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A Study of Electrolytic Processes in Micro-Electroporation and Electroporation

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A Study of Electrolytic Processes in Micro-Electroporation and Electroporation

By

Arie Meir

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor in Philosophy in Biophysics in the Graduate Division of the University of California, Berkeley

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Abstract

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Doctor of Philosophy in Biophysics

University of California, Berkeley

Professor Boris Rubinsky, Chair

Tissue ablation with minimally invasive surgery is important for treatment of many diseases and has an increasing role in treatment of solid neoplasms. A variety of biophysical and biochemical processes are used for this purpose. They include thermal ablation with heating, cooling or freezing, electroporation (pulsed electric fields), injection of chemical agents, photodynamic effects, sonoporation effects and many others.

Electrolysis, the passage of a low magnitude direct ionic current through the tissue, between two electrodes, is a biochemical/biophysical process that has been considered for tissue ablation since the 19th century. Electrolysis affects the ionic species in tissue, which change into compounds that can ablate cells. The advantage of electrolysis in comparison to other ablation techniques can be attributed to its simplicity and low cost of instrumentation, which might make it a suitable treatment modality for resource constrained communities where more expensive medical treatment is often not available.

Electrolytic effects have been conveniently ignored by earlier work focusing on pulsed electric fields. The induced fields were considered brief enough and the resulting currents small enough to ignore changes in pH occurring due to electrolytic processes arising in the tissue. Recent research findings however, indicate that under some practical conditions, the observed effects which were believed to be driven by reversible electroporation can be traced to electrolysis.

This dissertation is primarily focused on electrolytic effects occurring during electric stimulation of living tissues and the development of innovative research tools that enable characterization and research of these effects. The dissertation covers three main parts: first the fundamental phenomena and the necessary conditions are characterized. The second part focuses on research tools design and development. These tools allow to study electrolytic effects during electro-stimulation via imaging (EIT,MRI) and mathematical models. Finally, the last part focuses on application development – two novel applications are proposed which take the electrolytic effects into account. The first application leverages the electrolytic effects in order to make electroporation more efficient, and the second application prevents electrolytic effects by using capacitative coupling of the electrodes to the electroporated medium.
Dedicated to my family whom I love dearly.
This work would not have been possible without the help of trusted mentors and friends who were generous in lending a helping hand along the way:

First and foremost, my advisor, Professor Boris Rubinsky has been very supportive and understanding when the results of imaging experiments looked like shapeless blobs and the simulation results didn’t converge. Professor Rubinsky has become not only a mentor but also a friend and I am forever indebted to him for the intellectual toolkit he helped me acquire while working under his wing.

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My sister Vlada who helped me proof-read the dissertation and held me accountable to high standards of english grammar. Having said that, the responsibility for any mistake in this manuscript is purely my own.

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Chapter 1: Overview and Introduction

Overview and Thesis Structure

This dissertation is primarily focused on electrolytic effects occurring during electric stimulation of living tissues and the development of innovative research tools that enable characterization and research of these effects. The dissertation covers three main parts:

1. Fundamental phenomena - in this part the conditions under which the electrolytic effects become dominant are introduced.
2. Research tools development – in this part, two innovative research tools are designed and characterized. These tools allow to study electrolytic effects during electro-stimulation via imaging (EIT, MRI) and mathematical models.
3. Application development – in this part, two novel applications are proposed which take the electrolytic effects into account. The first application leverages the electrolytic effects in order to make electroporation more efficient, and the other application prevents electrolytic effects by using capacitative coupling of the electrodes to the electroporated medium.

The first part contains three chapters: Chapter 1 provides an overview of the thesis and outlines the overall structure of the work. Chapter 2 sets the stage for this work by introducing the two key electro-stimulation modalities relevant to this thesis: electroporation and electrolysis. Chapter 3 outlines the conditions when electrolytic effects become increasingly important to consider and presents some validated mathematical models that make electrolysis formally tractable.

The second part of the thesis contains two chapters: Chapter 4 introduces electrical impedance tomography (EIT) and demonstrates how EIT can be used as research tool to study electrolysis in tissue. Chapter 5 provides an overview of magnetic resonance imaging (MRI) and demonstrates how MRI can act as an instrument to monitor electrolysis.

The third part of the thesis focuses on applications: the first part of Chapter 6 outlines how combining the two electro-stimulation modalities, namely electroporation and electrolysis allows for an increased efficiency of electro-ablation procedures. The second part of Chapter 6 suggests a design of an electroporation device which prevents the side effects of electrolysis, potentially increasing the efficiency of electroporation based research techniques as well as suggesting new platform for solution sterilization.

The dissertation concludes with Chapter 7 which summarizes the conducted work, offers some remarks regarding the fundamental limitations of the proposed developments, and outlines potential directions for future work.
Underlying Motivation

Since Kurt Semm performed the first laparoscopic appendectomy in 1981, the field of minimally invasive surgery (MIS) has been gaining popularity in the eyes of modern medicine[1]. Reduced infection risks, quicker recovery time, and shorter hospital stays – all these are used as arguments by clinicians preferring MIS over open surgery. In practice, besides demanding a high level of virtuosity from the surgeon, MIS procedures are often limited by the available tools, tools that help achieving the desired clinical outcome with a high degree of control and precision. It is the development of new tools that advances the realm of possibility in the scientific and engineering domain.

This work focuses on a particular area of MIS: tissue ablation. A major factor preventing cutting edge research in the tissue ablation field to reach the clinic seems to revolve around the lack of efficient tools. The general motivation behind this work was to understand the limitations of currently available instruments and develop a set of new conceptual and practical tools which go beyond the shortcomings of their predecessors, and potentially enable conducting new research and clinical procedures.
Chapter 2 : Background and Related Work

Tissue Ablation – review of different modalities

Tissue ablation, the destruction of undesired tissue region in a focused and controlled manner has become an important tool in the armamentarium of modern medicine. The goal of a typical tissue ablation procedure is to induce cellular death in a highly specified region of the organism, causing minimal collateral damage to surrounding tissues and organs. The way that tissue ablation is normally administered is by applying energy and/or chemicals to the target location. Over the last several decades, multiple ablation approaches have been developed aiming to maximize efficacy and minimize associated side effects. Although the focus of this work is limited to electrically-based ablation approaches, i.e. electroporation and electrolysis, for the sake of completion, some of the more commonly used modalities are briefly mentioned in this work. Widely used ablation methods include the following:

- **Electrolytic ablation** – this approach involves running a small direct current through the ablated region. Damage mechanisms suggested by the literature include electro-osmosis of water[2], extreme pH changes[2], and the release of electrolytic by-products such as e.g. chlorine into the tissue[3].

- **Irreversible Electroporation** – this ablation modality involves applying a series of brief, high magnitude pulses of electric-field in the target area. Under some conditions, the electric field causes a significant increase in the membrane’s permeability to external ions and molecules, leading to the disruption of the cell’s homeostasis and eventual cell death.

- **Chemical ablation** – in this approach, a chemical agent (e.g. Bleomycin) is typically injected into the target tissue. The agent then diffuses into cells causing biological damage and eventually leading to cellular death. The efficiency of purely chemical methods is often limited due to the selectivity of the cellular membrane which prevents the drug from penetrating into the cytoplasm, however multiple methods exist which combine chemical elements with other, physical ablation methods.

- **Cryo-ablation** – this approach involves freezing or causing rapid freeze/thaw cycles on the target cells. In a typical procedure, liquid-nitrogen cooled cryoprobe is placed within the neoplasm, alternating with rapid thawing using helium or argon gas infusions. The damage mechanism in cryo-ablation is multidimensional: proteins undergo cold denaturation, metabolic heat absorption preventing enzymatic and other cell functions, and formation of ice-crystals which rupture cell membranes[4].

- **Radio-Frequency Ablation** – in this modality, a high frequency alternating current is run through the ablated tissue generating local heat and leading to protein denaturation[5, 6].

- **High-Intensity Ultrasonic Ablation[7]**.

- **Interstitial laser coagulation**[8].
Electrolysis

Introduction and Historical context

Electrolysis, in the biomedical context typically refers to the passage of a low amperage direct ionic current through the tissue, between two electrodes. Fundamentally, it is a biochemical/biophysical process that can be used to ablate unwanted or abnormal tissue. As a consequence of the process of electrolysis, the ionic species in tissue change into compounds that can ablate cells. Tissue ablation with electrolysis was practiced since the beginning of the 19th century. In his 1886 book “A treatise on Electrolysis and its applications to therapeutical and surgical treatment in disease”,[9], Robert Amory writes “Crussel of St Petersburg, (Med. Ztg. Des Vereins, 1841) worked perseveringly on this subject [electrolysis] for seven or eight years, and addressed a memoir to the French Academy of Sciences. In 1849 he reported cases of the cure of cancer, ulcers and two cases of cataract in which he used the galvano puncture. The committee of the academy, to whom this paper was referred, reported that the evidence presented was not sufficient to warrant the expression of an opinion concerning the merits of the method. This discouraged, Crussel subsequently limited himself to the study of the effects of calorific action.” Indeed, while known for over two centuries, electrolysis is used much less frequently for tissue ablation than the other biochemical/biophysical processes mentioned earlier.

Modern research into electrolysis as an ablation approach can be traced back to the work of Humphrey and Seal in 1959. In their work [10], they have reported encouraging results when applying a low DC current to sarcoma tumors in mice. A notable wave of systematic research into electrolytic ablation began in the late 1970’s, through the work of Bjorn Nordenstrom and his colleagues[11, 12]. Nordenstrom and his group treated lung metastases (carcinoma) and several primary lung tumors. Their approach was often applied when all other therapy modalities – surgery, radiation or chemotherapy, were prohibitive or exhausted. Nordenstrom mentions that tumors with diameter larger than 3cm did not always respond well to therapy. A notable case included a 4cm metastasis and three smaller metastases. After being treated with electrolysis, the tumors vanished and the patient lived for at least 20 more years [13]. These efforts led to important research on fundamental and clinical aspects of electrolysis. Important work was published on understanding of the effects of electrolysis on tissue through histology, mathematical modeling of the process of electrolysis and clinical work, see e.g. [14-31].

Focusing on the clinical context, a significant body of work has been reported from Chinese research groups, where electrochemical therapy is seen as an effective complementary approach in the treatment of tumors due to its associated low cost compared to other alternatives. Although the results originated in China were published in multiple sources [32, 33], they were received in the West with a degree of skepticism, perhaps due to dubious methodology. This has been gradually changing over the last 15 years and various publications originating in Europe, USA and Australia have been using similar techniques to these described in the original Chinese works [34-37]. According to available data, up to 2002, more than 15,000 clinical human studies have
been performed in China. A report by Nilsson and colleagues summarizes the results of these experiments [23].

Some of the findings made previously, and several research techniques that were developed, have contributed to the inspiration of this work. Specifically, it was shown that the electrolysis induced pH changes, can be used to reliably monitor the extent of tissue ablation [38]. This finding has led to several basic studies on quantifying the process of electrolysis through the use of transparent gels with pH dyes [28, 39, 40].

**Mechanism**

**Electro-Chemical Model**

When two electrodes are immersed in a conductive medium, and a direct current is applied between the electrodes, several electrochemical reactions occur around the electrodes as well as in the bulk of the medium. These reactions depend on the composition of the electrodes themselves, and for the sake of simplicity, the electrodes are initially assumed to be inert, i.e. practically insoluble, such as e.g. platinum. In such a case, the key electrode reactions that take place include the decomposition of the water, as well as oxidation and reduction of the substances dissolved in the medium.

The chemical reactions considered, are part of water electrolysis, where both the anodic and the cathodic reactions are accounted for. At the anode we have:

\[ 2H_2O \leftrightarrow O_2 + 4H^+ + 4e^- \]  

and

\[ 2Cl^- \leftrightarrow Cl_2 + 2e^- . \]  

Anodic reaction represented by (1) is the decomposition of water, which causes oxygen evolution and the production of protons, which causes reduction in the pH level. Anodic reaction represented by (2) describes the oxidation of chloride ion leading to chlorine gas formation. Another reaction (3), which is sometimes not accounted for in the simpler models of electrolysis, is the further acidification caused by the chlorine reacting with the water. In this reaction hypochlorous acid is produced and the reaction can be written as:

\[ Cl_2 + H_2O \leftrightarrow HClO + H^+ + Cl^- . \]

The reasoning behind the simplified models is based on experimental results as well as theoretical estimations of the relative importance of these two phenomena [41]. The available data suggests that chlorine generation in medium (tissue) can be considered a secondary effect in comparison to the spreading of hydrogen and hydroxide ions.
At cathode we have:

\[ 2H_2O + 2e^- \leftrightarrow H_2 + 2OH^- \].

The reaction represented by (4) describes the decomposition of water into hydrogen gas and hydroxide anions which result in increased pH level.

**Damage Mechanisms**

Due to the complexity of the phenomena occurring around tissue electrolysis, the precise damage mechanism still remains a matter of academic debate. There are however several key factors which clearly impact the overall damage to the tissue. These include pH changes, electric fields, metal ion dissolution and chlorine.

1. **pH Changes** – most electrolysis related reports agree that extreme pH gradients are formed during the electrolysis process. The acidic pH at the anode was reported to be as low as 1 [2, 20]. At the anode, the basic front has been reported to reach a level of pH as high as 13 [2, 33]. The extreme pH levels cause protein denaturation and eventually lead to the collapse of the cellular structure and cellular death [2, 42, 43]. Theoretically-based analytical models were used to calculate the expected pH levels evolving during electrolysis. Hydrogen and chlorine transport around inert electrodes of different geometries were studied by [41, 44]. The evolution and transport of electrolytic by-products, including potentially toxic species were studied by [21, 22, 45].

2. **Electric Fields** – the polar nature of water molecules under the influence of the electric-field forming in the tissue during electrolysis leads to the migration of interstitial water (electro-osmosis) from the anode towards the cathode. This causes edema around the cathode and dehydration around the anode [2, 46]. Moreover, the electric field causes a redistribution of the ionic specifics and other charged components dissolved in the tissue. This leads to changes in the transmembrane potential of the cells constituting the tissue. These changes have been shown to affect the ion exchange across the membrane which in turn could lead to changes in conditions necessary for many essential enzyme-regulated reactions [2, 47].

3. **Metal Ion Dissolution** – in the case where the electrodes are not made of inert metal such as platinum, additional electro-chemical reactions occur at the electrode surfaces which typically leads to the erosion of the electrode(s) and the introduction of the metal ions into the tissue. Typical metals used for electrode fabrication include copper, rhodium or brass, and the metal ions might have toxic effects on the tissue [48]. Miklavcic et.al. [20] report metal ion deposition, gold in their case, as not the major cause of tissue damage. Their work also ruled out the thermal effects of electrolytic therapies, as the temperature has not changed significantly under their experimental conditions [18].
4. **Chlorine Evolution** – Several studies have considered the evolution of toxic chlorine as the dominant factor in the damage mechanism [3, 49], but the methodology does not seem to allow making a conclusive statement.

A schematic representation of a typical electrolytic therapy setup is shown in Figure 1:

![Figure 1: Schematic of a typical electrolysis system. Adapted from [50].](image)

The power source is connected with wires to the undesired tissue. According to the stimulation protocol, direct current is run through the tissue which causes propagation of acidic and basic fronts around the anode and the cathode, respectively. The current meter is connected in series with the tissue and it is used to monitor the administered charge dosage.

**Electric Parameters**

Due to lack of monitoring/imaging tools, the primary parameter characterizing an electrolytic therapy procedure is the dosage, i.e. the amount of charge “injected” into the tumor. The dosage is typically measured in Coulomb, corresponding to a unit of electrical charge, equivalent to the amount of charge moved by a current of 1 Ampere during 1 second. In a clinical setting, the charge dosage is correlated with the volume of the tumor, e.g. in Nordenstrom’s works the average reported dosage was 80C for each centimeter of the tumor diameter [2, 12]. Different strategies to stimulation can be current driven or voltage driven. Regardless of the stimulation used, the amount of charge moved can be measured.

**Existing Research Methods and Tools**

Despite a degree of encouraging clinical results, electrolysis has not enjoyed the same degree of research attention as other electro-therapeutic methods such as e.g. electroporation. One of the possible reasons for this might be the lack of high quality research tools allowing for systematic studies of the involved phenomena. At present, the two key methods used to study electrolysis are mathematical models and pH sensitive dyes. In addition, *in vitro* and *in situ* animal models allow for visualizing of the
effects due to the haematin formation. In this section, the available research tools are reviewed along with their limitations and drawbacks.

Mathematical Models

Mathematical modeling is a powerful tool in developing effective treatments using electrolysis. The geometry and the electric parameters of a therapeutic session could be evaluated and optimized using a low-cost computer model, before resources are committed for fabrication and experimental efforts. Models are useful in order to understand the relative importance of different factors, but their main limitation is that they by definition represent a very simplified version of physical reality. This aspect becomes especially important when dealing with any biological system due to the incredible level of complexity associated with even the simplest living things. For example, experimental data shows that at very low current densities ($\mu A/cm^2$) the production and the transport of the electrolytic by-products is low enough for cells to be able to counteract any macroscopic changes in the tissue [2, 3, 48]. For a mathematical model to predict this kind of behavior, it would have to encompass an extremely complex, analytic and formal description of the physiological functionality of a cell. This makes the modeling process prohibitively cumbersome. This makes mathematical modeling a necessary, but not sufficient tool in studying electrolysis.

