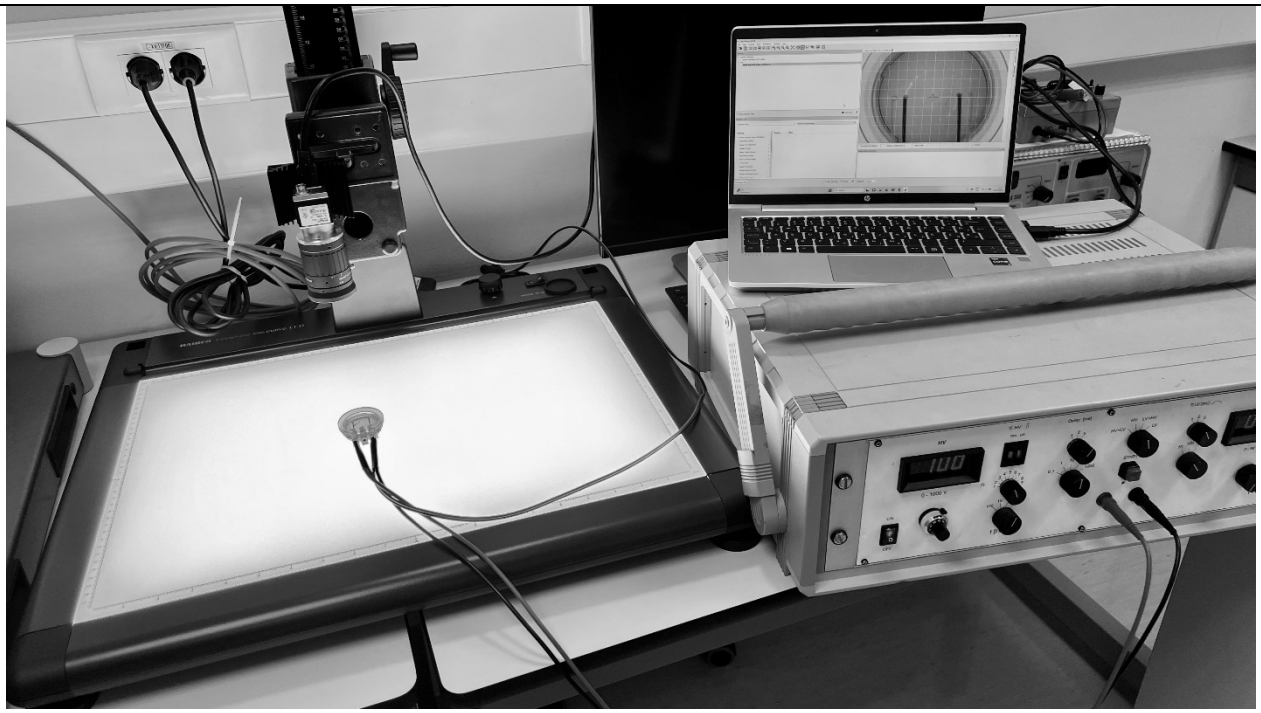

Figure 2: Experimental setup showing the camera rig and the agarose sample under treatment in a petri dish (diameter of 34 mm) with a custom-made cover/electrode holding guide, pulse generator, and laptop computer for controlling the camera and recording video data.

Protocol:

We will begin by mixing an indicator solution using purified water, methyl red, bromothymol blue, and phenolphthalein disodium salt in the following quantities: 150 mL water, 0.012 g methyl red, 0.060 g bromothymol blue, and 0.050 g phenolphthalein disodium salt. We will then prepare a 0.60 % agarose solution using two different ready-made solutions; the first will be a representation of an unbuffered medium and will consist of 0.9 % saline, the second will be prepared by taking 1 molar phosphate-buffered saline that will act as an example of a buffered medium. Note that PBS consists of saline, potassium chloride, dibasic sodium hydrogen phosphate, and monobasic potassium hydrogen phosphate and has a pH of 7.4. Both solutions need to be diluted in a solution-to-purified water ratio of 5-to-1, since we would like our agarose phantoms to model in vivo tissue conductivity of skeletal muscle of about 0.25 S/m.

Having prepared the buffered and unbuffered agarose solution, the two solutions should be quickly heated in a microwave oven almost to the point of boiling, after which (but before pouring into petri dishes) the solutions must be supplemented with the indicator solution in the ratio of 9-to-1 (medium-to-indicator). Following the addition of the indicator solution and agitation, agarose can be measured out into small petri dishes with a pipette (3 mL per dish) and await hardening (about 10-15 minutes should suffice).

Once hardened, you will deliver to the agarose phantoms either a typical gene electrotransfer (GET) protocol (4 x 5 ms delivered at 1 s-1) [8], or a typical short, biphasic (HFIRE) protocol (5 μs positive/negative phase, 5 μs interphase delay, 500 μs interpulse delay, 200 pulses per train, train repetition frequency 1 s-1, 10 trains in total) using the pulse generator and a pair of needle electrodes. Note the total "on time" of the two protocols is the same – 20 ms. Different amplitudes of the pulses can be used to study the effects of lower/higher pulse current (the phantom conductivity is constant) – try experimenting with a few voltages!

You will record the videos of the experiments using the fast camera and subsequently analyse the pH front spatial progression and colour changes (in qualitative terms) by comparing the observed front appearance and migration (at a single chosen voltage, to save time):

- with model results seen earlier during the theoretical introductory part of the lab work for the GET protocol considering the buffered and unbuffered medium, and
- for the two different protocols, i.e., the monophasic GET and biphasic HFIRE as experimentally recorded in the buffered and unbuffered medium.

[1]Skeletal muscle conductivity exhibits a wide range of values that can fall anywhere between 0.04 and 0.8 S/m. We chose 0.25 S/m for the model and experiment as a rough midpoint off that interval [9].

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NOTES & RESULTS

L13

Simultaneous measurement of sarcomere shortening and calcium transients in primary rat cardiomyocytes exposed to electrical pulses

Vid Jan, Marko Stručić
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiments: 90 min
Max. number of participants: 3
Location: Cell Culture Laboratory 1
Level: Advanced

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

The aim of this laboratory practice is to observe the effects of electroporation – using monopolar single electric pulse or a burst of bipolar high frequency irreversible electroporation pulses (H-FIRE) on Ca^{2+} homeostasis, contractility and recovery of adult rat ventricular cardiomyocytes.