Mathematical models reported in the tissue electrolysis literature differ by their level of complexity with regards to the physical phenomena considered. Transport mechanisms might include passive diffusion, electric-field driven migration, and convection. Simplifying assumptions such as saturation of “input species” for the reaction around the active electrode areas can be made. Electrochemical reactions often result in evolution of gas species. Gas bubbles can have an effect on the conductivity of the electrolyte. This effect is typically ignored [21, 22, 45] to make the model more comprehensible. The kinetics of the electrochemical reactions are modeled based on experimental measurements presented in the literature. Electro-osmotic effects influence the transport of water molecules in the tissue and are chosen to be ignored by some simpler models. For experimental validation this can be compensated to a certain extent by maintaining a steady influx of solution into the anode area [21]. The purpose of this section was to provide an overview and set a context of the entire thesis. More details and specific mathematical models will be developed in a later section.

pH Sensitive Dyes

Besides the analytical tools offered by mathematical models, the second primary approach to study electrolysis related phenomena involves the use of pH sensitive dyes. pH level plays many critical roles in cell, enzyme and tissue activities, including proliferation and apoptosis, drug resistance, ion transport, endocytosis and muscle contraction [51]. On an intracellular level, an intimate connection between cellular functions and pH means that precise measurement of pH level can provide critical information relevant to physiological and pathological processes. On this scale, pH can
be measured qualitatively using fluorescent pH sensitive detection probes, some of which are non-destructive to cells. In order to obtain more precise information regarding pH levels, more sophisticated instrumentation is required, such as e.g. multiple wavelength excitation/emission [51].

While pH sensitive probes are commonly used in microscopic studies, when it comes to monitoring electrolysis on a macroscopic scale, they suffer from several drawbacks. To begin with, commonly used pH indicators (e.g. phenolphthalein) are not appropriate for in vivo imaging, due to their toxicity [52]. Secondly, in the case of a deep in-body tumor, there is no way to visually observe the dye, which complicates animal studies. And thirdly, the cost of pH sensitive dyes, while not prohibitive, still contributes to an overall bill of materials associated with doing research.

**Visual Observation**

In animal models, the electrolytic stimulation causes the formation of haematin, a dark-brown pigment which causes color change around both the anode and the cathode [42]. The acidic haematin contains methaemoglobin whilst the dark area around the cathodic lesion consists of haemochromogens [49, 53]. The spreading of chlorine causes a grey coloured zone close to the anode, which is considerably smaller than the heamatin zone [49]. While the altered color provides some degree of feedback regarding the electrolysis procedure, it is not practical to use this method for in vivo models when the ablated tumor is located deep inside the body and is not available for visual inspection.

One of the problems that can be recognized from animal work is the lack of effective imaging methods to monitor the progress of electrolysis. This is especially true for deep in-body tumors when the effects cannot be observed visually. The focus of this thesis is based on these premises, as a new method of imaging electrolysis is suggested and developed in later sections.

**Electrolysis Applications**

The primary application of tissue electrolysis is to ablate tissue for treatment of localized malignant and benign tumors [23]. Despite the large numbers of patients treated successfully using this ablation modality (more than 10,000 according to [32, 33]), electrochemical therapy is still far from being universally accepted as a clinical treatment approach. This discrepancy can be attributed to the lack of systematic basic preclinical studies as well as reliable clinical trials. The goal of this work is to advance basic as well as clinical research of tissue electrolysis by extending the toolkit available to researchers working in the field.
Electroporation

Introduction and Historical Context

Electroporation or electro-permeabilization commonly refers to a temporary or permanent increase in the cellular membrane’s permeability to external substances, specifically as a result of applying electric field to the cell. Under some conditions, the effect of the external stimulation can be reversed and the cell survives the “shock”. This modality of electroporation is referred to as reversible electroporation. When the applied stimulation is too extreme, the integrity of the cell membrane is compromised, and the cell is no longer able to maintain the necessary conditions for its vitality. This leads to cellular death and the associated phenomenon is referred to as irreversible electroporation. In order to position this work in the context of a larger body of scientific knowledge, a brief review of the history of electroporation research is included herein.

While biomedical usage of electroporation has become a focus of research relatively recently, in the later part of the 20th century, anecdotal, observational reports have been made as early as the 18-th century. J.A. Nollet’s book, published in 1754 [54] mentions visible effects on human skin, resulting from a brief exposure to electrical sparks. The absence of thermal effects is one of the key elements distinguishing electroporation from other ablation methods. This distinction, coupled with the technical limitations of static electricity generators available at the time, supports the hypothesis that the observed skin lesions were caused primarily by electrical means. This leads some sources to consider Nollet’s work as the first reported observation of electroporation reported in literature [55]. A later study by G.W. Fuller, published in 1898 [56], reports observational findings of discharging a high voltage source through a water sample, leading to bactericidal effects, without significantly increasing the temperature. These two representative studies show that effects known to be today as results of electroporation were observed before the 20th century, which is when electroporation began developing into a specialized research field. For additional information regarding earlier electroporation related studies, refer to first chapter of [55].

It was not until later in the 20th century, that the bactericidal action of electric fields has motivated a series of three seminal studies by Sale and Hamilton, often cited as the scientific basis of electroporation [57-59]. The authors have studied the effects of different electric parameters on microorganisms. Their first work has systematically shown that a high enough electric field can lead to death of microorganisms. It was demonstrated that the effect was independent of the growth stage, pH or heating [57]. The second work in the series has identified the loss of the membrane function as the main mechanism leading to the cellular death. This conclusion was supported by evidence showing the leakage of cellular content into the medium [58]. The third paper of the series has shown that the applied electric field leads to an increase of the transmembrane voltage. The conclusion was made that the elevated transmembrane potential induced by the external field may cause “conformational changes in the membrane structure resulting in the observed loss of its semipermeable properties” [59].
The two decades following Sale and Hamilton’s work in 1967-1968, have focused primarily on reversible electroporation. The works of U. Zimmerman’s group [60, 61] are noted as some of the first systematic studies into the effects of various electric parameters as mentioned by Ivorra and Rubinsky [55]. Zimmerman’s group has measured the effect of field amplitude and electric pulse length on the cell membrane breakdown. In their work, the degree of cytoplasm leakage into the medium was used to estimate the degree of membrane integrity. Specifically, they have reported that for pulse length of 50-100 us, maximal amount of leakage (and by proxy maximal permeabilization) occurs at electric field of 2.6 kV/cm for human red blood cells. For bovine red blood cells, a similar effect occurred around 2.8 kV/cm, and the transmembrane potential reached was around 1.1V [61]. In an attempt to understand the fundamental mechanism leading to membrane permeabilization, several studies have focused on the electric breakdown of bilayer lipid membranes, using them as a simplified model of the cellular membrane [62, 63]. This work led to the development of theory stating that permeabilization occurs due to the formation of transient pores [63]. A notable study reported by Kinosita and Tsong in 1977 [64] showed using osmotic mass transfer experiments, that pore size of red blood cells could be modulated in a controlled manner, and that the pores reseal, allowing extracellular molecules to be transferred into the cytoplasm. During the 1980s, some of the first biomedical applications were developed, and the term “electroporation” was coined to describe the phenomenon when Neumann with colleagues have successfully transfected mouse lymphoma cells with foreign DNA [65]. Other prototypical applications included first reports of cell fusion [66] and electrochemical therapy treatments combining electroporation with cytotoxic chemical agents for focused ablation [67].

The last decade of the 20th century has seen the transition of electroporation from a basic research field into a basis for practical applications. In 1991, Titomirov has reported the introduction of plasmid DNA into living tissue [68]. Another breakthrough was reported in 1991 by the group of L.M.Mir [69, 70] which leveraged reversible electroporation to facilitate the penetration of anti-cancer drugs such as bleomycin into malignant cells. To date, electrochemotherapy, a term coined by Mir’s group to refer to the combination of chemical cytotoxic agent with electroporation, is an established approach used clinically to treat cancer patients [55, 71]. Additional applications included a transdermal drug delivery mechanism [72], and standardization of electroporation as a method for gene transfections [55].

It is interesting to note that most of the research efforts in the electroporation field were focused on the reversible electroporation modality. While irreversible electroporation was used since the 1960s in the food sterilization field, in the biomedical arena, the irreversible aspects associated with electroporation were typically treated as an undesired effect. In 2004, irreversible electroporation was proposed as a purely electrical ablation method by Davalos and Rubinsky [73]. By designing a treatment protocol that avoids thermal effects, it became possible to induce purely electrical damage to the tissues, leading to a higher degree of selectivity and precision for the interventional procedure [74].
Mechanisms of Electroporation

Over the last several decades, numerous models have evolved in an attempt to explain the mechanism behind the experimental observations of electro-permeability. One of the challenges associated with studying electroporation is the small (nano-meter) length scale of the phenomenon, and the short (micro-seconds) time-scale on which critical events are occurring. To date, there is no consistent, direct evidence of the electroporation process, e.g. in a form of membrane level imaging. While a single, coherent and comprehensive model which explains the phenomenon has yet to be developed, there are several features of the electroporation process that various theories attempt to explain with alternating degree of completeness. Reviewing these key features, one could summarize them in the following list:

1. **Induction step** [75]: Microsecond-millisecond ranged electrical field pulses are applied to a cell, leading to rapid (within single microseconds) elevation in the transmembrane potential to a critical threshold value in the 0.2-1V range.

2. **Expansion step** [75]: Membrane permeability changes locally and the membrane undergoes a time-dependent transition, depending on the conditions of the applied stimuli.

3. **Stabilization step** [75]: Once the external electric-field is removed, the membrane either recovers from the induced “shock” and reseals, or, alternatively, the membrane loses its integrity, leading to loss of homeostasis and the leakage of cytoplasm into the medium.

Depending on the electroporation modality, the process can take two time-courses: in the case of **reversible electroporation**, the cell is able to recover from the insult – in this scenario, the transmembrane potential drops to its resting state within single microseconds, and the membrane slowly undergoes the **resealing** [75] step, returning to its original condition where it is permeable only to small molecules. The typical time scale of the resealing process is on the order of seconds or minutes [75]. During the resealing time, the membrane contains putative pores which cause it to be increasingly permeable to external ions and molecules [76]. It is exactly this condition that is sought by the various gene-therapy applications aiming to gain access to the cytoplasm.

Alternatively, in the case of **irreversible electroporation**, the cell is not able to maintain its homeostasis and the membrane disintegrates leading to cellular death in one of several possible mechanisms [77]: Membrane rupture due to increasingly large pore, membrane rupture due to osmotic swelling, loss of ionic balance or the leakage of crucial internal cytoplasm components into the medium.

A commonly cited theory attempting to explain the fundamental mechanism of electroporation is referred to in the literature as the aqueous pore theory [78]. Not surprisingly, it revolves around the concept of pores, which are hypothesized to develop and evolve in the membrane as the result of applying the external electric field. In order
to appreciate and visualize the conceptual framework induced by this theory, it might be valuable to recall the typical structure of the lipid bi-layer membrane:

![Lipid bi-layer membrane structure](Image source: HeartZine)

Fundamentally, the lipid bi-layer membrane is made of two adjacent layers of phospholipid molecules (Figure 2). Each phospholipid molecule contains two parts: hydrophobic triglycerine heads, and two hydrophilic hydrocarbon chains attached to it. When the phospholipids form a bi-layer membrane, the hydrophilic “tails” are organized symmetrically facing each other, creating a unique structure that makes biological membranes impermeable to most water-soluble molecules and ions.

While the bi-layer membrane is impermeable to water soluble molecules, it is permeable to water and various lipid-soluble molecules. The two layers of charged (polar) triglycerine heads are exposed to a typically conductive medium and cytoplasm. Furthermore, these two layers and separated by the hydrocarbon chains (typical distance of 5-10 nm). Due to its imperfect impermeability, the membrane behaves like a leaky capacitor. Strong electric field applied across the membrane causes transient currents and charge reorganization. This charge reorganization changes the energy of the bi-layer membrane which in turn causes a structural change in the membrane. It is these structural changes that are believed to lead to the pore formation in the bi-layer membrane.

The key factor driving the formation of membrane pores is the transmembrane potential [76, 78]. Elevated transmembrane potential leads to increased propensity of the ongoing thermal fluctuations to cause a structural change in the membrane, referred to as the hydrophobic pore and presented in part (a) of Figure 3. If the external stimulation field is maintained, the theory predicts the hydrophobic pore will tend to expand as shown in part (b) of Figure 3, eventually reaching a critical radius which makes a different structural configuration energetically preferred. At this stage, the pore goes through a transition and becomes hydrophobic. This is manifested by the polar heads on the two adjacent layers connecting to each other and forming the structure
presented in part (c) of Figure 3. In this state, the membrane becomes permeable to external ions and molecules [65].

Finally, once the external stimulus is removed, assuming that the effect is reversible, the membrane reseals the pores and returns to original condition, shown in part (d) of Figure 3. If the effect is not reversible, the bi-layer membrane loses its structural integrity and never returns to its original state.

Electrical Parameters of Electroporation

The two key parameters which have major effect on the outcome and extent of electroporation procedure are: electric-field magnitude and pulse length. Additional parameters such as number of pulses, pulse frequency and pulse polarity are used to optimize electroporation protocols.

Electric-field magnitude

Based on experimental data reported in [63], electroporation was observed when the trans-membrane potential exceeded a threshold which was on the order of 200 mV. This implies that a certain threshold for the applied electric field exists, to induce electroporation. Besides determining whether electroporation would occur or not, the magnitude of the electric-field has an effect on the distribution of the pores across the cell. Kinosita et.al. [80] showed that to a certain extent, by modulating the strength of the electric field, the spatial distribution of the pores around the cell membrane can be controlled. To support this conclusion they leveraged voltage sensitive dyes to monitor the distribution of pores across the cell membrane in unfertilized sea urchin eggs.
Pulse length

The second key parameter determining the extent of electroporation is the duration of the applied pulse of elevated electric-field. Data presented in [81] supports the hypothesis that longer pulses of electric field lead to a higher degree of permeabilization. To make their conclusion, Gabriel and Teissie, the authors of [81], have utilized ethidium bromide and propidium iodide, two commonly used fluorescent dyes, to show that longer simulation pulses applied to Chinese hamster ovary cells result in higher dye intake.

Number of pulses and pulse frequency

Controlling the number of pulses and the temporal distance between pulses (i.e. the frequency) can be used to reduce the amount of Joule heating generated during the procedure. This can be important in both reversible and irreversible modalities of electroporation: in the reversible case, excessive heating can lead to thermal damage of the cells we are trying to electroporate, leading to lower yield, e.g. in gene transfection. Alternatively, in the irreversible case, excessive heat can cause damage to heat conducting tissues such as blood vessels, as well as increase the overall region which undergoes partial damage, making the intervention less focused. Additional evidence to support the importance of number of pulses was presented by L.M. Mir’s group [82]. In this study an increased expression of a gene delivered via electroporation was correlated to the number of electric pulses applied.

Pulse polarity

Pulse polarity is a parameter largely overlooked by the electroporation research. Most of the commonly used electroporation protocols involve a series of unipolar DC pulses applied to the sample. An interesting feature of electroporation is the inherent asymmetry of the process [83]. This may be explained by considering the natural resting trans-membrane potential, which is typically measured in the negative 100mV range. The existence of this negative resting potential makes the anodic side of the membrane experience the supra-threshold electric field earlier than the cathodic side of the membrane. An experimental study by Tekle et.al. [84] presents markedly higher efficiency transfection results by using bi-polar electroporation pulses. The next section on AC electroporation provides a brief overview of an alternative approach to stimulation.

AC Electroporation

To apply a pulse of electric field, earlier works have simply charged a capacitor to the desired voltage and then discharged it into the sample using a switch controlling the duration of the pulse [85]. The shape of such a pulse follows the classic exponential decay time-course and some electroporation devices today still offer exponential-decay shaped pulses (e.g. Gene Pulser XCell by BioRad). Having said that, most electroporation works reported these days in the literature employ unipolar, typically rectangular pulses. The advantage of using rectangular pulses is that it greatly simplifies the required electronics: depending on the amplitude and the power...
requirements, one could generate precise electroporating fields using nothing more than a micro-controller and a relay. This simplicity however, comes at a price. The inherent asymmetry of electroporation [83] suggests that a bipolar pulse sequence might lead to more effective applications, e.g. transfection. Over the last two decades, several works were reported which brought to front the electrolytic effects of electroporation. Although these effects are largely ignored in electroporation studies, when electric field is applied to a conductive sample, current runs through the volume of the sample and certain electrochemical reactions occur both around the electrodes and in the bulk of the sample. While this topic will be addressed and thoroughly discussed in the next section of this thesis, it is worth mentioning here that one of the typical by-products of electrolysis is the generation of gases. These gases are generated next to the electrodes and form bubbles. These bubbles create undesired effects, e.g. clogging of channels in micro-electroporation applications as reported in [86]. In their work, Ziv et.al. have showed that an AC electric-field at a specific range of frequencies led to electroporation, yet prevented the formation of undesired bubbles [86]. Another key complication of DC electroporation came to surface during clinical applications: in vivo electroporation of regions located in proximity to musculature causes muscle contractions [87, 88]. Davalos’ group have addressed this problem by replacing the DC pulse stimulation with Radio-Frequency AC electric-field [89]. Other, perhaps less dramatic advantages include improved transfection yields due to “sonication” effect [90], and more symmetric permeability [84].
Joule Heating and Other Related Effects

Most of the thermal ablation methods leverage the fundamental ohmic heating principle, which states that when electric current is run through a conductor, heat is dissipated in the conductor volume. Since most biological tissues are comprised of aqueous solutions of ionic compounds, from an electric standpoint they constitute a conductor. This means that electric field applied to a biological sample would lead to current flow, and therefore to heating. As a typical example, in RF ablation, the increase in temperature caused by the heating is used as the key mechanism to cause tissue damage. The main challenge with this method of damage infliction is the lack of selectivity. Because different tissues have different electric properties, they conduct electrical currents to different degrees. Some tissues, such as blood vessels and bile ducts, act as disproportionately effective conductors, creating heat sinks that can confound planned heating of target ablation zones. This possibility necessitates close follow-up imaging, and in some cases, a repeat RFA with larger treatment volumes—increasing the risk of damage to healthy tissue and resulting patient morbidity [6]. In contrast with thermal ablation methods, the key advantage of electroporation is its fundamentally different mechanism which acts on a molecular level on the membrane of the cell. In their seminal work, Davalos and Rubinsky [74] have shown that there exists a range of electrical parameters for which the electric field generated in the tissue causes irreversible electroporation without significant raise in temperature. This has essentially opened a way for a new ablation modality: Non Thermal Irreversible Electroporation (NTIRE), which has later grown to become a commercial product (NanoKnife by AngioDynamics). Besides being an undesired side-effect in irreversible electroporation, in reversible electroporation as well, ohmic heating associated with current running through the sample complicates the interpretation of results as the effect of heating cannot be eliminated. For this reason, transfection protocols, as well as other applications, are designed around a very low duty cycle. Specifically, this means that the protocol tends to involve a series of pulses separated enough from each other to allow the heat generated during each pulse to dissipate during the low part of the duty cycle.

In addition to the undesired joule heating, the second key group of effects occurring during electroporation falls into the electrochemical category. When current runs through a biological sample, certain reactions occur both around the electrodes, and in the bulk of the sample. These reactions were historically ignored in electroporation studies, due to the perceived insignificance of their effect. However, some earlier works [91], as well a series of more recent reports [28, 92, 93] bring awareness to the importance of considering electrolytic effects, as they can confound the interpretation of experimental findings. The topic of electrolysis is central to this thesis and it is systematically reviewed in the previous section.
Nano-Second Pulses

While the classical electroporation regimes employed pulses in the microsecond–millisecond range, a seminal work by Schoenbach’s group has demonstrated the first sub-microsecond in vitro study using very high electric field magnitudes [94, 95]. The differentiating factor of this line of research is based on the hypothesis that by using ultra-short pulses, the external membrane of the cell could be bypassed and the electroporation effect could be applied directly to the internal organelles. Later works, however, both theoretical and experimental, report results indicating that even in the nano-pulse regime, the external membrane of the cell still gets porated, although perhaps to a lesser extent [96]. An interesting point to note is that the discrepancy between the initial results which detected no pores at the membrane, and the later contradictory findings, stemmed from the limitations of the available tools used by electroporation researchers.

Research Methods and Tools

Numerous approaches to study electroporation were developed over the years. This section provides a brief overview of the different types of approaches in order to establish a context for their contribution.

Mathematical Models

Experimental observations of lipid bilayer membranes [78] have inspired multiple attempts to develop analytical models which would allow to predict the dynamics and extent of the electroporation process. A review of electroporation theories by Chen and colleagues [97] identifies three key conclusions based on the experimental electroporation data:

1. Membrane rupture is a stochastic process [63].
2. Increase in transmembrane potential corresponds with an increased probability of membrane to undergo rupture [98].
3. The outcome of the electroporation process is determined by the amplitude of the applied electric field [99].

Earlier models tried to approach electroporation from a deterministic standpoint and develop a precise, mechanistic description of the membrane rupture phenomenon. Representative examples include e.g. the theoretical work of Crowley [100] which attempted to estimate the rupture voltage by balancing the pressure exerted on the bilayer membrane by the electric field, and the elastic pressure of the membrane itself. The model predicts rupture voltages around 5V which is far beyond the observed voltage range (0.2V-1V) in lipid bilayers.

Another work taking the deterministic approach looked at the cellular membrane as a planar layer of dielectric fluid “locked” between two planar layers of conductive fluid. Michael and O’Neil [101] have considered the electrohydrodynamic instability of the
insulating fluid and its dependence on two types of perturbations: symmetric and asymmetric. A symmetric perturbation occurs when the two conducting fluids on both sides of the insulator are perturbed in phase with one another. Similarly, the asymmetric perturbation occurs when the two conducting fluids are out of phase with one another. The elasticity of the lipid membrane induces a constraint on the system which rules the asymmetric perturbations as unnatural since they would put the membrane in tension. This is to compare with symmetric perturbations which put the membrane in compression and are considered feasible. Based on these assumptions, the authors were able to solve for the critical transmembrane potential that would lead to rupture. While the solution is within the range of observed experimental data (0.375V), this model fails to explain the stochastic nature of the observations.

More recent models have tried to address and capture the inherent stochastic nature of the electroporation phenomenon. One of the more commonly cited group of models falls under the umbrella of the aqueous pore theory. This family of models accounts for the surface tension of the lipid bilayer membrane and the free-energy changes associated with having a water-filled pore evolve in the membrane [78, 102]. According to this theory, at the resting state (no external stimulation), the change in energy in a lipid bilayer membrane due to the formation of pores is given by

\[ \Delta W_p(r) = 2\gamma r - \Gamma \pi r^2, \]

where \( \gamma \) is the “edge energy”, i.e. the energy associated with a unit length of a pore edge, required to form the pore. \( r \) represents the radius of the pore, and \( \Gamma \) is a coefficient that quantifies the energy associated with surface area of the membrane. Observing equation (5), it is noteworthy that the formation of a circular edge contributes twice on the right side: firstly, there is an energy gain associated with the formation of the pore. Secondly, the loss of membrane area leads to energy loss which can be seen in the second term [78]. When an external transmembrane potential is applied to the lipid bilayer, the associated change in energy can be viewed as a change in the energy of a capacitor whose cross-section area has changed by the formation of the pore. Taking into account the membrane’s altered capacitance with its associated energy leads after modifying (5) to:

\[ \Delta W_p(r) = 2\gamma r - \Gamma \pi r^2 - 0.5 C_{LW} U^2 \pi r^2, \]

where \( C_{LW} \) is the new, post-pore formation capacitance of the membrane, and \( U \) represents the externally applied transmembrane voltage. Equation (6) attempts to formally capture the relationship between the pore radius and the transmembrane voltage, and the change in energy associated with the bilayer membrane [63]. By studying equation (6), it is possible to explain several multiple features of the electroporation process, such as e.g. its stochastic nature and the rupture potential.

Besides the macroscopic, energy-based models, asymptotic approaches to modeling the electroporation phenomenon have gained popularity in the literature [103-105]. The basic idea is to model the population of the pores evolving on the surface of the membrane and use the aggregated variables such as local pore density or number
of pores to estimate global, macroscopic properties, such as e.g. conductivity or mass transport coefficients.

**Molecular Dynamics Simulations**

While analytical models allow gaining a qualitative insight into the formation of water-filled pores in the membrane, they do not provide much information on the microscopic, molecular level. Molecular Dynamics Simulation (MDS) is a computational approach that enables to elucidate the micro-scale interactions between different molecules comprising the lipid bilayer membrane. The work of Tarek [106] has shown that water protrusions, or “fingers” penetrate the lipid bilayer from both of its sides until they unite and form a hydrophilic pore, as presented in Figure 4.

![Figure 4: Water "fingers", protrusions into the lipid bilayer shown by molecular dynamics simulation [106].](image)

A more recent work by Vernier’s team [107] has shown that under the influence of electric field, the water molecules, due to their natural dipole moment, reorient themselves in an energetically preferred configuration which further supports the computational model predicting water protrusions as well as the earlier analytical models.

**Dyes - General Purpose and Voltage-Sensitive**

Arguably the most common research method for detecting and measuring the dynamics of electroporation is optical microscopy aided by fluorescent dyes. Widely used dyes include Propidium Iodide, which is often used for cell viability essays. The operating principle is based on the observation that a normal cell, with an intact membrane, does not let the dye penetrate into the cytoplasm. If the membrane’s integrity has been altered e.g. via electroporation, the dye can then diffuse into the cell, and bind to the DNA inside it which causes it to emit light upon excitation. Another category of dyes is the voltage sensitive dyes used e.g. to get a spatial map of the transmembrane potential during the onset of electroporation [108].

While dyes seem to be the workhorse of the electroporation research field, recent advances in nano-pulse electroporation brought to light their inherent limitations. The fluorescent dyes based approach is limited by the mass transport process across the
cell membrane and depends on the dye size in relation to the pore size. It has been recently shown that smaller dye molecules could detect cell membrane permeabilization that is not observed with larger dyes [109]. Furthermore, if only a small amount of dye enters the cell, the emitted fluorescence is difficult to distinguish from the background with affordable and flexible imaging/detection systems. This issue has come to forefront with advances in nanosecond pulse cell permeabilization technology [110]. While there exists a report of successful fluorescence based imaging of nanopores [111], the authors had to preload the cells with a fluorophore and later use thallium as fluorescence enhancing agent. Moreover, when thallium is present in the extracellular bath, it can potentially permeate the cell through voltage gated potassium channels which further complicates the experimental setup due to the need to block the “classic” potassium channels by high concentrations of tetraethylammonium-like chemical agents.

The limitations of the dye-based methods have inspired a part of this thesis and led to a hypothesis that naturally present ions could be made to act as “markers” indicating the onset of electroporation. An appendix to this dissertation presents a systematic theoretical analysis and simulation results suggesting a new possible approach for studying electroporation on a single cell level [112].

Single Cell Electroporation

While bulk electroporation applications both in vitro and in vivo have become a widely spread tool at the bench and in the clinic, studying electroporation on a single cell level can provide some fundamental insights into the nature of the phenomenon. The approach was pioneered by the work of Huang and Rubinsky [113, 114] and it included the fabrication of a silicon chip with a small hole through which a cell was immobilized by applying negative pressure. In their work, the authors have detected the onset of electroporation by measuring the current through the cell. When the cell undergoes electroporation, the pores in its membrane allow external charge carriers to flow through the cytoplasm leading to increased current magnitude which can be easily detected with high temporal resolution. The limitation of this approach is in the bulky nature of the extracted information: since only the overall current flowing through the cell is measured, the spatial information pertaining to electroporation is lost. This shortcoming acted as one of the motivating aspects for this thesis: the outcome of the study attached in the appendix is a conceptual description and computational feasibility test for a research tool, which provides rich information in both the temporal and the spatial dimensions.

In recent years, single-cell electroporation has led to numerous publications, which focused on different design approaches for microfluidic-based devices for cell analysis, transfection or pasteurization [115].
**Imaging-Based Approaches**

While *in vitro* lab techniques have used optical microscopy with fluorescent dyes to detect electroporation, in the clinical setting, medical imaging is an extremely useful tool for monitoring the electroporation process. Most "classic" imaging modalities were applied to monitor electroporation, including ultrasound [116], CT [117] and MRI [118, 119]. In addition, a novel approach towards visualizing electroporation was developed in Rubinsky’s group by Granot et.al [120, 121]. Granot’s work was based on the hypothesis that electroporation causes a change in the conductivity of the affected tissue, due to the additional paths that ions can take after the membrane’s permeability has increased. The authors have used Electrical Impedance Tomography, a low-cost imaging approach which uses multiple AC current stimulations and voltage measurements in order to reconstruct a possible map of conductivity inside the tissue. This thesis was partially inspired by Granot’s work and has extended its conceptual reach to a single cell systems undergoing electroporation.

**Applications of Electroporation**

A pivotal point in the history of electroporation was the seminal study published by Neumann in which he has shown that it is possible to transfect mouse lyoma cells. The new wave of research which followed has transformed electroporation from a scientifically interesting electrophysiological phenomenon into a standard bench technique used in many biological labs.

Electroporation applications can be roughly classified into two categories: *reversible* electroporation and **irreversible** electroporation. Reversible electroporation applications include:

1. *In vitro* DNA transfection [122]
2. Cell fusion [123]
3. Electro-insertion of proteins into cellular membrane [124]
4. *In vivo* transdermal drug delivery [72, 87]
5. Electrochemotherapy [125, 126]
6. Localized *in vivo* gene therapy [82]
7. DNA transfer for vaccination [127]

The applications of the **irreversible** modality of electroporation have been developed and used since the 1960s, but have been focused on the food industry, and used for sterilization and pre-processing of foods [55]. The work of Rubinsky’s group brought to focus a new ablation modality which leverages the non-thermal regime of irreversible electroporation: NTIRE [73, 74]. Some of the more recent works included a feasibility study of singularity induced electroporation [79, 128]. The potential applications of such technology might include sterilization of liquids using ultra-low energy consumption. Another water sterilization targeted application was reported by Liu et.al. [129]. The
authors have used nano-sponges to generate very high electric fields using low level voltages, and have shown low energy consumption sterilization when compared to the state of the art.
Chapter 3: Fundamental Models

Mathematical Modeling of Electrolysis

The considered geometry of the problem can be seen in Figure 5. The radial axis is aligned with the direction of the electric field. This model geometry was chosen for a set of tools available through the COMSOL numerical analysis toolkit as it allows for an easier experimental validation. Validation of the model is demonstrated below.

![Figure 5: Electrolysis 1D model in cylindrical coordinates susceptible for numerical analysis and experimental validation.](image)

Our model is based on previous work reported in [22] and [28]. To estimate the electric field, as well as the concentration fields in this configuration, we solve two key equations: the Laplace equation, and the Nernst-Planck equations for four species: $Na^+$, $Cl^-$, $H^+$ and $OH^-$. The chemical reactions considered, are part of water electrolysis, where both the anodic and the cathodic reactions are accounted for. At the anode we have:

$$2H_2O \Leftrightarrow O_2 + 4H^+ + 4e^- \quad (7)$$

and

$$2Cl^- \Leftrightarrow Cl_2 + 2e^- \quad (8)$$

and at cathode we have:

$$2H_2O + 2e^- \Leftrightarrow H_2 + 2OH^- \quad (9)$$

Anodic reaction represented by (7) is the decomposition of water which causes oxygen evolution and the production of protons which causes reduction in the pH level. Anodic
reaction represented by (8) describes the oxidation of chloride ion leading to chlorine gas formation. At the cathodic side, the reaction represented by (9) describes the decomposition of water into hydrogen gas and hydroxide anions which result in increased pH level.

Each one of the reactions (7)-(9) is represented by a Nernst-Planck mass-transport equation:

\[
\frac{\partial C_i}{\partial t} = -\nabla N_i + R_i, 
\]

where \( C_i \) represents the concentration of the species \( i \) : \( \{H^+, OH^-, Na, Cl\} \). \( N_i \) represents the molar flux of the species \( i \) and is given by the equation:

\[
N_i = -D_i \nabla C_i + \frac{z_i}{|z_i|} u_i C_i \nabla \phi. 
\]

\( D_i, z_i \) and \( u_i \) represent the diffusion coefficient, the charge number, and the mobility of the species \( i \), respectively. \( \phi \) represents the local electric potential in the electrolyte. \( R_i \) represents the source term, resulting from the production of the species \( i \) as a by-product of a chemical reaction occurring in the system. In our model the two non-zero terms representing source terms come from the evolution of protons and hydroxide ions, thus we have:

\[
R_H = R_{OH} = k_{w,b} C_{H_2O} - k_{w,f} C_H + C_{OH}, 
\]

\[
R_{Na} = R_{Cl} = 0. 
\]

The constants \( k_{w,b} \) and \( k_{w,f} \) represent the rate constants of water hydrolysis in the backward and forward direction, respectively [28]. The ionic mobility is related to the diffusion coefficient via the Nernst-Einstein relationship:

\[
D_i = \frac{RT}{|z_i|F} u_i, 
\]

where \( R, T \) and \( F \) stand for universal gas constant, temperature and Faraday’s constant, respectively. To solve for the concentration of the species, we use the electro-neutrality hypothesis stated as:

\[
\Sigma_{i=1}^4 z_i C_i = 0. 
\]

Finally, to solve for the electric field, Faraday’s law coupled with the conservation of current in the electrolyte contributes the last equation:

\[
\Sigma_{i=1}^4 z_i D_i \nabla^2 C_i + \nabla \left( \Sigma_{i=1}^4 z_i C_i \Sigma_{i=1}^4 z_i \nabla \phi \right) = 0. 
\]

while electrolysis has been conveniently ignored.

**Boundary Conditions and Implementation Approach**

To solve the equations, boundary conditions are prescribed based on the chemical reactions occurring at the surface of the electrodes.
The partial current density $I_1$ matches the proton production and oxygen evolution next to the anode, described by reaction (17):

$$I_1 = I_1^{eq} \left\{ \exp \left[ -\frac{F(\phi + E_1^{eq})}{2RT} \right] - \left( \frac{C_{H^+}^0}{C_{H^+}} \right) \exp \left[ \frac{F(\phi + E_1^{eq})}{2RT} \right] \right\}. \quad (17)$$

The partial current density $I_2$ matches the chlorine production next to the anode, described by reaction (18):

$$I_2 = I_2^{eq} \left\{ \left( \frac{C_{Cl^-}^0}{C_{Cl^-}} \right) \exp \left[ -\frac{F(\phi + E_2^{eq})}{2RT} \right] - \exp \left[ \frac{F(\phi + E_2^{eq})}{2RT} \right] \right\}. \quad (18)$$

The partial current density $I_3$ corresponds to the hydroxide ion production next to the cathode, described by reaction (19):

$$I_3 = I_3^{eq} \left\{ \left( \frac{C_{OH^-}^0}{C_{OH^-}} \right) \exp \left[ -\frac{F(\phi + E_3^{eq})}{2RT} \right] - \exp \left[ \frac{F(\phi + E_3^{eq})}{2RT} \right] \right\}. \quad (19)$$

In equations (17)-(19), $I_j^{eq}$ represents the current exchange density for reaction (17) at equilibrium. $E_j^{eq}$ represents the potential difference between the solid electrode phase and the liquid electrolyte phase. $C_j^0$ represents the initial concentration of the species $j$.

The system of equations (10)-(16) was solved with the boundary conditions (17)-(19) over a time interval of $[0..T]$. The initial conditions were given by considering no concentration gradients in the bulk of the electrolyte. The concentrations of protons and hydroxide ions were set to correspond to neutral pH of 7. The concentration of both sodium and chlorine, were set to 0.16 mol/liter modeling standard physiological saline of 0.9% w/v.

In general, to solve for the electric field, the conservation of current equation is solved:

$$\nabla \cdot \vec{j} = 0. \quad (20)$$

where $\nabla \cdot ()$ is the divergence operator, and $\vec{j}$ stands for the local current density vector. The current density has a conductive and a displacement components and is given by:

$$\vec{j} = (\sigma + \varepsilon_0 \varepsilon_r \frac{\partial}{\partial t}) \vec{E}, \quad (21)$$

where $\vec{E}$ represents the local electric field, $\sigma$ is the conductivity, $\varepsilon_0$ is the vacuum permittivity and $\varepsilon_r$ is the relative permittivity of the material. The electric field is linked to the potential field by the relationship:
\[
\vec{E} = -\nabla U.
\]  
(22)

In our model, for the 1-dimensional case, equation (22) simplifies to

\[
\vec{E} = \frac{(V_1 - V_2)}{d},
\]
(23)

where \(V_1\) denotes the potential at the anode, \(V_2\) denotes the potential at the cathode and \(d\) is the distance between the electrodes.