THEORETICAL BACKGROUND
Despite a long history of using electric pulses on the cardiac tissue for defibrillation and pacing, effects of high-voltage (supraphysiological) electric pulses at the level of a single cardiomyocyte (or even neurons and excitable cells in general, for that matter), remain relatively unexplored. It is known that during electroporation, cell membrane is damaged, its conductivity is increased, and cell homeostasis disrupted [1]. Voltage-gated channels can also be affected, although the consensus on the mechanisms and the consequences of electroporation on voltage-gated channels is not yet reached. Interestingly, in a recent molecular dynamics study, the voltage sensor area of the voltage-gated channels was shown to be permanently damaged under the influence of high electric fields [2].
During electroporation, Ca2+ enters the cell in an uncontrolled way through the permeabilized membrane. Thus, the precise calcium balance, needed for normal cardiomyocyte function is disrupted [3]. This can lead to undesired and unpredictable behaviours. For example, excitation-contraction coupling (pairing cardiomyocyte depolarization with mechanical contraction) depends largely on Ca2+ signalling and disturbances of Ca2+ homeostasis can severely impact muscle physiology and give rise to various myopathies and cardiac disorders. Under physiological conditions, cardiomyocytes can efficiently remove the excess intracellular Ca2+, which enters during the action potential plateau phase to prepare for the next cycle. However, with electroporation, the uptake of Ca2+ can be much higher than in normal working conditions; moreover, the cell membrane is damaged and does not reseal for minutes after the treatment. Furthermore, ATP, necessary for membrane resealing and operation of pumps, following electroporation leaks out of the cell [4].

This labwork is conducted by

Vid Jan

Tina Turk

The efficiency of electroporation depends on the parameters of the electric field, cell size, geometry, position, and physiological characteristics of the medium surrounding the cell. Different fluorescent dyes (e.g. Rhod-2, Fluo-4, Fura-2, Fura-3,...) can be used to investigate the influence of these parameters on electroporation and the same dyes can be used to monitor how electroporation affects Ca2+ transients [5].

EXPERIMENT

We will simultaneously monitor sarcomere shortenings, i.e., contraction and Ca^{2+} transients of rat cardiomyocytes with IonOptix-upgraded Zeiss Axiovert microscope which applies Fast Fourier Transform to measure sarcomere length while at the same time measuring signal from fluorescent Ca^{2+} sensitive indicator Rhod-2 AM. As cardiomyocytes are paced, Ca^{2+} concentrations inside the cells change, due to its importance in action potentials, while supraphysiological electric pulses, i.e. electroporation, cause changes in Ca^{2+} concentrations due to perturbations in membrane permeability (due to electroporation). Once Ca^{2+} enters the sarcoplasm of cardiomyocytes, it binds to the dye and changes its fluorescence intensity more than 100-fold (Figure 1).

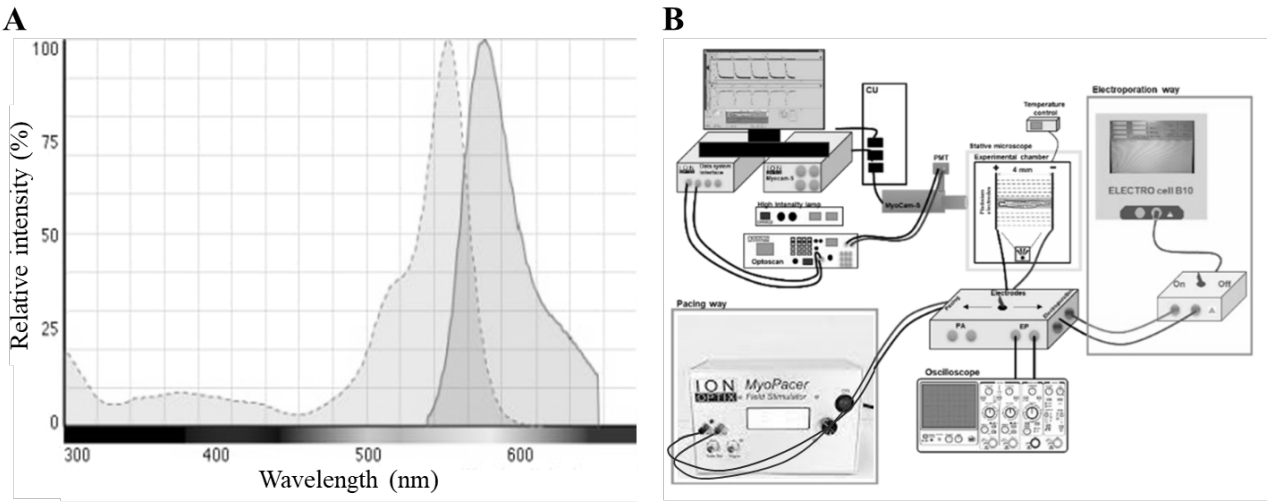

Figure 1: (A) Fluorescence excitation (blue) and emission (red) spectra of Rhod-2 AM for different concentrations of Ca2+ (image from https://www.thermofisher.com/order/fluorescence-spectraviewer?SID=srch-svtool&UID=14220ca#!/). (B) Schematic of the experimental setup (adapted from Chaigne, S. et al., 2022 [3]). (C) Simultaneous acquisition of calcium transients and sarcomere shortenings with IonOptix system.

Protocol: The experiments will be performed on adult rat ventricular cardiomyocytes, isolated from female Wistar rats, weighing 250-350 grams [6,7]. Before measurements, cardiomyocytes will be incubated in Tyrode buffer with 2 µM Rhod-2, AM for 20 min at 37 °C (Thermo Fisher Scientific). After the incubation time, cardiomyocytes will be washed with fresh Tyrode to remove excess dye and then seeded on 25 mm square coverslips for imaging.