The field equation was solved for the geometrical configuration of Figure 5 subject to Dirichlet boundary condition (voltage), imposed at the electroporation electrodes.

Solving the Laplace equation makes it possible to estimate the associated Joule heating \(P\), which is the heat generation rate per unit volume, caused by the electrical field:

\[
P = \sigma |\nabla \phi|^2.
\]
(24)

In our model, for the 1-dimensional case, equation (24) simplifies to

\[
\vec{P} = \sigma (V_1 - V_2)^2,
\]
(25)

where \(\sigma\) represents the conductivity of the sample we are electro-stimulating.

| \(C^0_{H^+}\) | Initial concentration of protons | 1e-7 mol/liter |
| \(C^0_{OH^-}\) | Initial concentration of hydroxide ions | 1e-7 mol/liter |
| \(C^0_{Cl^-}\) | Initial concentration of chlorine ions | 0.16 mol/liter |
| \(C^0_{Na^+}\) | Initial concentration of sodium ions | 0.16 mol/liter |
| \(C^0_{H_2O}\) | Initial concentration of water | 55.5 mol/liter |
| \(D_{H^+}\) | Proton diffusion coefficient | 6.25e-9 \(m^2/s\) |
| \(D_{Na^+}\) | Sodium diffusion coefficient | 0.89e-9 \(m^2/s\) |
| \(D_{Cl^-}\) | Chlorine diffusion coefficient | 1.36e-9 \(m^2/s\) |
| \(D_{OH^-}\) | Hydroxide diffusion coefficient | 3.52e-9 \(m^2/s\) |
| \(I^{eq}_{1}\) | Current exchange density for reaction (1) | 1e-8A/m |
| \(I^{eq}_{2}\) | Current exchange density for reaction (2) | 1e-5A/m |
| \(I^{eq}_{3}\) | Current exchange density for reaction (3) | 1e-6A/m |
| \(E^{eq}_{1}\) | Potential difference for reaction (1) | 1.23V |
| \( E^\text{eq}_2 \) | Potential difference for reaction (2) | 1.36V |
| \( E^\text{eq}_3 \) | Potential difference for reaction (3) | -0.828V |
| \( k_{w,b} \) | Backward reaction coefficient | 2.7e-5 [1/s] |
| \( k_{w,f} \) | Forward reaction coefficient | 1.5e11 liter/(mol s) |
| \( T \) | System temperature | 300K° |

Table 1: Electrolysis Modeling Parameters Numerical Simulation Results

Experimental Procedure and Results

To validate the \textit{in silico} model, we have compared the numerical simulation results with the observed propagation of the pH sensitive dye (Hagen RC wide range). An optical image of the Petri dish containing agar gel impregnated with 1% pH sensitive dye was stimulated using 1mA direct current for 15 minutes. The electrodes were positioned in accordance with the cylindrical coordinate system: a centric anode (graphite rod) and a distributed anode at the periphery of the Petri dish (copper tape glued to the internal sidewall of the dish). Every 4 minutes an optical image of the Petri dish was acquired. These images are presented in parts (a)-(d) of Figure 7. Part (f) of Figure 7 presents a color legend provided by the pH sensitive dye manufacturer for mapping the color to a pH range. The radius of the acidic front propagating from the anode was measured from the optical images and plotted vs. time. Part (e) of Figure 7 compares between the simulated front and the measured front using pH sensitive dye and a good qualitative agreement is observed.
Figure 6: pH evolution in 1D.
The purpose of this section is to motivate taking a deeper look into electrolysis. By presenting the context for studying electrolysis, we create a natural transition into developing tools and applications of electrolysis based approaches.

The Rising Importance of Electrolysis

Electrolytic effects have been conveniently ignored by earlier works focusing on pulsed electric fields. The induced fields were considered brief enough and the resulting currents small enough to ignore changes in pH occurring due to electrolytic processes arising in the tissue. Recent research findings, however, indicate that under some practical conditions, the observed effects which were believed to be driven by reversible electroporation can be traced to electrolysis [92]. In a series of works, Marshall's lab have shown both in silico and in vitro that electro pulsed stimulation causes significant local changes in pH which introduces additional fundamental effects into the picture [28, 40, 92, 93].

To demonstrate why this is becoming an increasingly interesting problem one needs to consider advances in micro-electromechanical technology which have made it possible to design a single cell micro-electroporation chip [114]. This development has paved the way to the flow-through single cell micro-electroporation chip [130]. Microelectroporation is an important component in many labs on chip devices [131]. Attempts to further miniaturize electroporation have led to devices that electroporate on
only part of the cell membrane [132], and to nano-electroporation [128]. Literature survey indicates a growing interest in micro-electroporation, and a thorough review of flow-through micro and nano/electroporation devices can be found in [133].

However, in the context of micro-electroporation, as the size of the effective electroporation volume reduces, geometric considerations begin to play an important role. The effective electroporation volume decreases proportionally to $s^3$, whereas the active surface area approximately decreases proportionally to $s^2$; where $s$, is a typical dimension for electroporation. This leads to increased dominance of the mass transport phenomena prompted by electrolysis, since the relative concentrations of the reaction products are higher in smaller scale electroporation chambers.

**Practical demonstration of electrolysis**

An example of electrolysis observed while applying electroporation can be observed in Figure 8. A planar inter-digital electrode was covered in an agar gel impregnated with a pH sensitive dye. After a few electroporation pulses using a standard protocol, the gel changes color indicating a local change in pH.
Chapter 4: Monitoring Electrolysis by Electrical Impedance Tomography

Introduction to Electrical Impedance Tomography (EIT)

EIT Concepts

To illustrate the basic concept of EIT, it is easier to start with a simple example: given a resistor of unknown value, it would be possible to determine the resistance by injecting a known current through the resistor and measuring the resulting voltage that develops on it. Ohm’s law defines a linear relationship between the voltage and the current and the unknown resistance values can be extracted. In a more complex example, when the resistor is replaced with a sample that has a finite volume of non-homogenous resistivity, the problem becomes more complicated yet the fundamental approach remains the same: inject known currents through the sample and measure the developed voltages. By capturing the relationship between the currents and the voltages into a system of algebraic equations, it is possible under some conditions to solve for the unknown quantity, i.e. the local impedance. This is the way EIT is typically characterized: a technique that produces an image of spatial distribution of electrical impedance based on a set of measurements obtained with an electrode array at the periphery of the object [134]. Due to the differential impedance properties of different tissues, as well as the differential impedance properties of normal tissues and pathologies, EIT has been used as a tool in the biomedical imaging community for research and diagnostics [135].

The way a typical EIT system works is by injecting a series of known currents through pairs of electrodes attached to the tissue sample and the voltages arising at the other electrodes are measured. Under the assumption that the tissue sample contains no sources of charge, the governing relationship that connects the local impedance with the electrical potential in the volume is given by Laplace’s equation. The boundary conditions for the equation depend on the stimulation protocol, and typically include the injected stimulation currents. When the local impedance is known, calculating the potential that develops on the boundary as a result of a known current injection is called the Forward problem. The forward problem is a relatively straight-forward one, and can be solved e.g. using finite element based methods. The reverse, however, also referred to as the Inverse problem, which involves solving for the unknown impedances based on known injected currents and periphery measurements, turns out to be mathematically ill-posed and does not have a unique solution for every input [136]. This means that two different internal conductivity distributions can result in identical boundary measurements. Moreover, small changes in the impedance can result in large swings in the observed voltages measured at the periphery.

To overcome the ill-posed nature of the problem, a typical approach involves the inclusion of prior knowledge, which often appears as a regularization term in the system of equations that is solved. The prior knowledge can "encode" some properties of the solution, e.g. a common prior knowledge that is used for EIT reconstruction assumes that the impedance distribution is a slowly changing spatial function, i.e. that the
impedance doesn't change much between two neighboring tissue parts. This type of prior knowledge constraints the absolute values of its gradient of the impedance function, however it also comes at a price of reduced resolution. Small inhomogeneities become difficult to detect and borders between two regions with different impedance become blurred. What this means is that EIT produces images with inherently lower resolution compared to this of CT or MRI modalities. This limitation alongside other drawbacks have prevented EIT from becoming an ubiquitous tool. Despite the speed and the low cost compared to other imaging modalities, EIT was not able to compensate for the inherently low resolution. One of the results of this thesis included demonstrating the feasibility of EIT to image electrolytic processes, which might expand the range of application of EIT-based techniques and make them more available in a research and/or clinical environment.

Mathematical Aspects of EIT

The next sections describe the more practical sides of EIT, and they were adapted from [137].

Forward Problem

EIT can be conceptually divided into two parts: the forward problem and the inverse problem. Nature solves the forward problem for us - given a conductivity map, we inject known current patterns into a pair of electrodes and measure the voltages developing on other electrodes. Various measurement protocols have been devised, among which adjacent or opposite measurement protocols seem to be widely used [135]. Mathematically speaking, since we assume no internal current sources, solving the forward problem is equivalent to solving the Laplace equation for a given geometry with boundary conditions determined by our injection patterns:

\[ \nabla \cdot (\sigma(x,y,z)\nabla U(x,y,z)) = 0, \]  

(26)

where \( U(x,y,z) \) is the electric potential sampled at a given point in our domain, and \( \sigma(x,y,z) \) is the conductivity at a given point. Equation (26) stems from a continuum form of Ohm’s law which can be written as:

\[ \vec{J}(x,y,z) = \sigma(x,y,z)\vec{E}(x,y,z), \]  

(27)

where \( \vec{J}(x,y,z) \) denotes the current density vector at a given point in space and \( \vec{E}(x,y,z) \) denotes the electric field in that point. Equation (27) can be derived from (26) by taking the expression of the electric field as the gradient of the potential scalar field and presenting the current conservation law (Kirchoff’s law) in its continuum’s version:
\[
\n\nabla \cdot \mathbf{j}(x, y, z) = 0. \tag{28}
\]

For simplicity, we assume here that the conductivity \( \sigma \) is isotropic, \textit{i.e.} it can be represented as a single scalar. An extension to the general case is straight-forward. Typically, an analytic solution for (26) is tractable only for a limited number of cases, and in the general case, numerical methods are used in practice. A useful tool, commonly applied to solve (26) is the Finite Element Method (FEM), which approximates the problem by basically breaking down the domain into a collection of small elements of the appropriate dimension, and assuming that each element is small enough so that the potential across it can be considered constant. For each single element, the problem can be easily solved \cite{138}. The boundary conditions for (26) typically include electrical insulation (zero normal current density) for most of the domain and prescribed current density for the injection electrodes, typically denoted as

\[
\sigma \nabla U(x, y, z) \cdot \mathbf{n} = j_0, \tag{29}
\]

where \( j_0 \) is the value of the current density prescribed by the stimulation protocol and the geometry in the case of stimulation electrodes, and zero in the case of insulated boundary.

The following implementation of the mathematical model of EIT has been adopted from \cite{137}. In the implementation of the model described above, we use \( M \) electrodes that are described by the complete electrode model (CEM) \cite{139}. This model states that for each electrode \( e_i \), \( i = 1, 2, \ldots , M \), the injected current \( I_i \) equals to the flux of the current density under the electrode:

\[
\int_{e_i} \sigma \frac{\delta U}{\delta n} = I_i \tag{30}
\]

where \( n \) is the normal vector to the surface under the electrode. The voltage measurement electrodes always have \( I_i = 0 \) and for the current injection electrodes, it may take any value, including zero according to the prescribed current stimulation protocol. The current stimulation protocol is defined as a column vector of all the currents injected through the electrodes: \( I = [I_i] \), \( i = 1, 2 \ldots , M \). We name the current injection electrodes \( E_i \), and the set of voltage measurement electrodes \( V_i \). For areas that are not directly under any electrode, we assume perfect insulation, \textit{i.e.} no current enters or leaves the tissue.

\[
\sigma \frac{\delta U}{\delta n} = 0 \tag{31}
\]

The CEM model takes into account the conductance of the electrodes (a.k.a the shunting effect), as well as their impedance. The potential on the electrode \( e_i, V_i \) is not identical to the potential under the electrode \( U \) but rather:
\[ V_i = U + z_i \sigma \frac{\delta U}{\delta n} \]  

where \( z_i \) is the electrode’s contact impedance, assumed to be greater than 0. Another condition is a reformulation of the conservation of charge and it states that the total current, through all the electrodes is zero:

\[ \sum_{i \in E_i} I_i = 0 \]  

Finally, in order to find a unique solution, we add a condition that the electrode voltages satisfy the equation

\[ \sum_{l \in E_v} V_l = 0 \]  

Finite element method was used to solve for the voltages on all the electrodes \( V^p = [V_l^p], l = 1, 2 \ldots M \), which result from applying the stimulation current pattern \( I^p \). The tissue is divided into small triangular elements of different sizes, with a total of \( N \) nodes. An example of what a standard mesh may look like can be seen in Figure 9. In the figure, there are 32 electrodes represented by green round circles. Notice that the size of the elements near the electrodes is much smaller. This is designed to increase the accuracy of the solution, i.e. to accommodate for the fact that the current density is...
typically higher around the electrodes. We define a set of basis functions \( \psi_k, k = 1, 2 \ldots N \) where \( \psi_k \) gets the value of 1 at a node \( k \) and 0 at every other node.

In a way aligned with numerous finite element methods, we now formulate our problem in terms of the basis functions \( \psi_k \), i.e. we are looking for an approximate solution to the electric potential \( U, \bar{U} \) which is defined as:

\[
\bar{U} = \sum_{k=1}^{N} a_k \cdot \psi_k \tag{35}
\]

so that for the correct choice of \( a_k \), Laplace’s equation and the prescribed boundary conditions would be satisfied with minimal error. A possible criterion for qualifying the error would be requiring that over the entire domain \( \Omega \), which is the tissue we examine, the weighted average of the errors disappears. The weighting can be done by taking \( N \) weighting functions, \( w_k, k = 1, 2 \ldots N \) and imposing a constraint:

\[
\int_\Omega [\nabla (\sigma \nabla \bar{U})] w_k = 0 \tag{36}
\]

An approach often used by EIT literature [140], a.k.a. the Galerkin method, suggests taking the weighting functions \( w_k \) to be identical to the basis functions themselves \( \psi_k \), so that for every index \( k = 1, 2 \ldots N \) we have:

\[
\int_\Omega [\nabla (\sigma \nabla U)] \psi_k = 0 \tag{37}
\]

Using vector calculus identity that states that

\[
\nabla (a \cdot b) = b \nabla a + a \nabla b \tag{38}
\]

we can expand equation (37) and get

\[
\int_\Omega [\nabla (\sigma \nabla U)] \psi_k = \int_\Omega \nabla [(\sigma \nabla U) \psi_k] - \int_\Omega (\nabla \psi_k) (\sigma \nabla U) = 0 \tag{39}
\]

Applying Gauss theorem on (39) leads to transforming the first expression on the RHS to:

\[
\int_\Omega \nabla [(\sigma \nabla U) \psi_k] = \int_\Omega (\sigma \nabla U) \psi_k \cdot n = \int_\Omega \sigma \cdot \psi_k \frac{\delta \bar{U}}{\delta n} \tag{40}
\]

Introducing equation (40) into (38) yields

\[
\int_\Omega (\nabla \psi_k) (\sigma \nabla U) - \int_\Omega \sigma \cdot \psi_k \frac{\delta \bar{U}}{\delta n} = 0 \tag{41}
\]

Remembering that the approximated solution \( \bar{U} \) is given by equation (35) and we now require it to satisfy all the same constraints that are satisfied by the solution \( U \). If we
use equation (31) this means that the second integral in equation (41) vanishes almost for all elements, besides perhaps the elements adjacent to the electrodes, where CEM induced equation (32) holds. Expanding the approximated solution using equation (35) and substituting the expanded form into equation (41) we get:

\[
\int_{\Omega} (\nabla \psi_k)(\sigma \nabla \sum_{j=1}^{N} a_j \cdot \psi_j) - \sum_{i=1}^{M} \int_{\delta \Omega} \psi_k \frac{V_i - \sum_{j=1}^{N} a_j \cdot \psi_j}{z_i} = 0
\]

Expanding (42) further we arrive at:

\[
\sum_{j=1}^{N} a_j \left[ \int_{\Omega} (\nabla \psi_k)(\sigma \nabla \psi_j) + \sum_{i=1}^{M} \int_{\delta \Omega} \frac{\psi_k \psi_j}{z_i} \right] - \sum_{i=1}^{M} \frac{V_i}{z_i} \int_{\delta \Omega} \psi_k = 0
\]

Using the same approach to reformulate the boundary conditions we introduce the approximate solution \( \bar{U} \) and get another set of linear equations:

\[
l_i = \int_{e_i} \frac{V_i - \sum_{j=1}^{N} a_j \cdot \psi_j}{z_i} = V_i \int_{e_i} \frac{1}{z_i} + \sum_{j=1}^{N} a_j \int_{e_i} \psi_j = 0
\]

To make sure that the resulting system of equations has a solution, we will note that the number of the unknowns is \( N + M \) and the unknowns are the coefficients of the approximation \( a_j, j = 1,2,..N \) and the voltages at the mesh nodes \( V_i, i = 1,2,..M \).

We formulate the system of equations in a matrix-based form:

\[
\begin{bmatrix}
K & K' \\
K' T & K''
\end{bmatrix}
\begin{bmatrix}
a \\
V
\end{bmatrix}
= Y
\begin{bmatrix}
a \\
V
\end{bmatrix} = \begin{bmatrix} 0 \\
I \end{bmatrix}
\]

(45)

where the coefficient matrices are given by:

\[
K_{kj} = \int_{\Omega} \sigma \nabla \psi_k \nabla \psi_j + \sum_{i=1}^{M} \int_{\delta \Omega} \frac{\psi_k \psi_j}{z_i}
\]

(46)

\[
K_{ki} = \frac{1}{z_i} \int_{\delta \Omega} \psi_k
\]

(47)
\[ K_{kl} = \begin{cases} \frac{S_i}{z_i} & , \ l = i \\ 0 & , \ l \neq i \end{cases} \]

The matrix \( Y \) will denote the coefficient matrix \( \begin{bmatrix} K & K' \\ K'^T & K'' \end{bmatrix} \) and we observe that by inverting its inverse we can solve the forward problem, i.e. given the impedance distribution and the stimulation pattern, we can find what is the external voltage that is measured on the boundary.

**Inverse Problem**

While the forward problem is well-defined in mathematical sense and admits a single solution given the appropriate boundary conditions, it is the inverse problem that makes EIT useful, but also difficult. Solving the inverse problem means that given a set of measurements, one has to find the conductivity pattern leading to it. The problem is ill-posed [134], which means that without any additional constraints, multiple solutions (conductivity maps) might lead to the same set of measurements. To deal with this, additional constraints are introduced in the reconstruction algorithm, typically taking the form of a regularization term [141], which implies certain conditions on the behavior of the solution. A typical regularization term can be Total Variation (TV) regularization, which implies a certain degree of smoothness on the solution. While this additional constraint makes the EIT algorithm converge and reach a solution, this imposes a fundamental limit on the resolution of the system as it biases the reconstruction scheme to prefer solutions that do not exhibit sharp conductivity transitions between domain boundaries (e.g. between the extracellular fluid and the cytosol). This leads to a natural question of value of such a solution, since in bio-medical scenarios, sharp transitions between conductivity values arise quite regularly and it is very important to image them for accurate representation of the studied phenomena. To address the issue of sharp transition fidelity, a front-tracking reconstruction approach can be applied [142]. In the front-tracking approach, instead of attempting to find the impedance of each image pixel, the technique reconstructs the shape of the boundary. This approach allows incorporating the prior knowledge of the solution’s properties into the reconstruction scheme. Front tracking methods have been applied to other FEM based reconstruction problems in optical tomography [143] as well as in EIT [144].

To make things more concrete, let us present a possible solution for the inverse problem. We would start with an initial guess \( \sigma_0 \) for the conductivity, solve the forward problem and compare the results of the calculated voltages to the voltages that are measured by the system. The current guess for conductivity \( \sigma_i \) is then changed until the error in the electrode voltages becomes small enough and we assume that the
error in $\sigma$ is also small. The value of $\sigma$ at iteration $i + 1$ is labeled as $\sigma^{(i+1)}$ and is computed iteratively from the formula:

$$\sigma^{(i+1)} = \sigma^{(i)} + \delta\sigma$$  \hspace{1cm} (49)

where the best estimate for $\delta\sigma$ is what we are actively seeking. To obtain a reliable solution, we would like to match several current patterns to their voltage measurements. Different current patterns result in diversified current densities inside the body and that in turn provides more information on the conductivity of various areas in the tissue. Fortunately the computational cost of calculating the voltages from a given current pattern is not high. Once we have the inverse of $Y$, we can easily find the voltage resulting from different current patterns. In this model, we assume that half of the electrodes are used for current injection and half are used for voltage measurement. If we use $M$ electrodes we obtain $\frac{M}{2}$ voltages on the measuring electrodes. We take one of the voltage measurement electrodes to be the reference electrode (ground), and we measure all the other electrodes with reference to it. To obtain these relative voltages from our voltage vector $V$ we use a $\left(\frac{M}{2} - 1\right) \times M$ measurement matrix:

$$Q = \begin{bmatrix}
1 & 0 & -1 & 0 & 0 & 0 & 0 & \cdots & 0 \\
1 & 0 & 0 & 0 & -1 & 0 & 0 & \cdots & 0 \\
1 & 0 & 0 & 0 & 0 & 0 & -1 & \cdots & 0 \\
1 & 0 & 0 & 0 & 0 & 0 & 0 & \cdots & 0 \\
1 & 0 & 0 & 0 & 0 & 0 & 0 & \cdots & 0 \\
\vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \ddots & \vdots \\
1 & 0 & 0 & 0 & 0 & 0 & 0 & \cdots & -1 \\
1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0
\end{bmatrix}$$  \hspace{1cm} (50)

Note that all the even numbered columns are zeros since we are not using the voltages on the current injecting electrodes. The column vector $T$ then gives the voltages calculated from the forward solution run on the current estimate of the conductivity $\sigma$:

$$T = QV$$  \hspace{1cm} (51)

The vector $H$ contains the observed measurements, which we get from the physical instrumentation system. By comparing $T$ and $H$ we would like to arrive at $\delta\sigma$ and ultimately the estimate of $\sigma$. Unfortunately, the inverse problem, as was mentioned earlier, is ill-posed and so we need to apply a regularization approach to obtain a reliable solution [136, 145]. Since the set of equations is underdetermined, there are many possible solutions that will satisfy the equations. We therefore seek a solution that will also satisfy another condition that emanates from some a priori knowledge. In mathematical terms, we would like to minimize some penalty function simultaneously with the solution of the equations. This allows us to choose one of the many possible conductivity distributions that satisfy the equations according to an additional criterion.
The subject of choosing a good criterion is the topic of much research in EIT [146] and depends on the specific a priori knowledge available for every application. One general criterion which is used very often is the smoothness criterion that was mentioned earlier in this chapter. It is usually beneficial to assume that the conductivity changes very slowly so that the conductivity image appears smooth. This can be represented mathematically as a very small gradient, so minimizing the spatial derivatives of the conductivity function is commonly used for regularization. Sometimes the a priori knowledge we have is very reliable, however there are cases where we have only limited knowledge since the problem is not defined very well, or perhaps the physical system is not well understood. We therefore need some coefficient, usually called the regularization parameter that will define the importance of the regularization matrix with respect to the solution of the set of equations. If we define $H$ as the solution to the set of homogenous equations, instead of minimizing $\|H(\sigma)\|^2$, we now would like to find $\sigma$ so that $\|H(\sigma)\|^2 + \alpha\|L\sigma\|^2$ is minimized, where $L$ is the regularization matrix and $\alpha$ is the regularization parameter. As the regularization parameter increases, the solution will tend to minimize the regularization matrix rather than the set of equations. This is a good example of why choosing the regularization matrix and the regularization parameter is extremely important. In fact, when the regularization parameter is too high, the solution represents the a priori information and may disregard the measurements altogether, so that every solution is essentially similar, no matter what the measurements were. We use a regularization matrix $L$ and a regularization parameter $\alpha$ to obtain

$$\delta \sigma = (J^TJ + \alpha LL^T)^{-1}(J^TH - T) - \alpha LL^T\sigma$$

(52)

$T$ is written here in the vector form and $H$ is the measured voltage vector. $J$ is the Jacobian, the changes in the measured voltages caused by small changes in the conductivity of each element. To compute it, we look at each component from the voltage measurement vector $T$, name it $t_i$, and each element’s conductivity $\sigma_j$ and compute. The value of $t_i$ is a function of $I$, $Y^{-1}$ and $Q$, however we need to take specific vectors to obtain $t_i$ for a certain $i$. We need to define two zero padded vectors so we can use the matrix $Y^{-1}$. These vectors both have $N$ zeros: $\tilde{I} = [0\ I]$. $T$ and $Q_k$ which is the zeros padded version of the $k$-th row of $Q$. where $k = k(i)$ is the matching row for $t_i$. Then the Jacobian is

$$J_{i,j} = \frac{\delta t_i}{\delta \sigma_j} = \frac{\delta (Q_kY^{-1}\tilde{I})}{\delta \sigma_j}$$

(53)

Since $Q$ and $I$ are independent of $\sigma_j$, we take the partial derivative of $Y^{-1}$ and remember that it is symmetric.

$$J_{i,j} = Q_k \frac{\delta Y^{-1}}{\delta \sigma_j} \tilde{I} = -Q_k Y^{-1} \frac{\delta Y}{\delta \sigma_j} Y^{-1} \tilde{I} = -(Y^{-1}Q_k^T)\frac{\delta Y}{\delta \sigma_j} Y^{-1} \tilde{I}$$

(54)
We can see from this result that most of the terms needed for the Jacobian are already available. First, \[ \begin{bmatrix} 0 \\ \nu \end{bmatrix} = Y^{-1} \tilde{I}. \] Furthermore, the matrix \( K \) of equation (45) is the only part of \( Y \) that depends on \( \sigma \), so the partial derivative is also quickly obtained. Finally, we have found a solution \( \sigma \) that satisfies the equations derived above and at the same time agrees with our a priori knowledge of the problem, as it is represented by the regularization matrix.

**EIT Instrumentation**

The implementation of a typical EIT system is depicted in Figure 10. The sample and the surrounding electrodes are represented by a circle in the right part of the diagram. The electronic components that are implemented on a printed circuit board are represented by the shaded area in gray. This main board is connected to the electrodes and sample on one side and to an amplifier and a laptop on the other. The system contains 32 stainless steel electrodes surrounding a circular liquid container (diameter = 65 mm) where 15 electrodes are used for current injection, one for current sink, and 16 for voltage measurements. To generate the AC signals we have used a signal generator based on a set of low cost micro-controllers (μC), which have square signals as an output. To obtain a more narrowband sinusoidal signal we used second order low-pass filters (LPFs) with a quality factor (Q) of 4, which were centered at the appropriate frequency. A different filter was used for each current source and tuned appropriately for the frequency of that current source. To make sure that the signal has a constant current, or at least a relatively constant output current, without complicating the system very much, we used 100 k output resistors. Such a large output resistance makes the resistance of the entire system quite constant, since the resistance of the sample we are imaging is usually not very large. The sum of the output resistance and the system’s actual resistance is thus always close to that of the output resistance and does not vary a lot. This simple scheme does create some error due to the impedance of the sample and the electrodes. However, in the experiments presented here with saline solutions and gels, such error is much lower than 1% and does not create significant problems. After injecting the AC signals, we need to measure the voltage at all of the voltage measurement electrodes. The demodulation process is performed by a commercial lock-in amplifier (Model 7280 BFP, Signal Recovery, Oak Ridge, TN, USA). The task of this amplifier is to measure the voltage at a specific frequency, which is specified by a reference signal. The measurements with such an amplifier are in phase with the reference signal, so our measured signal has both the amplitude and phase information. To measure all of the signals with a single lock-in amplifier we have used a 1:15 multiplexer (MUX) to select one of the current generation signals as the reference signal (Ref) for the demodulation. An additional 2:16 multiplexer was used to select the voltage measurement pairs. The measurements are read by a PC laptop (IBM thinkpad T43) that is also responsible for controlling the multiplexer’s signals.
Original Study: EIT of Electrolysis

Research Context

Tissue ablation with minimally invasive surgery is important for treatment of many diseases and has an increasing role in treatment of solid neoplasms. A variety of biophysical and biochemical processes are used for this purpose. They include thermal ablation with heating, cooling or freezing, electroporation, injection of chemical agents, photodynamic effects, sonoporation effects and many others. Electrolysis, the passage of a low magnitude direct ionic current through the tissue, between two electrodes, is a biochemical/biophysical process that has been considered for tissue ablation since the 19th century [9]. Electrolysis affects the ionic species in tissue, which change into compounds that can ablate cells. The advantage of electrolysis in comparison to other ablation techniques can be attributed to its simplicity and low cost of instrumentation, which might make it a suitable treatment modality for resource constrained communities where more expensive medical treatment is often not available [147].

The work of Nordenstrom and colleagues [12, 148] is among the early modern work on electrolysis. Important recent work has been published on understanding of the effects of electrolysis on tissue through histology, mathematical modeling of involved electrochemical processes, and clinical work, e.g. [21, 31, 149-157] and [14, 15, 18, 20]. Several findings were made and several research techniques were developed that have
inspired this paper: it was shown that the electrolysis induced pH changes can be used to reliably monitor the extent of tissue ablation [38]. These findings have led to several basic studies on quantifying the process of electrolysis through the use of transparent gels with pH dyes [155, 158, 159].

While minimally invasive thermal ablation is now commonly used in surgery, “it has become common since the advent of modern imaging” [160]. Electrolysis is currently limited by the lack of an effective means to monitor the extent of tissue ablation deep in the body. The finding that pH changes are indicative of electrolytic tissue ablation [38], and the interesting results obtained with pH dyes marked gels [28, 92, 93], have suggested to us a way to monitor and image electrolysis. The pH fronts developing during the electrolysis process [28, 149] are caused by evolution of protons (H+) and hydroxide (OH-) ions. While the relationship between pH level and electric conductivity is not straight-forward and depends on relative concentrations of other ions, the increased concentrations of protons and hydroxide ions would affect the local electrical conductivity of the tissue being electro-treated. The smaller relative sizes of the proton and the hydroxide ions could lead to a higher mobility compared to that of other ions typically found in biological solutions – we address this question further in the later discussion section. Historically, one of the foci of our lab’s work has been in the field of electroporation, i.e. the effect of applying brief pulses of high-magnitude electric field to a tissue [161, 162]. The central hypothesis of this work is based on numerous empirical observations by our group of conductivity changes during electro-stimulation occurring even when the stimulation voltage was low and did not cause electroporation. We hypothesize in this work that the pH change-induced changes in electric conductivity, could be used to detect, monitor or image the process of electrolysis deep in the body, in real time. It was further hypothesized that one possible technique to image electrolysis induced pH changes in real time, deep in the body, is Electrical Impedance Tomography (EIT). Electrical impedance tomography is used in a variety of scientific fields, from geology, to semiconductor characterization, to medical imaging. EIT produces an image of the electrical properties of the examined media. In a typical EIT application, electrodes are placed around the volume of interest, and small, sinusoidal currents are injected into the tissue, while voltages are measured on its boundary. Using the finite element method, the complex impedance of the analyzed domain is modeled, and a solution for the approximate impedance configuration that fits the measurements is obtained [163-165]. During the last four decades, substantial basic and applied research has been done in the field of EIT. Our group has focused on applications for EIT in the context of monitoring minimally invasive surgery procedures such as cryosurgery [166], tissue viability [162, 167] and electroporation [161], [168].

The primary goal of this study is to explore the hypothesis that changes in pH during electrolysis can be detected with EIT, for possible applications in monitoring tissue ablation with electrolysis. To explore the hypothesis we conduct an experimental study using a pH dye stained physiological saline agar-gel based phantom as a model for a living tissue, from an electrochemical standpoint. To investigate the hypothesis, we compare EIT reconstructed images to optical images acquired using pH-sensitive dyes embedded in the agar phantom that is exposed to electrolysis. In addition to validating the EIT-based approach using pH-sensitive dyes, we demonstrate a biological
application of our EIT work by comparing a spatial map of bacterial viability exposed to electrolysis with the EIT image of the phantom during electrolytic treatment. Our results are promising, and invite further experimental explorations.

**Methods and Materials**

**Tissue Model**

Our tissue model consists of a physiological saline based agar gel phantom with electrical conductivity designed to simulate that of a tissue. To construct the phantom, 0.5% Bacto-Agar (Fisher Scientific) was mixed with 0.9 g/l Sodium Chloride (Fisher Scientific) in distilled water. The solution was then brought to a boil and poured into the Petri dishes. The conductivity of the agar phantom was measured to be approximately $0.14 \text{ S/m}$ which is close to the range of hepatic tumor conductivity [169]. During the experiments, the EIT electrode holder was placed in the Petri dish with the electrodes galvanically coupled to the gel phantom (part (c) of Figure 11).

**Experimental Model**

To test the feasibility of EIT as a means to monitor the onset and extent of electrolysis in tissue, we have devised the following experiment: 1) A reference EIT image of the tissue phantom is taken, 2) Electrolytic stimulation is applied, 3) Another EIT reference image of the tissue phantom is taken. We leverage the differential nature of EIT images to represent the changes in conductivity, which are used as surrogates to regions of altered pH level. As a control study, we use pH sensitive dyes in order to estimate the boundary of the region where the pH has changed due to electrolysis. We use a digital camera (Casio Exilim EX-ZR100) to acquire optical images of the experimental chamber and correlate these images with the EIT reconstruction images. The results of several representative studies are presented in the following section: in each study we have repeated experimental steps 2 and 3 above, multiple times, in order to observe the evolution of the pH front over time.
Bacterial Model

Lyophilized *E.Coli* of HB101 strain (BioRad) were grown in LB broth overnight and plated on LB broth based agar gel filled petri dishes. The LB broth for the overnight growth consisted of 1% BactoTryptone (BD), 0.5% Yeast Extract (BD), 1% NaCl (Sigma Aldrich) and 1.5% Agarose (Sigma Aldrich). For pouring the plates, we have held the sodium salt from the broth, in order to control the conductivity of the resulting gel. 6mm glass beads (Sigma Aldrich) were used for plating to ensure uniform coverage. After plating, the beads were removed and the plates were incubated for 15 minutes at 37°. The conductivity of the gel was measured around 0.2 S/m. At the experimental stage, the petri dish was separated from its lid and the EIT electrode array was lowered into the gel. On top of the EIT chamber, a 2 electrode holder with auxiliary electrodes was introduced into the gel. For the bacteria-focused experiments, only the auxiliary electrodes were used for stimulation, as opposed to the pH-sensitive dye experiments where we have also used the EIT electrodes for electrolytic stimulation. The stimulation sequence was applied using specified current and time parameters, with EIT snapshots being taken in the process as a monitoring step. The results section includes the exact current and time parameters used for each study. After the stimulation, the petri dishes were covered and incubated for 24 hours. To evaluate viability, we have visually inspected the petri dishes for areas where bacterial growth was inhibited.
An EIT data acquisition system consists of a collection of electrodes, which are used to inject known sinusoidal AC current into the observed sample. Due to the sample's conductivity, a potential develops on the sample. Due to the low frequency of the stimulation current, we ignore phase information and approximate impedance by Ohmic conductivity (resistivity). This potential is measured on the boundary using the electrodes not used for current injection. A schematic of a typical EIT system is presented in part (a) of Figure 11. In this work, we have used the EIT system described in [121], with $N = 32$ electrode surrounded circular chamber. We used an adjacent stimulation scheme [135], leading to each data set containing $\frac{N(N-3)}{2} = 464$ independent measurements. While recent literature recommends against this stimulation scheme [170], the EIT system available to us in the lab has this stimulation scheme hardwired. While this clearly poses a limitation, and most likely leads to reduced image quality, we intend to address it in future work. After the data has been acquired, the data processing module of an EIT system attempts to reconstruct a conductivity map of the domain of interest from a set of known injected currents and measured resulting voltages, typically at the boundary of the geometric domain. In a typical EIT reconstruction algorithm, a map of impedance is guessed and the voltages resulting from injected currents calculated by solving Laplace equation in the domain. These voltages are compared to the measured voltage and the difference is then used as feedback for an iterative scheme. The guessed map of impedance is then updated, until the calculated and measured voltages agree within a certain tolerance. Here, we have used the EIDORS framework (v3.7.1) [138] with the complete electrode model (CEM). We modeled our electrodes as 2D needle electrodes surrounding a circular phantom area. The reconstruction has been done by using a Gauss-Newton solver (EIDORS function inv_solve_diff_GN_one_step) with total variation (TV) regularization [145, 171]. This approach works by attempting to minimize a cost function representing the overall voltage measurement discrepancy between the input (measured) voltages and the reconstruction algorithm's internal model. The total variation functional is an attempt to regularize the reconstruction by making sure that the jumps in the final conductivity are bounded. The value of the regularization hyper-parameter has been selected experimentally by taking a value that locally minimizes the residual error. This approach can be seen as a simplified version of the L-Curve method described in [172]. We used the value 0.003.
Experimental Setup

The system is composed of 32 stainless steel electrodes mounted on a holder (diameter = 75mm) lowered into a circular Petri dish (diameter = 85mm) chamber (part (c) of Figure 11). The chamber contains the pH dye infused agar gel phantom which is imaged using EIT and optical digital camera. All the EIT stimulation currents have had amplitude of 350μA and a frequency of 5kHz.