Insert laminin-coated glass coverslips into IonOptix stimulation chamber on fluorescence microscope (Zeiss Axiovert 200) and use 63×LD objective. Wait 5 min and then start the perfusion of Tyrode buffer at 37 °C. IonOptix MyoCam-S3™ Fast CMOS camera will be used to capture sarcomere shortening while IonOptix photomultiplier tube with be used to capture signals for Rhod-2AM. Measurements will be recorded and processed using IonOptix IonWizard software.

Using an IonOptix MyoPacer Field Stimulator pace cardiomyocytes on the coverslip with bipolar pulses at 2 Hz and 4 ms (2 ms positive phase followed immediately by 2 ms negative phase). IonOptix stimulation chamber is equipped with 2 platinum electrodes (4 mm spacing edge to edge) and our setup also includes a switch box to alternate between pulses from the IonOptix MyoPacer Field Stimulator for physiological

pacing and HF-IRE pulse generator (University of Ljubljana) for monopolar or bipolar supraphysiological pulses. Using a framing adapter, frame a cardiomyocyte that contracts in sync with delivered pacing pulses. Measure basal changes in sarcomere length and Ca2+ transients (capture at least 10 contractions). Afterwards use HF-IRE pulse generator and deliver one monopolar electric pulse of 100 µs or a burst of H-FIRE pulses (25 bipolar pulses, 2 µs positive and negative phase, 2 µs interpulse and interphase delay), which will represent an electroporation (EP) pulse. Start with 100 V/cm for monopolar pulses, and with 300 V/cm for H-FIRE pulses. 1 minute after delivered pulse, start pacing the cell again with 4 ms bipolar pulses. If cell still contracts, deliver another pulse with higher electric field (with increments of 50 V/cm). Continue in the same fashion until cell hypercontracts and does not respond to pacing 1 min after EP pulse delivery. With this approach we expect to observe different types of responses to EP protocol at different electric field strengths (shown in Figure 2). Compare results for sarcomere shortening and Ca2+ transients of cells treated with monopolar or H-FIRE pulses.

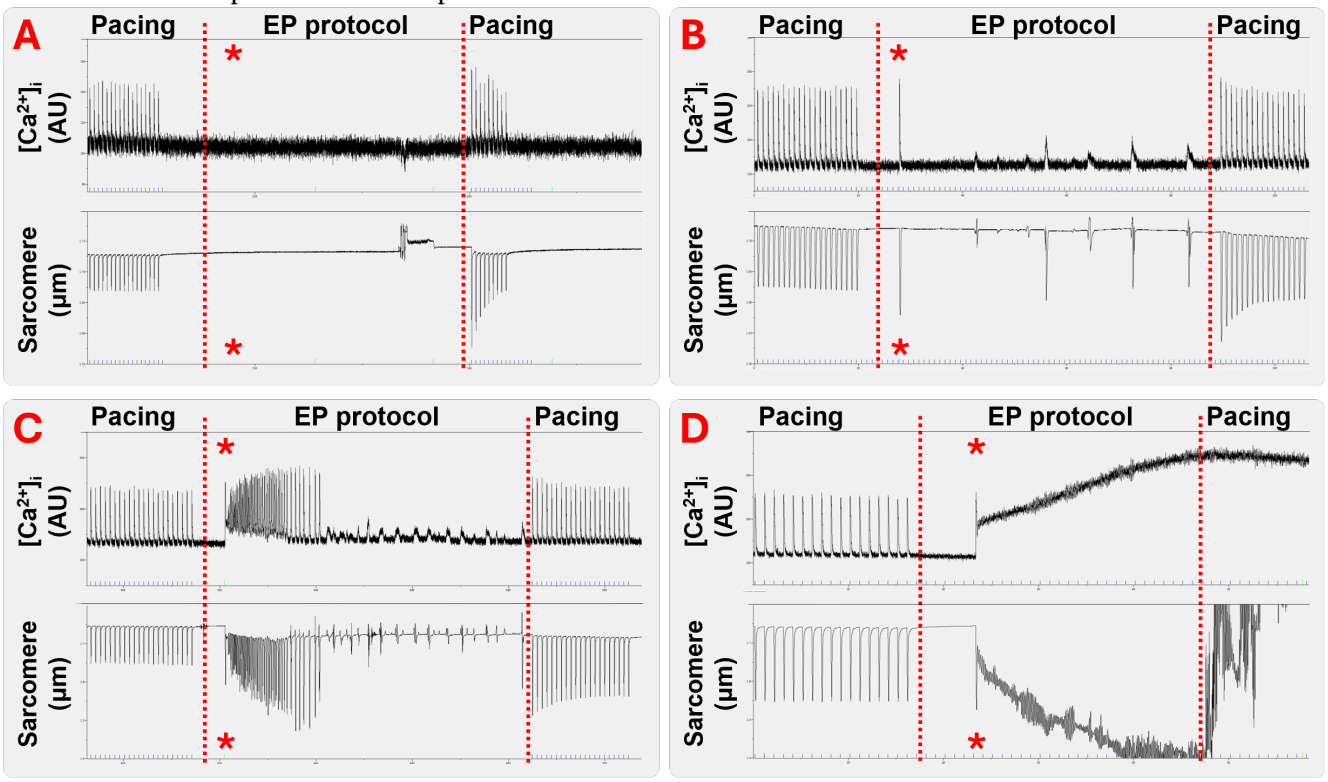

Figure 2: (A-D) Simultaneous measurements of calcium transients ([Ca]i), and sarcomere shortening. Primary rat cardiomyocyte was firstly paced at 2 Hz with 8 V bipolar pulses of 4 ms (Pacing). After that the cell will be exposed to either one 100 µs monopolar pulse or one burst of bipolar H-FIRE pulses (25 bipolar pulses, 2 µs positive and negative phase, 2 µs interpulse and interphase delay) (EP protocol). Each pulse or burst of pulses is marked with an asterisk (EP protocol). 1 min after EP protocols 4 ms pacing continued (Pacing after EP protocol). Normally we see different types of responses to electroporation, which correlate with the intensity of electric field at which EP protocol is performed. (A) At lowest electric fields there is no response to EP protocols. (B) When electric field strength is increased EP protocol induces a contraction of a treated cardiomyocyte. (C) When electric field strength is increased even further an EP pulse induces several spontaneous contractions and oscillations in calcium, but the cell can still be paced 1 min after EP pulse delivery. (D) At highest electric fields spontaneous contractions and high levels of calcium are observed and the cell becomes hypercontracted after EP pulse delivery. Cell is unresponsive to pacing 1 min after EP pulse delivery.

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NOTES & RESULTS

COMPUTER MODELING

C1

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

Anže Županič[1], Bor Kos[2]
[1]National Institute of Biology Ljubljana, Department of Biotechnology and Systems Biology
[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.

The aim of this laboratory practice is to learn how to use numerical modelling techniques to achieve suitable electric field distribution for successful electroporation-based treatments.

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 [1, 2]. 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 [3]. For irreversible electroporation (IRE), the electric field in the entire target volume needs to be above the irreversible electroporation threshold [4]. It is not necessary to electroporate the entire target volume by a single pulse or pulse sequence - sometimes a combination of several pulse sequences or a combination of different electrodes is required [5].

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.

This labwork is conducted by

Bor Kos

Peter Lombergar

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:

1.) Build the 3-d model by following the lecturer's instructions and take into account your tissue-specific electric properties.

2.) Solve the model and evaluate the initial solution.

3.) In the case that 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.

4.) Repeat the process, until the quality of your solution reaches the set goals. Compare the results with others who have used different tissue properties

5.) . Use a parametric study to find the lowest voltage which achieves the objective for the selected electrode geometry.

6.) Change conductivity model to electric field-dependent conductivity $\sigma=\sigma(\mathbf{E})$ [6].

7.) Repeat the parametric study and compare with previous results.

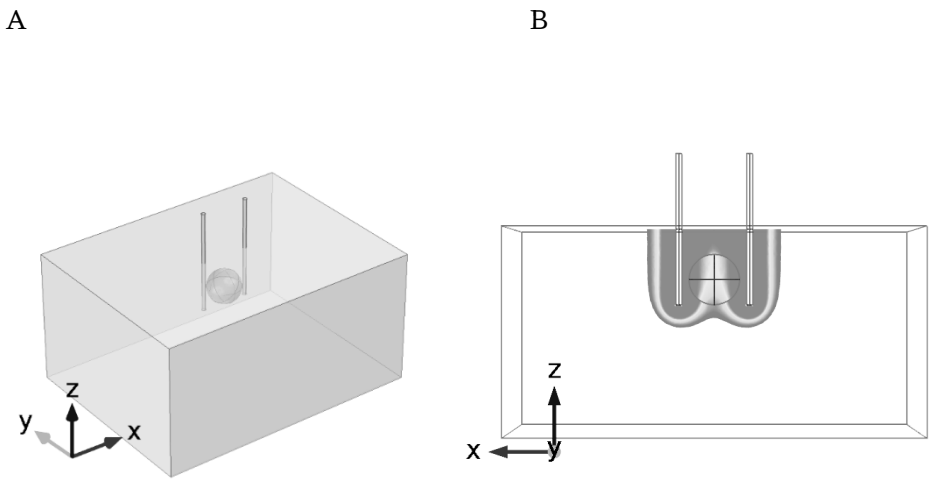

Figure 1: (A) Simple 3D model of tumor and needle electrodes in healthy tissue; (B) electric field over reversible threshold inside the healthy tissue while the tumor is not electroporated.

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NOTES & RESULTS

C2

Numerical modeling of thermal effects during irreversible electroporation treatments

Paulo A. Garcia[1], 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: Basic

PREREQUISITES
No specific knowledge is required for this laboratory exercise.

The aim of this laboratory practice is to model the electrical and thermal responses during a ninety 100-μs pulse irreversible electroporation treatment. This will be accomplished by coupling the Laplace, Heat Conduction, and Arrhenius equations using COMSOL Multiphysics (Comsol AB, Stockholm, Sweden). The IRE ablation zones and will be determined along with temperature increase due to Joule heating due to the pulses. Arrhenius integral will be used to assess potential thermal damage.

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 [1]. 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 [2]. 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 cause thermal damage, especially in critical structures (e.g. bile ducts, nerves and sensitive blood vessels) [3, 4].

This labwork is conducted by

Bor Kos

Peter Lombergar

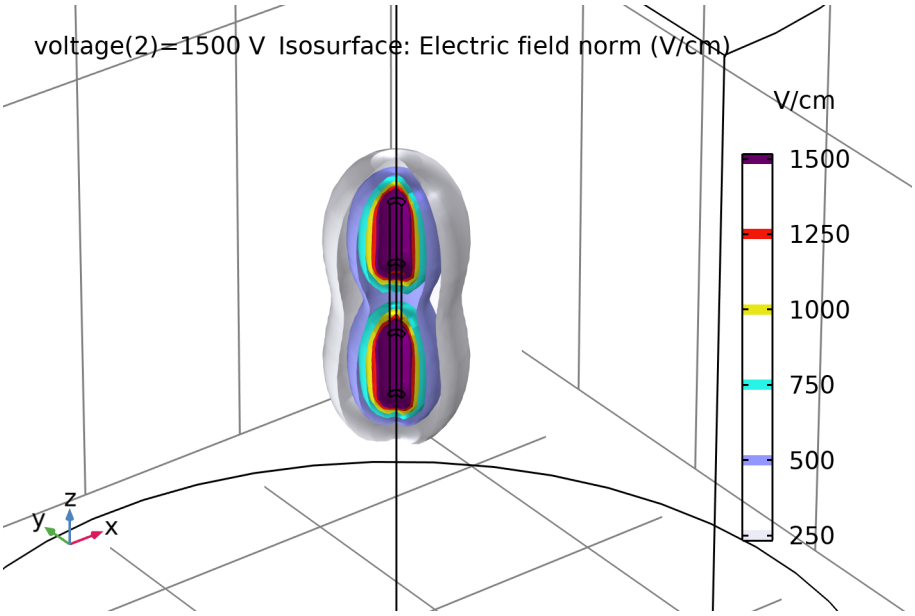

Figure 2: Electric Field distribution resulting from a bipolar electrode with an applied voltage of 1500 V.

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 $Q = \sigma|\nabla\varphi|^2$

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

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 [5]. 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.