Results and Discussion

Anode Centered Experiment

In this experiment, we have placed a thin, stainless steel rod (diameter = 0.6mm) in the center of the agar gel filled chamber. The central rod was connected using a copper wire to the positive terminal of the power supply and acted as the anode during this part of the experiment. For the cathode, all the 32 electrodes of the EIT were connected to each other by closing the switches $S_1...S_{32}$ presented schematically in the diagram on part (b) of Figure 11. The negative power supply terminal was then connected to the unified EIT electrodes. The EIT electrodes acted as a distributed cathode in that case. As a control study we have employed two pH sensitive dyes: 1% phenolphthalein (Sigma-Aldrich), which turns pink/purple above pH 8.8 and acts as a basic indicator, and 2.4% pH indicator (Fresh water test-kit, API), which turns yellow at pH 6.0. Both pH indicators were added to the agar gel phantom before its solidification.

A photographic picture of our experimental chamber is presented in part (c) of Figure 11. The protocol of our experiment involved taking a control set of images: EIT and optical, before every electro-stimulation step. The electro-stimulation parameters, including time and stimulation current, are specified in Table 2 of the Study Appendix. These parameters are typical to tissue ablation electrolytic processes, at the lower range of the parameters [2, 12]. Figure 12 summarizes the results of our experiment by showing a sequence of image pairs: each EIT image is accompanied by its matching optical image, which we used as a validation method. We have also excluded the possibility that the changes in impedance are caused by the pH-sensitive dye by running a control study, where EIT data was acquired from agar gels without pH-sensitive dyes.

The current was delivered at 1mA, and the delivered charge dosage was 1.14C, which falls within a range of a typical electro-chemo therapy stimulation charge dosage [2, 12]. Parts (a)-(c) of Figure 12 show the EIT images at selected time points whereas parts (1)-(3) of Figure 12 show the corresponding optical images. Part (4) of Figure 12 shows the final result of the gel model after the EIT electrodes have been removed. It can be seen that the EIT images of the pH from near the central electrodes are in good correspondence with the pH indicator dye: the central spot around the anode grows over time in both the optical and the EIT images. The data shows a good qualitative correspondence between the EIT reconstructed images and their optical counterparts.
We have chosen to include representative images corresponding to times $t=1$ minutes, $t=3$ minutes and $t=19$ minutes. The contrast of the image in part (4) of Figure 11 was increased to show the altered pH indicator at the perimeter, close to the distributed anode.

The color-bar presented to the right of figure 12 helps interpret the EIT results: the EIT images are taken in differential mode, which means that the images show differences relative to a reference image taken before any electrolytic stimulation was applied. Warmer colors correspond to increased conductivity, while colder colors correspond to decreased conductivity in the sample.

The change in conductivity in the center of the gel phantom captured by the EIT system can be explained by noting the relatively small radii of protons ($H^+, 0.88 \text{ fm}$) compared to the radii of other physiological ions, such as chlorine ions ($Cl^- 167 \text{ pm}$) and $Na^+(116 \text{ pm})$. The smaller ions produced in the electrolytic reactions led to increased local mobility, which in turn led to increased conductivity around the anode. While the relationship between pH and change in conductivity depends on multiple factors, including the concentrations of various ions and their respective mobilities, the results indicate an observable correlation between changes in pH and changes in conductivity. To facilitate the comparison of results, we superimposed the margin of the altered region in the pH dye infused gel onto the EIT image in part (d) of Figure 12. The margin of the marked region indicates the boundary of the highest pH of 6. For our stimulation conditions, the area with altered pH takes a circular shape. The region within the marked boundary corresponds to pH range of 1-6. Our future work is envisioned to explore the relationship between pH and the electric conductivity.
Figure 12: Anode Centered Electrolysis Experiment. EIT images: (a) after 1 minute, (b) after 3 minutes, (c) after 19 minutes, (d) after 19 minutes with overlaid outline of the pH altered region. Optical images: (1) after 1 minute, (2) after 3 minutes, (3) after 19 minutes (4) after 19 minutes with increased contrast.
Cathode Centered Experiment

In this part of the experiment, we have reversed the roles of the anode and the cathode. The same pH indicators were used as before: 1% phenolphthalein, which turns pink/purple above pH 8.8 and acts as a basic indicator, and 2.4% pH indicator (Fresh water test-kit, API), which turns yellow at pH 6.0. As in the previous section, both pH indicators were added to the agar gel phantom before its solidification. The protocol of this experiment involved taking another control set of images: EIT and optical, before every electro-stimulation step. The electro-stimulation parameters, including the current and the stimulation duration are specified in Table 2. Figure 13 presents the results of the experiment by showing a sequence of image pairs: each EIT image is accompanied by its matching optical image, which we used as a validation method. The overall charge dosage was $2.16C$. While this dosage falls within a range of a typical electro-chemo therapy procedure, it is a larger charge dosage compared to the anode centered experiment. We have administered more charge in the cathode-centric experiment because the altered pH front indicated by the pH-sensitive dye (phenolphthalein) was growing slower in the cathode-centered case. A possible explanation to this difference is the relative size of the $H^+$ and the $OH^-$ ions, and we discuss this discrepancy in more details in a later section (Bacterial Sterilization Model). Parts (a)-(c) of Figure 13 show the EIT images at selected time points, whereas parts (1)-(3) of Figure 13 show the corresponding optical images. Part (4) of Figure 13 shows the final result of the gel model after the EIT electrodes have been removed. It can be seen that the EIT images are in good correspondence with the pH indicator dye: the central spot around the cathode grows over time in both the optical and the EIT images. Moreover, while it is too subtle to see in the optical images, the EIT imaging clearly shows a circular feature at the periphery of the EIT chamber. This peripheral region with reduced pH level can be clearly distinguished in part (4) of Figure 13 by its distinguished yellowish color. It can only be seen after the EIT electrodes have been removed. The data shows a good qualitative correspondence between the EIT reconstructed images and their optical counterparts. We have chosen to include representative images corresponding to times $t=2$ minutes, $t=6$ minutes and $t=36$ minutes. An accumulation of liquid, presumed to be water, can be observed around the cathode in the form of a growing bubble. We have attributed it to the osmotic effects of electrolysis reported by other researchers. Part (4) of Figure 13 shows the optical image after the EIT electrodes have been lifted at the end of the experiment. The contrast of the image in part (4) of Figure 13 was increased to show the altered pH indicator at the perimeter, close to the distributed anode.

The change in conductivity in the center of the gel phantom captured by the EIT system can be explained by noting the relatively small radii of hydroxyl ions ($OH^-$, 110 pm) compared to the radii of other physiological ions, such as chlorine ions (Cl$^-$, 167 pm) and Na$^+$ (116 pm). The smaller ions produced in the electrolytic reactions had led to increased local mobility, which in turn led to increased conductivity around the cathode. This behavior is similar to the anodic case, although the degree of change in conductivity is different, possibly due to the difference in relative sizes of protons and hydroxyl ions. To facilitate the comparison of results, we superimposed the margin of the altered region in the pH dye infused gel onto the EIT image in part (d) of Figure 13.

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The margin of the marked region indicates the minimal pH of 8.8. For our stimulation conditions, the area with altered pH takes a circular shape. The region within the marked boundary corresponds to pH range of 8.8-14.
Figure 13: Cathode Centered Electrolysis Experiment. EIT images: (a) after 2 minute, (b) after 6 minutes, (c) after 36 minutes (d) after 36 minutes with overlaid outline of the pH altered region. Optical images: (1) after 2 minute, (2) after 6 minutes, (3) after 36 minutes (4) after 36 minutes with increased contrast.
Two Internal Electrodes Experiment

In this part of the experiment, instead of using the EIT electrodes as a distributed electrode, we have utilized two graphite electrodes made of pencil lead (Pentel super HB 0.7mm). The electrodes, mounted in a horizontal holder were placed perpendicularly to the gel phantom. The electrodes were inserted 5mm deep into the gel. We have used a 5% pH indicator (RC Hagen wide range). As in the previous experiments, the pH indicator was added to the agar gel phantom before its solidification.

The protocol of this experiment involved taking another control set of images: EIT and optical, before every electro-stimulation step. The electro-stimulation included a sequence of direct current injections at 2mA of the following durations: [1min, 1min, 1min, 1min, 1min, 1min, 1min, 5min]. Figure 14 presents the results of the experiment by showing a sequence of image pairs: each EIT image is accompanied by its matching optical image, which we used as a validation method. The delivered charge dosage was $1.44C$, which falls within a range of a typical electro-chemo therapy stimulation charge dosage [2, 12]. Parts (a)-(c) of Figure 14 show the EIT images at selected time points whereas parts (1)-(3) of Figure 14 show the corresponding optical images. It can be seen that the EIT images are in good correspondence with the pH indicator dye: the central spot around the anode (red) grows over time in both the optical and the EIT images, and the same is observed for the spot around the cathode (blue).

We have chosen to include representative images corresponding to times t=1 minutes, t=3 minutes, t=6 minutes and t=12 minutes. It is notable that both the optical and the EIT approaches are able to image the collision of the basic and the acidic fronts (parts (d) and (4) of Figure 14).
Figure 14: Two Electrodes Electrolysis Experiment. EIT images: (a) after 1 minute, (b) after 3 minutes, (c) after 6 minutes, (d) after 12 minutes. Optical images: (1) after 1 minute, (2) after 3 minutes, (3) after 6 minutes (4) after 12 minutes.
Bacterial Sterilization Model

To confirm the efficacy of our method in a biological model, we have used EIT for imaging electrolysis in an agar dish plated with *E. Coli* bacteria. The liquid bacterial culture was first plated as described in our methods, and then a current of 2mA was administered using the auxiliary electrodes. The total administered charge dosage was 5.4C, which falls within a range of a typically delivered charge during an electro-chemo therapy stimulation [2, 12]. Figure 15 shows a comparison between the EIT imaging data and a bacterial viability pattern captured using an optical, digital camera after 24 hour growth period. Parts (a)-(c) of Figure 15 indicate two growing regions of increased conductivity, around the auxiliary electrodes through which electrolytic stimulation was applied. We have chosen to include representative images corresponding to times t=15 minutes, t=30 minutes and t=45 minutes. Part (d) of Figure 15 shows the optical image of the viability pattern taken 24 hours post-stimulation. It is interesting to note that both the EIT images as well as the optical image exhibit asymmetry with regards to the anodic and the cathodic regions under our experimental conditions. The brighter upper spots in the EIT images, in particular in the one shown in part (c) of Figure 15 indicates that the conductivity of the anodic region has changed to a larger degree than the conductivity of the cathodic region. This discrepancy can be attributed to the relative radii of protons (H⁺, 0.88 fm) and hydroxide ions (OH⁻, 110 pm). Due to their relative smaller size, the protons are more mobile, hence contributing to a larger extent to the conductivity increase around the anode. The increased mobility causes the bactericidal pH region around the anode to be larger than around the cathode. This is supported by the viability observations presented in part (d) of Figure 15. To clarify, the circular pattern of dots around the bacterial culture dish corresponds to the EIT electrodes imprinted in the gel when the EIT chamber was lowered.
Figure 15: Bacterial Viability Experiment. EIT images: (a) After 15 minutes, (b) after 30 minutes, (c) after 45 minutes, (d) optical image of growth patterns after 24 hour incubation.

Limitations and Future Work

While our results show a degree of correlation between EIT measurements and optical measurements obtained using pH-sensitive dyes, several key questions arise which need to be answered before EIT can be successfully used in clinic as an imaging technique for electrolysis. These include inquiries into the nature of the relationship between pH and conductivity, or the differential changes around the cathodic and the anodic regions. Since other imaging techniques for electrolysis have not been reported in the literature, our current benchmark is optical pH-sensitive dye images, but other imaging modalities such as MRI might potentially result in a better comparison standard.
There are several limitations inherent to our work: first, our tissue model is limited to an agar gel based phantom, which is a simplistic way to represent a biological tissue. Further experiments are envisioned with living tissues replacing the gel phantom. In addition to the inherent limitation of the model, the EIT system we have employed in this work is outdated and was used as a first order, crude approximation, in order to establish if further experimental effort can be justified. Clearly, optimizing EIT acquisition and reconstruction parameters would result in better image quality, which might increase the likelihood of developing a clinically viable imaging modality. Assuming that the method works on a living tissue, it could potentially be applied in a clinical setting: in this scenario, EIT might become a monitoring method for electrolytic ablation in the treatment of breast cancer, see e.g. [173]. This could allow a more controlled real-time monitored treatment at a low cost, which could provide a new set of medical tools to resource constrained communities. The key limitations are foreseen to be the tissue types where the therapy could be applied. For example, when the tissue exhibits high baseline conductivity, the changes in pH might not affect the conductivity to such an extent that can be detected using an off-the-shelf EIT system. In a highly conductive tissue, the pH changes can fall under the noise margin of EIT measurements. We have limited this work to conductivity corresponding to a hepatic tumor, in which detection feasibility was demonstrated. We intend to explore the relationship between pH and conductivity, which will allow us to characterize the conductivity regions in which our approach could be potentially useful.

Demonstrating the proof of concept for a new application of the EIT technology was the main goal of this study, i.e. our purpose was to show that EIT is sensitive enough to image electrolysis and we do not claim any improvements in the technology itself. We believe that using EIT for this new application, i.e. monitoring electrolysis, could become an enabling technology for planning and monitoring low-cost minimally invasive surgery procedures involving electrolysis. The configuration employed in this study and the *E.Coli* experiments have an immediate application in relation to the use of electrolysis and electrolytic products to sterilize wounds and destroy microorganisms on the surface of the body, the skin [9, 174]. Placing an EIT array around a surface wound, treated with electrolysis, could provide a means to monitor the treatment. While EIT has numerous technical limitations, the application in the configuration tested which focuses on the outer surface of the body is less restricted by the typical constraints of EIT. The microorganism study employed here is directly relevant and illustrative of this application. Another field of application is in relation to the combination between reversible electroporation and electrolysis [92, 93, 175, 176]. We have recently shown that generating electrolytic products, prior, during or after reversible electroporation can be seen as a new method for tissue ablation [175, 176]. The ability to monitor the extent of electrolysis could provide greater control over tissue ablation with this combination, with possible applications to treatment of melanomas. Reversible electroporation combination with bleomycin is a tissue ablation technology known as electrochemotherapy, also used primarily for treatment of melanoma [177]. Reversible electroporation is also an important technology for gene vaccination [178]. However, it has been recently found that electrolytic products can be generated during reversible electroporation for insertion of genes into cells. These products may cause cell death and be detrimental to gene therapy protocols [92]. Using EIT to detect the production of
electrolysis during reversible electroporation may benefit gene vaccination; in particular, in configurations in which the electroporation is near the surface of the body, the skin.

Given the scope of our work, we see how it suffers from the generic limitations of EIT i.e. nonlinear current spread, and the uncertainty associated with the position of the electrodes. Having said that, these are challenges that are not unique to the application we suggest and we do not claim to solve them in this paper. Furthermore, as discussed earlier, the configuration analyzed here may be less susceptible. Our wish in this work was to demonstrate that EIT can reliably detect pH changes. We see this study as a first step towards developing a novel imaging approach and this paper was focused on validating the fundamental feasibility of the method.

Study Conclusion

In summary, we report experimental findings that support the hypothesis that electrolysis induced pH-changes lead to local conductivity changes in a physiological gel tissue model. It is these changes in conductivity that can be captured in real time by EIT. Our results indicate the feasibility of using EIT as a means to monitor dynamic changes in local pH level of a biological sample during an electrolysis process. Our work uses agar-based gel model with conductivity in the range of a biological tissue, and is validated vs. optical images utilizing pH indicator dyes. In addition, we demonstrate the relevance of our work in the biological context by correlating bacterial viability data with EIT measurements. Our results are promising, and invite further experimental explorations.

Study Appendix

<table>
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<tr>
<th>Experiment</th>
<th>Current</th>
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<td>19min</td>
</tr>
<tr>
<td>Cathode centered</td>
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<td>2.16C</td>
<td>36min</td>
</tr>
<tr>
<td>Two electrodes</td>
<td>2ma</td>
<td>1.44C</td>
<td>12 min</td>
</tr>
<tr>
<td>Bacterial viability</td>
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<td>5.4C</td>
<td>45min</td>
</tr>
</tbody>
</table>

Table 2: Study Experimental Parameters
Chapter 5: Monitoring Electrolysis by Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) overview

Fundamental Concepts

Magnetic Resonance Imaging (MRI) utilizes the principle of Nuclear Magnetic Resonance (NMR) as a foundation to produce highly detailed images of the human body. The patient is placed inside a strong magnetic field created by a powerful magnet. The hydrogen atoms in the human body then align themselves with this magnetic field - that is, they behave like tiny bar magnets. A transmitting coil, emits a radiofrequency (RF) pulse. This causes the hydrogen atoms to alter the direction of their orientation. The transmitting RF pulse is then switched off. The hydrogen atoms begin to return to their equilibrium state before the stimulation. The nature of this relaxation process is a function of time and it depends on the T1, and T2 relaxation times of the local tissues. As this re-alignment occurs, the protons induce an electrical signal, which is detected by a receiving coil. From this received signal, the intensity of this signal and the exact location from where this signal originated can be determined with the help of sophisticated electronics, three additional magnetic field gradients, and computer equipment.