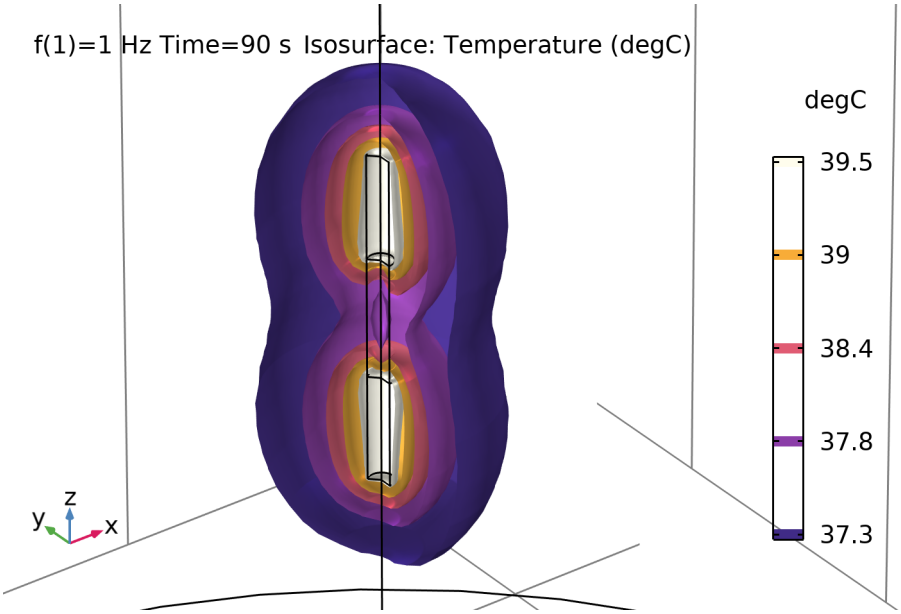

Figure 3: Temperature distribution after a ninety 100-μs pulse IRE treatment in liver tissue at 1 pulse per second with 1500 V pulses.

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:

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

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 [5, 6]. 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 [7, 8]. In order to convert the damage integral to a probability of cell death, $P(\%)$, we will use:

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

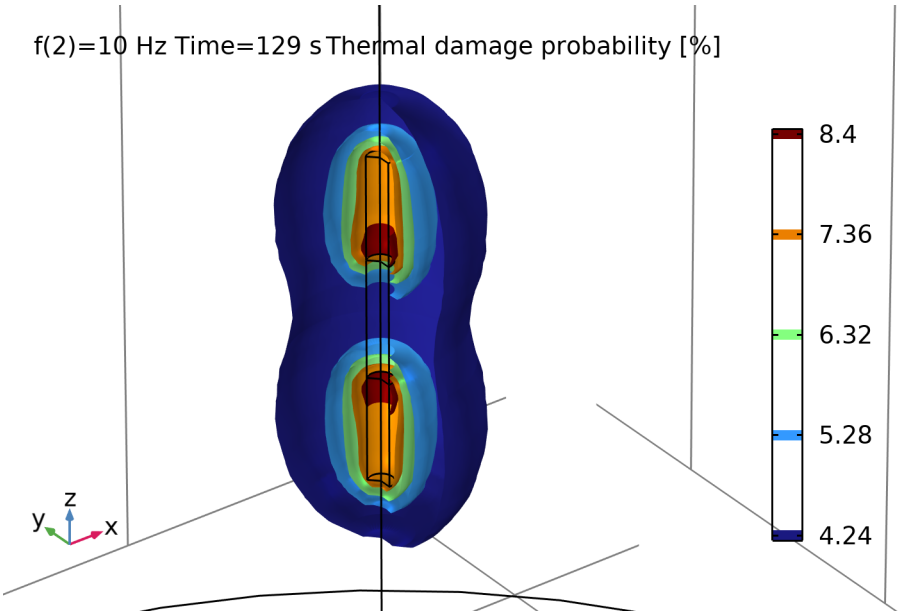

Figure 4: Thermal damage probability of cell death due to excessive thermal effects as a result of Joule heating after protocol with 1500 V pulses delivered at 10 Hz repetition frequency.

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.

Protocol:

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.
7) Investigate a HFIRE protocol of your choice

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NOTES & RESULTS

C3

Molecular dynamics simulations of membrane electroporation

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 (P18-A2)
Level: Basic

PREREQUISITES
No specific knowledge is required for this laboratory practice.

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.

THEORETICAL BACKGROUND
The application of strong electric fields to cells or tissues permeabilizes the cell membrane and produces aqueous-filled pores in the lipid bilayer (along with other chemical and structural changes of the membrane) [1]. Electroporation is witnessed when the lipid membrane is subject to transmembrane voltage (TMV) of the order of few hundred millivolts, which is induced during exposure to an electrical pulse. Such TMV is sufficient to produce an electric field within the membrane of the order of ~ 10^8 V/m. The electroporation 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 [2,3].
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 [4]. 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 [5,6].

This labwork is conducted by

Mounir Tarek

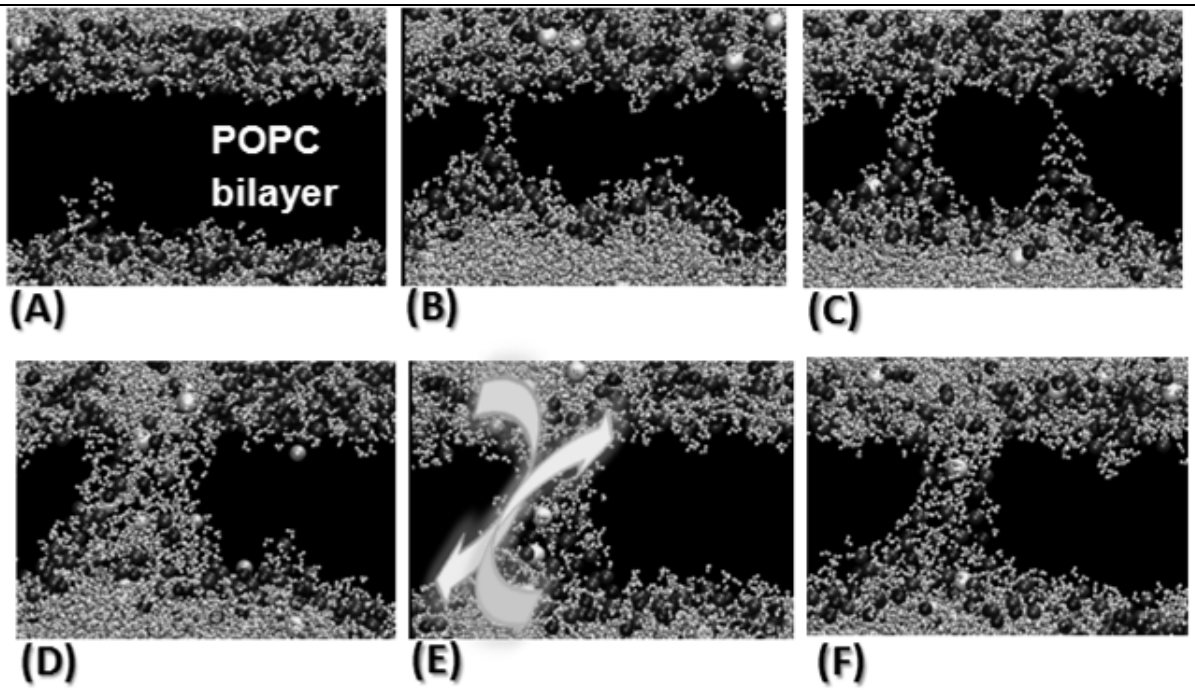

Figure 1: Configurations from the MD simulation for a large POPC bilayer subject to a transverse electric field. (A) Bilayer at equilibrium. (B-C) Formation of water wires at the initial stage of the electroporation process. (D-F) Formation at a later stage of large water pores that conduct ions (yellow and cyan spheres) across the membrane and that are stabilized by lipid head-group (magenta and blue spheres) [3].

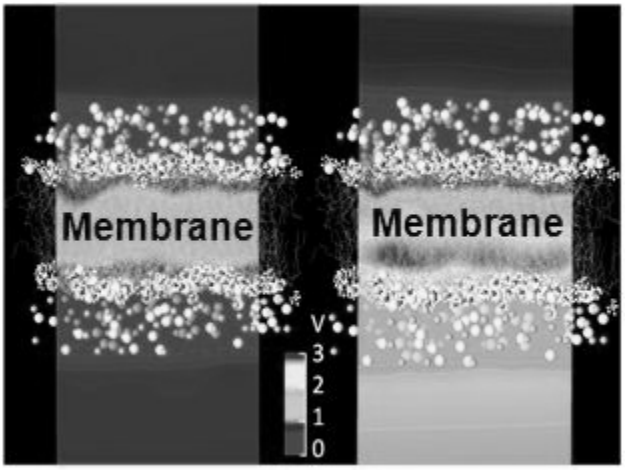

Figure 2: Electrostatic potential maps generated from the MD simulations of a POPC lipid bilayer (acyl chains, green; head groups, white) surrounded by electrolyte baths at 1 M NaCl (Na+ yellow, Cl- green, water not shown) terminated by an air/water interface. Left: net charge imbalance $Q = 0$ e (TMV=0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

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 a few nanoseconds generated with or without a TMV induced by unbalanced ionic concentrations in the extracellular and intracellular will be provided. The students will (1) determine the distribution of the electric 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 (4) measure the intrinsic conductance of such pores.

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NOTES & RESULTS

HARDWARE DEVELOPMENT AND MEASUREMENT

H1

Measurement of electroporation pulses with oscilloscope, and voltage and current probes

Matej Reberšek
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiment: 60 min
Max. number of participants: 10
Location: Laboratory of Biocybernetics
Level: Basic

PREREQUISITES

No specific knowledge is required for this laboratory exercise.

The aim of this laboratory practice is to learn how to use standard measurement equipment to measure or monitor the delivery of electroporation pulses. During the laboratory practice we will also learn what are the electrical parameters of electroporation pulses, what should we report in our studies concerning the measurement and what are some possible complications during the pulse delivery or measurement.

THEORETICAL BACKGROUND

Electroporation is initiated by the delivery of electrical pulses to biological cells. Electrical pulses may vary in pulse parameters such as pulse shape, amplitude, duration and polarity [1,2]. We may deliver different number of pulses, use combination of different pulses or vary pulse repetition rates. We also may deliver pulses in bursts or in different directions relative to the cell. The process of the electroporation is strongly dependent on the pulse parameters of the delivered electrical pulses. In order to control the process of the electroporation and to exactly specify the experimental method, and thus enable the reproduction of experiments under the same conditions, we should exactly determine and describe these electrical parameters. When reporting electroporation methods we should: 1) describe exactly how the electric pulses were measured; 2) provide time-domain waveforms of the electric pulse at the electrodes; and 3) calculate or otherwise determine to what electric field the cells were exposed to [3].

EXPERIMENT

Oscilloscope, and voltage and current probes will be used to monitor the delivery of the electroporation pulses to the load. We will first learn how to set the three main controls (vertical, horizontal and trigger) for adequate data acquisition. We will learn how to use measuring tool to automatically measure the pulse parameters, how to use sequencing to measure several pulses with low pulse repetition rate and how to set acquire to measure bursts of pulses.

This labwork is conducted by

Matej Reberšek

Tomaž Leskovar

We will monitor the delivery of microsecond and nanosecond pulses to the load [4-6]. Learn how to detect disconnection and improper impedance matching of the load, and how a point of measuring and improper wiring may affect the measuring and the delivery of the pulse.

Several different commercial and prototype electroporators will be available during the exercise.

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[1] Reberšek M., Miklavčič D., Bertacchini C., Sack M. Cell membrane electroporation –Part 3: The equipment. *IEEE Electr Insul M,* 30: 8-18, 2014.

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NOTES & RESULTS

H2

Development of pulsed power generators for electroporation

Matej Reberšek, Eva Pirc
University of Ljubljana, Faculty of Electrical Engineering

Duration of the experiment: 60 min
Max. number of participants: 6
Location: Laboratory for Physiological Measurements
Level: Advanced (Electrical Engineer)

PREREQUISITES
Basic to advanced knowledge of electrical engineering.