MRI utilizes numerous parameters to control the image contrast including tissue relaxations times T1 and T2, number of hydrogen protons or proton density (PD) and pulse sequence parameters, mainly TR and TE. These factors can be used to explore different tissue characteristics. By transmitting a carefully designed sequence of RF pulses, T1 and T2 relaxation times can be made to contribute to the contrast among different tissues. The three gradient magnetic coils, along with three-dimensional orthogonal axes in the tissue are used to localize and spatially encode the RF signal emitted from the tissue. Specific values of TR and TE are selected to produce T1W, T2W, and PD images from this signal. The selection of TR and TE weighs the MR image so that one contrast source predominates over the other two.

Original Study: Magnetic Resonance Imaging of Electrolysis

Study Context

Our work draws from a 1957 paper by Meiboom, Luz and Gill who studied proton relaxation times in water as a function of pH [179]. During the last several decades, Magnetic Resonance Imaging (MRI) has been used to study pH changes in many biomedical settings with various methods and for various applications. For example, the effect of intracellular pH, as well as blood and tissue oxygen tension on T1 relaxation in the rat brain were studied in [180]. Measurements of pH changes due to ischemia in the brain, in relation to amine and amide protons were reported in [181, 182]. Measurements of pH changes due to kidney failure with an MRI-CEST pH responsive contrast agent, Iopamidol were presented in [183]. An evaluation of a range of MRI-active pH indicators for food applications is found in [184, 185]. In [186], it was shown
that calf muscle T2 changes correlate with pH, PCr recovery and oxidative phosphorylation. Most relevant to this study is the work of Schilling et al, who have found that changes in intracellular pH, affect the relaxation time of T2 in brain tissue [187]. While an abundance of papers exist on the use of MRI for detecting changes in pH, no publication on the use of MRI for monitoring the development of pH fronts during electrolysis has been found by our literature search. The primary goal of this study is to explore the hypothesis that pH fronts produced by electrolysis can be detected with MRI, for possible application in monitoring cell ablation with electrolysis. As briefly illustrated by the above literature citations, there are many methods that could be used for detecting pH changes in tissue with MRI. Inspired by references [179], [187], we chose to explore our hypothesis with basic T1 weighted and T2 weighted based sequences for water.

To explore the hypothesis, we conduct an experimental study using a pH dye stained physiological saline agar-gel based phantom as a model for a living tissue, from an electrochemical standpoint. In the study, we compare images obtained with MRI to optical images acquired using pH-sensitive dyes. The MRI imaging sequences used were T1 weighted (T1W), T2 weighted (T2W) and Proton Density (PD). The optical images were acquired using pH-sensitive dyes embedded in the agar phantom that was exposed to electrolysis. In addition to validating the MRI-based approach using pH-sensitive dyes, we demonstrate a biological application of our MRI work by comparing a spatial map of bacterial viability exposed to electrolysis with the MRI image of the phantom during electrolytic treatment. Our results are promising, and invite further experimental explorations.

Experimental design

The experiment was designed to allow for a comparison between different images of pH fronts produced by the electrolysis of a physiological saline solution phantom. The images were generated by various MRI sequences and compared with: a) optical images acquired using pH-sensitive dyes embedded in a physiological saline agar solution phantom treated with electrolysis and b) bacterial E.Coli model, grown on a phantom and treated by applying the same electrolysis protocol. Each experimental study was done separately. In addition, we have also collected a set of discrete measurements using a micro pH probe.

Tissue model

Our tissue model consists of a physiological saline based agar gel phantom with electrical conductivity designed to simulate that of a tissue. To construct the phantom, 1% Bacto-Agar (Fisher Scientific) was mixed with 0.9 g/l Sodium Chloride (Fisher
Scientific) in distilled water. The solution was then brought to a boil and poured into 85 mm diameter Petri dishes. The same dish dimension was used in all the studies. The conductivity of the agar phantom was measured to be approximately 0.14 S/m, which is close to the range of hepatic tumor conductivity [169].

**Experimental procedure**

The experimental setup is shown in part (a) of Figure 16. We have used two disposable graphite electrodes made of pencil lead (Pentel super HB 0.7mm), to avoid contamination with products of electrolysis from the electrodes. The electrodes, mounted in a horizontal holder were placed perpendicularly to the gel phantom in the Petri dish. The electrodes were inserted 7mm deep into the gel at a distance of 2 cm between their centers. The electrodes are connected to constant voltage batteries. We used 3V, 6V and 9 V batteries. The electrolysis process lasted 15 minutes. While typical electrolysis stimulation is administered using a fixed current source, we have used a fixed voltage source and have taken current measurements during the procedure (data not shown) for charge dosage estimation purposes. The overall delivered charge dosages over the 15 minutes stimulation period were approximately 0.9C, 1.8C and 2.9C, for 3V, 6V and 9V, respectively. These charge dosages fall within the range of a typical electrolytic ablation therapy stimulation [2, 12, 148]. Identical experiments were done separately for MRI imaging, pH dyes based optical imaging, discrete pH measurements and the E.Coli viability model.

**MRI Experimental Model**

The phantom models were scanned, before and after electrolysis in a clinical 1.5 T MRI system (Philips Achieva SE) using a SENSE pediatric coil. The specific MRI parameters of each sequence are listed in Table 3. The mean acquisition time for each sequence was 3 minutes. Images shown in the paper were taken in the following order: T1W, T2W and PD. For later comparison with the MR images produced after electrolysis, parts (b), (c) and (d) of Figure 16 show the baseline images for the sequences introduced above, respectively. In repeated experiments (results not shown) we changed the order and the time after the end of electrolysis at which the various sequences were taken and found no measurable effect of the time at which the images were acquired on the dimensions of the affected region.
Table 3: Study Experimental Parameters

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Coil</th>
<th>TR [ms]</th>
<th>TE [ms]</th>
<th>FOV [mm]</th>
<th>Slice thickness</th>
<th>Num. slices</th>
<th># of excitations NSA</th>
<th>Recon. Matrix size</th>
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<tbody>
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<td>1000</td>
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<td>120</td>
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<td>4</td>
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<tr>
<td>PD</td>
<td>SENSE-PED-HEAD</td>
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<td>30</td>
<td>120</td>
<td>2 mm</td>
<td>4</td>
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</tbody>
</table>

Figure 16 Experimental setup and control images for different experimental modalities used in this work. a) Experimental setup, b) T1 control study, c) T2 control study, d) PD control study, e) Phenolphthalein 1%, f) Hagen wide range pH indicator dye, g) E.Coli control study.
**pH-Sensitive Dye Model**

As a control study, we used 2 distinct, pH sensitive dyes in order to estimate the boundary of the region where the pH has changed due to electrolysis. The dyes employed were 1) Phenolphthalein 1% by Sigma (turns pink in pH range of 8.2-12) and 2) Nutrafin pH wide range by Hagen (indicates pH by color in the range of 4.5-9). The dye manufacturer instructions prescribed the concentration of the last dye. We used a digital camera (Casio Exilim EX-ZR100) to acquire optical images of the experimental chamber before and after electrolysis and correlate these images with the images acquired using MRI and bacterial viability. For later comparison with images produced after electrolysis, parts (e) and (f) of Figure 16 show the baseline images prior to electrolysis for the two dyes used, respectively.

**Discrete measurements with micro pH probes**

To produce numerical values for pH we have used a MI-4146 Micro-combination pH probe (Microelectrodes, Inc. 40 Harvey Rd., Bedford, NH) with a response time of 5 seconds to 15 seconds in a stirred solution and a pH meter pH150 Oakton (625 East Bunker Court., Vernon Hills, IL). The experimental procedure is similar to that used in the MRI study and the pH sensitive dye study. We have marked a line through the diameter of the Petri dish and marked off every 5 mm (see part (h) of Figure 16). The two electrodes were placed at a distance of 2 cm between them. Similarly to the previous experiments, we applied voltages of 3V, 6V and 9V for 15 minutes. After the electro-stimulation, the electrodes were removed, and pH measurements were made, starting with the marking furthest away from the dish center. To minimize and evaluate the effect of diffusion on the systematic errors of this method, in one set of the experiments the reading was performed from left to right, and in another set of experiments the reading was performed from right to left. The pH probe was placed over the mark and into the gel, making certain that the tip was covered. We waited until the reading was stabilized, usually between 15 and 25 seconds. The reading was recorded and the pH probe flushed and moved over to the next mark until the entire dish was transversed. One transverse reading took about 20 to 25 minutes. (No new electrolytic products were produced during the measurement period, however the pH changed during the measurements because of diffusion). While the actual data recorded is with a precision of 0.1, the accuracy in relation to the colorimetric dye and MRI measurements is low. Therefore, the discrete pH measurement should be viewed as a rough indication of the actual values. We also considered measuring pH during the electro-stimulation process, however, we found, in other studies, that the insertion of an active pH probe during electrolysis distorts the reading because of the effect of the current on the pH probe.
**Bacterial Model**

Lyophilized *E. Coli* of HB101 strain (BioRad) were grown in LB broth overnight and plated on LB broth based agar gel filled petri dishes. The LB broth for the overnight growth consisted of 1% BactoTryptone (BD), 0.5% Yeast Extract (BD), 1% NaCl (Sigma Aldrich) and 1.5% Agarose (Sigma Aldrich). 6mm glass beads (Sigma Aldrich) were used for plating to ensure uniform coverage. After plating, the beads were removed and the plates were incubated for 15 minutes at 37°. The conductivity of the gel was measured around 0.2 $S/m$. After electrolysis the Petri dishes were covered and incubated for 24 hours. We used a digital camera (Casio Exilim EX-ZR100) to acquire optical images of the areas where bacterial growth was inhibited and correlated these images with the images acquired using MRI and gels with pH dyes. For later comparison with images produced after electrolysis, part (g) of Figure 16 shows the baseline images for an untreated with electrolysis, cell growth plate.

**Study Results**

**MRI Experiment Results**

As described in our methods, the agar plates were scanned before the administration of electrolytic treatment with the following sequences: T1W, T2W and PD. Then the gel plates were electrolytically treated for 15 minutes using three different voltages: 3V, 6V, and 9V. After administering the treatment, the plates were immediately positioned in a pediatric head coil, and inserted into the MR scanner. MR sequences with the same pre-treatment parameters were then acquired. The MRI parameters are presented in Table 3. To facilitate comparison of results, Figure 17 brings together images obtained for the three voltages and the three MRI sequences. The three columns are for the voltages of 3V, 6V and 9V, from left to right, respectively. The rows from top to bottom are for the following sequences: T1W, T2W, and PD, respectively. All the images are for a standard Petri dish with the same diameter, 8.5cm. The electrolysis was administered via the same device, positioned at the same place for all the experiments, as constrained by the application rig in part (a) of Figure 16. The position of the electrodes can be seen in some images as the two black traces (void of signal) at the centerline of the Petri dish. The distance between the electrodes was 2 cm. In all the images the anode is on the left and the cathode is on the right.

The first row of Figure 17 shows images taken with the T1W sequence. The signal from the treated volume is isointense to hypointense. It is isointense for the lower voltage of 3V and becomes slightly hypointense with the increasing applied voltage. The pH change front is barely distinguishable for the 9V treatment. The second row shows results obtained with the T2W sequence. The margin of the electrolysis-affected region
is marked with dotted yellow line. Hypointense signal can be seen in the treated region, with the signal intensity decreasing with increasing voltage. The affected region near the anode is larger than near the cathode. The altered pH front appears diffused in the anode-affected region and well-delineated in the cathode affected region. The interface between the cathode affected region and the anode affected region is distinct and visible. It is also note-worthy that in the cathode-affected region, the intensity adjacent to the cathode decreases with increasing voltage. The images produced with PD sequences, presented in the third row, show a generally similar pattern to that described for the T2W sequence produced images. Images produced with the PD sequence show a hypo intense signal with lower intensity relative to the T2W sequence produced images.
Figure 17: Comparative MRI imaging results. Each row corresponds to a sequence modality (Top to bottom: T1, T2, PD). Each column corresponds to a stimulation voltage (Left to right: 3V, 6V, 9V).
For the pH dye experiments, we have infused the agar gel phantom described in our methods with two pH sensitive indicator dyes. Figure 18 shows results obtained from the pH dye experiments. To facilitate the comparison of results, Figure 18 brings together images obtained for the three voltages and results from the two-pH dyes infused gels. The three columns are for voltages of 3V, 6V and 9V, from left to right, respectively. The first row shows results obtained with phenolphthalein staining. The phenolphthalein stain produces a distinct pink color in the pH range from 8.2 to 12. The first row shows, as expected, an impression in only the cathode region on the right. The margins of the marked regions indicate a minimal pH of 8.2. For a voltage of 3V, the change of pH front takes a circular shape, most likely of a pH of 8.2. Increasing the voltage increases the size of the change in pH-affected area. Similar to the MRI images, the cathode-affected front collides with the anode produced front at a line between the electrode and cathode. The outer margin of the lesion that has a circular shape is most likely at a pH of 8.2, while the central line could be at any pH in the range of pH 8.2 to pH 12. It is interesting to note that immediately near the electrode for the 9V voltage the intensity of the image is reduced compared to a region further away from the electrode.

The second row shows the results of pH staining using the Hagen wide range pH testing kit. The cathodic region on the left is marked with a distinct blue color which indicates a basic pH in the vicinity of 8.3, while the anodic region on the right is marked with pink color which corresponds to pH level of 6.4. We have used the color-matching card provided by the manufacturer to establish the pH ranges. For 3V, the pH change affected regions have a circular shape. Increasing the voltage increases the size of the affected region. Just as for the other pH dye, and MRI images, the larger voltages lead to colliding pH fronts, which can observed as a straight line. Several interesting phenomena can be seen in the Hagen stained samples. First, for voltages of 6V and 9V, the areas immediately adjacent to the electrodes appear discolored relative to the surrounding stained areas. Furthermore, on the cathode side at 6V and 9V there is a drop of fluid, which was observed on the top of the gel. For 9V, some of the dye has leaked into this drop and stained it.
Figure 18: Comparative pH dyes and bacterial viability results. Each row corresponds to a control modality (Top to bottom: Phenolphthalein 1% pH indicator, Hagen pH indicator, E.Coli bacterial viability). Each column corresponds to a stimulation voltage (Left to right: 3V, 6V, 9V).
**pH probe experiments**

To correlate the pH data captured by MRI and the pH sensitive dyes, the pH measurements made by the pH probe are visualized graphically in the fifth row of Figure 19. The discrete pH measurements are presented on a single chart for each voltage. The effect of growing voltage is clearly seen: the extreme value of pH reached on both fronts is more deviant from the baseline normal pH. The data is presented in two forms, with the measurements made from left to right and from right to left. The precision of the pH reading is 0.1 and the precision of the data point location is +/- 0.5mm. The difference between the left to right measurement curve and the right to left measurement curve is indicative of the systematic error of this measurement.

**Bacterial Viability Experiment**

To demonstrate the relevance of our work to a biological model, electrolytic stimulation was applied to an agar dish plated with *E.Coli* bacteria. The third row in Figure 18 shows optical images of a bacterial viability pattern after treatment with 3V, 6V and 9V for 15 minutes, captured using a digital camera after 24 hour growth period. The anodic region on the right is marked with a clear bactericidal region increasing in area with increasing voltage. The cathodic region on the left is significantly smaller in terms of bactericidal area and is barely observable in the 3V image.
Figure 19: Comparative pH dyes and bacterial viability results. Each row corresponds to a control modality (Top to bottom: Phenolphthalein 1% pH indicator, Hagen pH indicator, E.Coli bacterial viability). Each column corresponds to a stimulation voltage (Left to right: 3V, 6V, 9V).
Discussion

As mentioned in the introduction, our hypothesis that MRI can be used to image the pH fronts during electrolysis is based on reports in references [179] and [187]. As can be observed in the T1W images (Figure 17 first row), the treated volume exhibits hypointense to isointense signal, which indicates that the effect of electrolysis is minimal on T1W signal. A T1-weighted sequence produces an image where the signal contrast is determined by the differences in T1 relaxation times. The tissue signal in a T1 weighted imaging mode is inversely proportional to its T1 relaxation time. A short echo time (TE) is used to minimize T2-weighting together with a short repetition time (TR). A T1-weighted image is typically characterized by dark fluid signal due to the long T1 relaxation time of water. This result is consistent with the original findings of Meiboom, Luz and Gill who studied proton relaxation times in water as a function of pH [179] and show that T1 in water does not change in the range of from pH 2 to pH 12.

Visible changes are produced by electrolysis in T2 weighted images (Figure 17, second row). In the T2-weighted imaging mode, the signal contrast is determined by differences in T2 relaxation times. The tissue signal in a T2 weighted image is proportional to its T2 relaxation time. A long repetition time (TR) is used to minimize T1-weighting together with a long echo time. The results in Figure 17 are also consistent with the original findings of Meiboom, Luz and Gill [179]. Their findings show that T2 in water is strongly affected by changes in pH and it increases symmetrically around pH 7 with an increase and decrease in pH. Shilling et al. [187] provide a mechanistic explanation for their observed changes in T2 with changes in pH in the brain, which is consistent with the findings of Meiboom et. al. by noting that “The effect of pH on spin-spin relaxation time (T2) might be explained by the fact that at pH 7.0, i.e., in the neutral environment, water molecules build larger hydrogen-bond mediated clusters than in the acid or base ranges. The reduced mobility leads to a prolonged correlation time for the dipolar interactions, which leads to a shortening of T2” [188]. Figure 17 shows that the electrolysis affected region near the anode is larger than that near the cathode. This difference makes physical sense and can be attributed to the relative radii of protons ($H^+$, 0.88 fm) and hydroxide ions ($OH^-$, 110 pm). Due to their relative smaller size, the protons are more mobile, hence contributing to a larger extent to the conductivity increase around the anode. The increased mobility causes the pH region around the anode to be larger than around the cathode.