The aim of this laboratory practice is to learn how to develop milli-, micro- and nanosecond square wave pulse generators. During the laboratory practice we will learn how to choose or calculate the values of the electrical components for a given load and pulse duration.

THEORETICAL BACKGROUND
Electroporation is initiated by the delivery of electrical pulses to biological cells. To generate electrical pulses for electroporation applications a pulsed power generator is required [1-3]. Pulsed power generators operate in two phases: a charge and a discharge phase. During the charge phase, energy is accumulated over a long period of time in an energy storage element such as capacitor. In the discharge phase, stored energy is quickly released into the load. Several different concepts are used to generate electroporation pulses. The most common method of generating micro- and millisecond electroporation pulses is a square wave pulse generator, in which an on/off switch is used to connect and disconnect capacitor to the load. To generate nanosecond square wave electroporation pulses pulse forming networks or lines are used because high-voltage power switches cannot turn off in nanoseconds [4,5].

EXPERIMENT
We will design and assemble two pulse generators: a square wave pulse generator and a Blumlein generator. The square wave pulse generator will enable generation of up to 1 kV micro- and millisecond electroporation pulses. And the Blumlein generator will enable generation of up to 1 kV nanosecond pulses. The output signals of the generators will be measured by oscilloscope, and voltage and current probes.

Square wave pulse generator: We will assemble this generator (Figure 1) by using: a high voltage power supply (V), high voltage capacitor (C), MOSFET switch (S), MOSFET driver (MD) and function generator (FG).

This labwork is conducted by

Matej Reberšek

Tomaž Leskovar

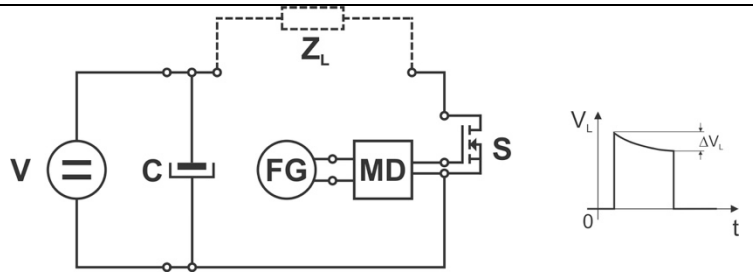

Figure 1. Schematics of the square wave pulse generator and its typical output waveform on load (Z_L).

To power the generator, we will use high voltage DC power supply MCP 350-1250 (FUG, Germany). To store enough energy in the capacitor for the pulse generation, we will calculate its minimal capacitance (equation 1). MOSFET switch will be chosen from the datasheets considering the pulse maximal output voltage and current, and rise, fall and turn off delay time. We will use 4422 (Microchip, USA) MOSFET driver circuit to drive the MOSFET switch and a function generator (HP, USA) to generate a control signal.

$$i_C = C\frac{dV_C}{dt};\ \ i_L = \frac{V_L}{Z_L} \xrightarrow{i_C=i_L;\ V=V_C=V_L;\ dV=\Delta V;\ dt=N\cdot t_P} C = \frac{N\cdot t_P}{\frac{\Delta V}{V}Z_L} \tag{1}$$

We will assemble the square wave pulse generator in four steps, by gradually increasing the requirements for pulse parameters and load (Table 1). We will vary pulse number (N), pulse duration (tP), relative voltage drop (ΔV/V) and resistance of the load (ZL). In the first step, we will assemble the generator for one short (10 μs) fully square (1%) pulse on high resistive (1 kΩ) load. In the second step, we will lower the resistance of the load and observe the operation of MOSFET switch and output pulse waveform. In the third step, we will improve the pulse waveform. And in the final step, we will improve the pulse waveform for prolonged pulse duration and number of pulses.

Table 1. Parameters of the pulses that will be generated and resistance of the loads that will be used in specific assembly step.

Step	N	t_P [μs]	ΔV/V [%]	Z_L [Ω]	C [μF]	S
1	1	10	1	1000		
2	1	10		50	1	
3	1	10	1	50		
4	8	100	5	50		

Blumlein generator: We will assemble this generator (Figure 2) by using: a high voltage power supply (V), resistor (R), transmission lines (T1 and T2), radiofrequency MOSFET switch (S), MOSFET driver (MD) and function generator (FG).

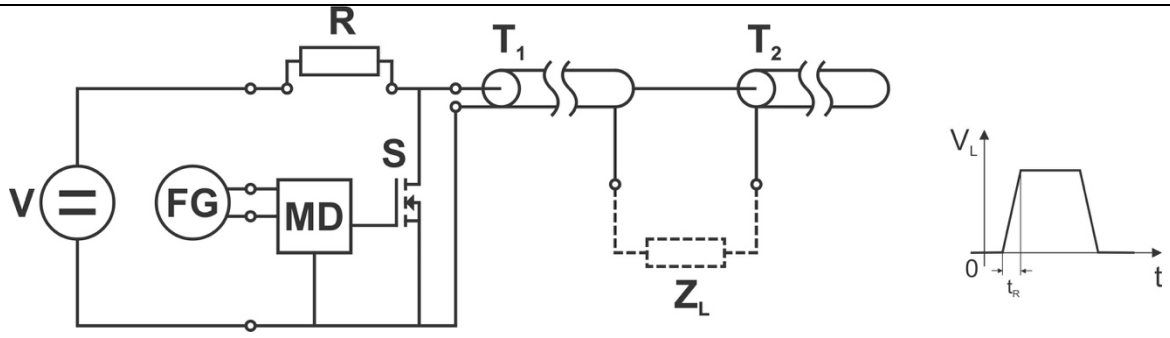

Figure 2. Schematics of the Blumlein generator and its typical output waveform on load (Z_L).

To power the generator, we will use high voltage DC power supply MCP 350-1250 (FUG, Germany). High-voltage and high-impedance resistor will be used to charge the transmission lines. The length of the transmission lines will be calculated (Equation 2) by propagation velocity (vP) of the signal in transmission line. High-voltage, high- frequency and high-current MOSFET switch (IXYS, USA) will be used to quickly discharge the transmission lines. We will use 4422 (Microchip, USA) MOSFET driver circuit to drive the MOSFET switch and a function generator (HP, USA) to generate control signal.

$$v_P = \frac{1}{\sqrt{\varepsilon \cdot \mu}} \overset{\mu_r=1}{\Longrightarrow} \frac{c}{\sqrt{\varepsilon_r}} \overset{polyethylene}{\Longrightarrow} \frac{3 \cdot 10^8 \frac{m}{s}}{\sqrt{2.25}} = 0.2 \frac{m}{ns}; \quad l = \frac{v_P \cdot t_P}{2} \tag{2}$$

The Blumlein generator will be assembled to generate 1 kV, 20 ns square wave pulses on 100 Ω load.

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[1] Reberšek M., Miklavčič D., Bertacchini C., Sack M. Cell membrane electroporation –Part 3: The equipment. *IEEE Electr Insul M,* 30:8-18, 2014.

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NOTES & RESULTS


E-LEARNING

E1

Electroporation of cells and tissues - interactive e-learning course

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Duration of the experiment: app. 90 min
Max. number of participants: 18
Location: Computer room (P9-B0)
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 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) [1-2].

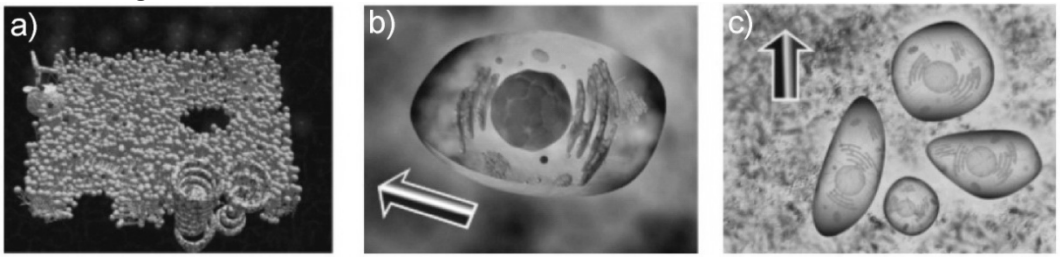

Figure 1: Introduction of small molecules through a cell membrane (a) into an electroporated cell (b) and into the successfully electroporated cells within an exposed tissue (c) [9].

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 [3-7], 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 to visualize the local electric field distribution in 2D and 3D dimensional tissue models.

This labwork is conducted by

Samo Mahnič-Kalamiza

Rok Šmerc

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 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 Erev ≤ E < Eirrev) with respect to the selected parameters such as: number and position of electrodes, applied voltage on the electrodes.

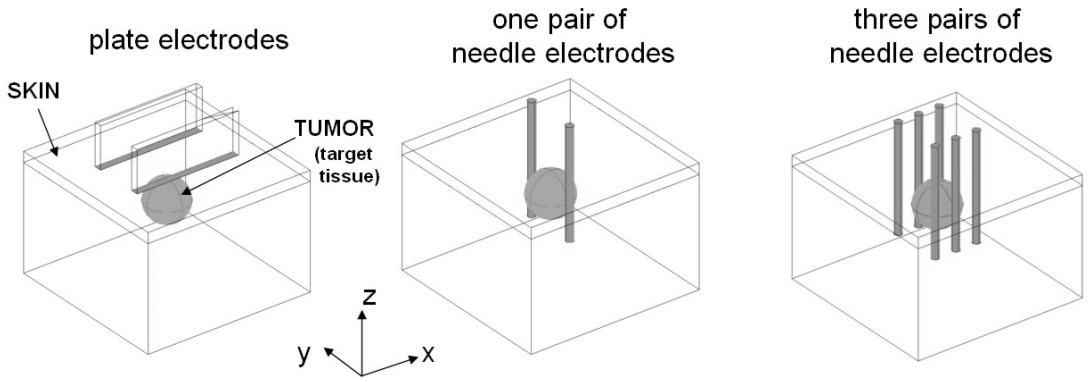

Figure 2: Plate electrodes vs. needle electrodes with respect to the target tissue (e.g. tumor tissue).

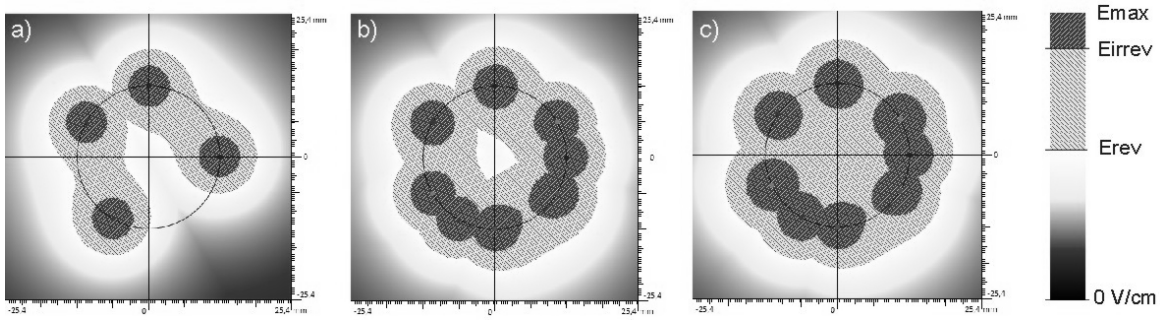

Figure 3: Electric field distribution within the tumor (inside the circle) and within its surrounding tissue (outside the circle) obtained with three different selection of parameters (number and position of electrodes and voltage applied): (a) 4 electrodes, (b) 8 electrodes and (c) 8 electrodes with increased voltage on electrodes so that the entire volume of tumor is exposed to the $E_{rev} \leq E < E_{irrev}$ [8-10].

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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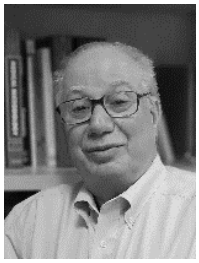

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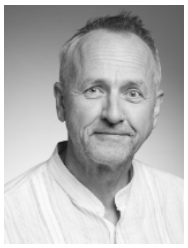

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