Proton Density (PD) is defined as the number of proton spins per unit volume of a tissue. Proton density may differ from the true water content due to short T2 components, which are not seen in MRI. Thus, PD-weighted imaging where the T1 and T2 effects are minimized leads to images whose contrast is determined primarily by the spin (proton) density. This requires a short TE and long TR. In Figure 17, the third row shows the process of electrolysis generated PD MRI images, which correspond well with the T2W images. This confirms that the observed images are related to the electrolysis caused diffusion of protons and hydroxide ions.
In Figure 18, the first and the second rows show the results of the process of electrolysis obtained with pH stained dyes. While the optical pH results cannot be quantitatively compared with the MR images, because the pH dyes have a restricted range, both the MRI and pH dyes images show similar phenomena and trends. The observed affected zone increase in both modalities with an increase in voltage, which is consistent with the increased production of electrolytic compounds with voltage. The anode and cathode electrolysis affected regions meet at the same location in both the pH dye images and the MRI images. The pH dye results, in particular the second row of Figure 18, show some additional interesting physical phenomena. The effect relates to the observed drops of water on the surface of the gel, during electrolysis with 6V and 9V. It is known that in an electric field, water moves by electro-osmosis from the anode to the cathode [150]. Therefore, during electrolysis, the gel near the anode tends to dehydrate while water accumulates near the cathode. This is the source of the water observed in the second row of Figure 18. We tentatively suggest that this electro-osmotic migration of water is responsible for the discoloration adjacent to the cathode observed with both MRI and pH dyes.

In Figure 18, row three shows viability results from electrolysis treated E.Coli, grown on the surface of the gel. This part of the work is clinically relevant because electrolysis is becoming an important method for sterilizing surfaces and wounds, considering the growing antibiotic resistances of microorganisms [189]. The pattern of cell ablation observed here is consistent with electrolytic ablation and further supports the idea that the MRI detected changes are relevant to electrolysis. Figure 18, row three shows that the extent of cell ablation increases with the voltage and charge delivered, as expected from a pH ablation process driven by electrolysis. It is also well established that the electrolytic products of the anode are more effective at cell ablation than the products of the cathode [150]. This is also confirmed in this study, which shows a much larger ablation zone near the anode than near the cathode.

A comparison between MRI images, pH dye based images and bacterial viability data shows that all the experiments, produce qualitatively similar results with respect to the effect of voltage on the affected area and with respect to the difference between the anodic and cathodic regions. A quantitative comparison is not possible because the pH dyes and the bacterial viability images represent limited ranges of pH. However, the results from the different imaging techniques show that increasing the voltage (charge delivered) increases the affected area in both the anode and cathode affected volume, the anodic front advances faster than the cathodic front, and the anode and cathode affected regions meet on the line perpendicular to the line connecting the electrodes.
Figure 19 was brought here to summarize the results. The first row shows the T2W MR image onto which we have superimposed the outline of the pH dye image (rows two and three) and the outline of the viability experiment (row four). It is interesting to notice that the interface between the anode and the cathode affected zones lies on the same line in the MRI image and the pH dye image – suggesting that they both represent the same phenomenon. The overall shape of the pH dye image is similar to the MRI image. The affected zone observed with MRI is larger than that observed with dyes, because the range of changes that can be observed with MRI is not restricted by a certain pH dye value. The extent of cell ablation is substantially less than the extent of the region in which MRI detects changes in pH. In the past, studies on the effect of electrolysis on cell death were carried out using pH probes or pH dyes. This study suggests that MRI could become a useful tool in fundamental research on the effect of electrolysis on cells.

To address the temporal resolution of the MRI based approach and its relevance in the context of a typical length of an electrolysis procedure, we would like to note that the resolution is limited by the time it takes to run a single scan, which is given by $N \cdot N_p \cdot T_r$, where $N$ represents the number of signals averaged (NSA excitations), $N_p$ represents the size of the encoding matrix and $T_r$ represents the time to repetition. When multiple echoes are acquired after each excitation, this allows for accelerating the scan time by a factor, and occasionally is referred to as the Turbo Spin Echo (TSE) or Turbo Factor. In such a case, the time given above should be divided by the Turbo Factor. For a typical choice of parameters, e.g. in our case study, the scan time falls in the range of 2-4 minutes. This is shorter than a typical length of an electrolytic treatment procedure. This further suggests that MRI could be a potential method to study electrolysis in tissue.
Chapter 6: Applications

1. Original Study: Combining Electrolysis with Electroporation [175]

Research Context

The field of minimally invasive tissue ablation in the context of surgery is an important alternative to surgical resection, often allowing for reduced surgery duration, improved access to the surgical site and enhanced focus on the target tissue. Of the variety of techniques for tissue ablation, some of the more commonly used methods employ electrically-induced phenomena. This paper focuses on two modalities of tissue electro-ablation: electrolysis and electroporation.

The process of electrolysis occurs at the electrode surfaces for electrodes submerged in an ionic conducting media [190]. New chemical species are generated at the interface of the electrodes as a result of the electric potential driven transfer between electrons and ions or atoms. The various chemical species produced near the electrodes diffuse away in a process driven by differences in electrochemical potential. In physiological solutions these chemical reactions also yield changes in pH, resulting in an acidic region near the anode and a basic region near the cathode. Tissue ablation is driven by two factors: a cytotoxic environment developing due to local changes in pH, as well as the presence of some of the new chemical species formed during electrolysis. Electrolysis is a chemical ablation mechanism, and the extent of ablation is a function of the concentration of the chemical species and the exposure time to these chemicals. The total amount of electrolytic products generated during electrolysis is related to the charge delivered during the process and therefore the total charge is used as a quantitative measure for the extent of electrolysis.

Electrolysis has been harnessed for tissue ablation in medicine since the early 1800’s [9], and the field has experienced a revival in the mid 1970’s with the work of Nordenstrom [148, 191]. Over the last two decades, substantial research has been done on tissue ablation by electrolysis, including cell and animal experiments, mathematical modeling, and clinical work [14-16, 20-28, 30, 39, 40, 192-194]. In the contemporary literature, electrolytic ablation is sometimes referred to as Electro-Chemical Therapy (EChT), and in this work these two terms are used interchangeably. Electrolytic ablation has been shown to exhibit several unique attributes. First, due to the chemical nature of the ablation process, the diffusion of chemical species in the tissue and the rate of chemical reactions dominate the time scale of the procedure. Second, the chemical products at the anode differ from those formed at the cathode, thus resulting in distinct mechanisms of ablation. Finally, electro-osmotic forces drive the migration of water from the anode to the cathode, further magnifying the contrasting physiological effects at the electrode surfaces. From an operational standpoint, electrolysis requires very low voltages and currents, providing advantages relative to other ablation techniques, e.g. reduced instrumentation complexity. It is, however, a
lengthy procedure, controlled by the process of diffusion and the need for high concentrations of electrolytically-produced ablative chemical species.

While electroporation also harnesses an electricity-induced phenomenon, it differs from electrolysis by employing a different set of biophysical principles. The bioelectric phenomenon of electroporation is characterized by the permeabilization of the cell membrane through the application of very brief, high-magnitude electric field pulses across the cell [57-60, 78, 195-198]. The effect on the cell membrane is a function of the electric field strength. Electroporation can be used to produce reversible pores in the lipid bilayer, allowing for the introduction of molecules such as genes and drugs into cells. The electric parameters, however, can be designed to produce irreversible defects, resulting in a cell membrane that does not reseal after the field is removed. Reversible electroporation techniques have been combined with anticancer drugs such as bleomycin to target cancerous tissues for successful clinical use in the field of electrochemotherapy [69, 199-201]. Reversible electroporation is also used in other medical and biotechnological applications, including transfection and introduction of molecules such as siRNA into cells that survive the permeabilization process [202, 203]. The use of irreversible electroporation for tissue ablation is a more recent addition to the armamentarium of tissue ablation techniques available to surgeons [204-207], resulting in direct cell death without the need to introduce drugs or other molecules to facilitate the treatment process. Electroporation specifically targets the cell membrane through the application of an electric field that develops instantaneously. A major advantage of tissue ablation by electroporation is the relative speed of the procedure in comparison to any other ablation technique. Furthermore, because the procedure affects primarily the cell membrane, the extracellular matrix is spared, and this ablation modality can be used to treat tissues, such as the pancreas, without concern for collateral damage [208]. Existing tissue electrically-mediated ablation approaches, however, have their respective disadvantages. Electrochemotherapy requires the injection of drugs into the tissue, while the high electric fields used in irreversible electroporation can cause muscle contractions and may affect the electrical function of the heart.

This study was inspired by a series of papers published by the group of Marshall and others [93, 209-212] indicating that the application of electroporation pulses in tissues will produce electrolysis at the electrodes. These previous research efforts have also shown that the products of electrolysis generated during electroporation can ablate cells. The produced cytotoxic environment can be detrimental to reversible electroporation processes, such as gene transfection, that require the cells to remain alive after the procedure. While Marshall’s group sought means to minimize the electrolytic ablation during reversible electroporation procedures [210], here it is proposed that another method of tissue ablation can be achieved that combines reversible electroporation fields with electrolysis – the use of multiple reversible electroporation field pulses (MREFP). Our hypothesis is that electrolytic ablation can be made more effective when the electric charge is delivered using MREFP.

By combining the effect of permeabilization of the cell membranes induced by reversible electroporation pulsed fields with the effects of electrolytic products
generated during the entire electro-stimulation procedure, the efficiency of the electrolytic ablation procedure is dramatically increased. This is estimated by measuring the total charge dosage delivered to the target tissue for a certain degree of ablation. A plausible explanation of the phenomenon could be attributed to the electrolytically produced chemicals that can pass through the pores in the cell membrane into the interior of the cell. Thus, in this new method of cell ablation, the electrical parameters typical to reversible electroporation protocols can be harnessed using a multiple pulse sequence to produce cell death with products of electrolysis without requiring drug injection.

The key parameter that affects the outcome of an electrolytic ablation procedure is the total charge delivered at the electrodes. The delivered charge dosage is a quantitative measure of the electrochemical reactions that occur at the electrodes and the amount of electrolytic products generated. In order to test our hypothesis, we compared the extent of tissue ablation produced by conventional electrolytic protocols to that produced by employing MREFP in acute, in vivo experiments in rat liver tissue.

Our study contains two sets of experiments: first, we performed a set of conventional electrolytic ablation treatments and used histology to show typical patterns of electrolytic damage as a function of delivered charge. Second, we performed another series of experiments, this time using MREFP as the stimulation modality. In that series, we again presented the pattern of ablation as a function of the total charge delivered. The results of those two data-sets allowed us to compare the differences in histology obtained after treatments with conventional electrolysis with those obtained after MREFP treatments. The goal of the comparison is twofold: a) to examine whether the general ablation patterns obtained by the two methods are the same and b) to evaluate the extent of the affected region as a function of the total charge dosage. Due to the challenges associated with quantifying the degree of the damage, we assessed damage effectiveness through a binary evaluation, by simplifying our model to differentiate between what we defined as “non-transverse” and “transverse” damage to the tissue under study. “Non-transverse” damage refers to the situation where the electro-stimulation causes a local lesion, typically in the vicinity of the electrodes, which does not span the bulk volume of tissue between the electrodes. “Transverse” damage refers to the situation where the lesion spans the region between the stimulation electrodes. In this first order study, we will consider our hypothesis as tentatively confirmed if the general ablation patterns are the same between the two methods and if the “transverse” damage is achieved with a smaller charge when the charge is delivered using MREFP. This is a preliminary study, and our main goal was to evaluate the justification of further experimental efforts which would then focus on detailed quantification of the effect, as well as process optimization. We humbly acknowledge that much additional research remains to be done to explore a much larger set of parameters in order to understand the effects of all the parameters involved in MREFP.
Methods and Materials

Animals

Eleven male Sprague-Dawley rats (250g-350g) were obtained from Harlan Laboratories through the Office of Laboratory Animal Care at the University of California, Berkeley. The rats have received humane care from a properly trained professional in compliance with both the *Principals of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals*, prepared and formulated by the Institute of Laboratory Animal Resources and published by the National Institute of Health. The University of California-Berkeley Animal Care and Use Committee and Office approved all procedures.

Experimental Procedure

The animals were anaesthetized throughout the entire procedure via the administration of vaporized isoflurane. The depth of anesthesia was assessed before surgery and throughout the surgical procedure. After the level of anesthesia was verified, the abdominal skin was shaved and an antiseptic was applied. A 3-cm midline abdominal incision was made, exposing the liver lobes. The experimental procedure is similar to that described by Edd et al. in [205], and the same experimental procedure was used for all animals in this study. Specifically, a set of 7 mm diameter, stainless steel plate electrodes (BTX Tweezertrodes, Harvard Apparatus, Holliston, MA) were applied across a liver lobe, as shown in the device illustration presented in Figure 20. The measured distance between the two electrodes was approximately 2 mm (+/- 0.15mm). The distance between electrodes was maintained for all animals tested. The electro-stimulation in the form of electrolysis or multiple reversible electroporation field pulses (MREFP) was applied to up to 3 distinct liver lobes per animal. The experiments were acute and the animals were euthanized without recovering from anesthesia.
In the first part of our experiment, focusing on conventional electrolysis, we began with typical parameters used for electrolytic ablation, which resulted in “transverse” tissue ablation spanning the entire thickness of the liver sample. We then systematically decreased the delivered charge dosage until we found electrolysis parameters that resulted in “non-transverse” tissue ablation which did not span the entire thickness of the liver sample. For the second part of the experiment, focusing on MREFP stimulation, we began with a set of parameters close to a typical reversible electroporation treatment used in electrochemotherapy. Using these parameters we have seen only partial, non-transverse damage to the tissue, which did not span the region between electrodes. We then systematically increased the delivered charge dosage to obtain transverse damage to the liver tissue sample. In both experiments, the parameters were designed so that thermal damage to the tissue would be avoided. Due to the natural variability of the liver tissue thickness, the different lobes were found to be within the 4.5mm-6mm range and all the lobes were compressed to the same thickness of 2mm (+/- 0.15mm).

The electrolysis experiments were performed using an Arbitrary Function Generator (Tektronix/AFG 3102, Beaverton, OR). The device was set to produce a constant current for a fixed period of time. The current magnitude used was typical of standard electrolytic ablation protocols reported in the literature [23]. The stimulation time was chosen to facilitate comparison with MREFP in the context of examining the hypothesis central to the study. The parameters used in the electrolysis part of the study were: a) one minute delivery of 8 mA current for a total charge dose of 1.25 C/cm², b) one minute delivery of 4 mA current for a total charge dose of 0.625 C/cm², and c) 30 seconds delivery of 4mA for a total charge per electrode surface area of 0.312 C/cm². For the reader’s convenience, the parameter values are summarized below in Table 4. The current was delivered in such a way that the anode was attached to the top
(convex) part of the liver lobe and the cathode was attached to the bottom of the liver lobe. At least three liver lobes were treated under each experimental condition.

In the second part of our study, multiple reversible electroporation field pulses (MREFP) experiments were performed using a square pulse generator (ECM 830, *Harvard Apparatus*; Holliston, MA). A constant electric field of 250 V/cm was generated in all the electroporation experiments by applying a potential difference of approximately 50V over a pair of electrodes separated by a 2mm (+/- 0.15mm) tissue sample. This field magnitude falls within the range typically associated with reversible electroporation [213], and it is similar to the field-magnitude used in tissue electrochemotherapy. The total charge delivered was calculated from the tissue data reported in [205] and the parameters of the experiments. The experimental protocol in this part of the study consisted of first administering eight 100 microseconds long pulses of 250 V/cm delivered at a frequency of 1 Hz across the liver lobe. During this stage, a total charge of 0.0001 C/cm^2 was delivered to the sample. To study the effects of electrolytic products generated during the delivery of reversible electroporation field type pulses, we increased the delivered charge dosage by employing the following parameters: a) ninety-nine, 100 microseconds long pulses of 250 V/cm delivered at a frequency of 1 Hz across the liver lobe for a total charge delivery of 0.0012 C/cm^2 and b) ninety-nine, one millisecond long pulses of 250 V/cm delivered at a frequency of 1 Hz [203, 204, 212, 214] across the liver lobe for a total charge delivered of 0.0124 C/cm^2. These parameters are summarized in Table 4 for the reader’s convenience. The same electrodes used to apply electrolytic ablation to the liver tissue were used in the MREFP experiments. Four liver lobes (not necessarily from the same animal) were treated under each MREFP experimental condition.

As a control study we have applied sham treatment to liver samples by pressing on them using the electrodes, but without applying any electrical power, treating a total of two additional liver lobes.

<table>
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<th>Electrolysis Group</th>
<th>Delivery time [s]</th>
<th>Current [mA]</th>
<th>Total charge per unit area [C/cm^2]</th>
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</thead>
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<td>60</td>
<td>8</td>
<td>1.25</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>4</td>
<td>0.625</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>4</td>
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</table>

<table>
<thead>
<tr>
<th>MREP Group</th>
<th>No. of pulses</th>
<th>Pulse length [ms]</th>
<th>Total charge per unit area [C/cm^2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>0.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>2</td>
<td>99</td>
<td>0.1</td>
<td>0.0012</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>1</td>
<td>0.0124</td>
</tr>
</tbody>
</table>

Table 4: Electrolysis and MREP Parameters used in study. All MREP groups employed an electric field of 250 V/cm and a pulse frequency of 1 Hz.
Liver Histology

Following the conventional electrolysis, as well as the MREFP procedures, each animal was observed continuously for half an hour while being under anesthesia and thermal incubation, to facilitate a similar exposure time to the electrolytic products. After half an hour, the animals were euthanized by a combination of a vaporized isoflurane overdose and a bilateral chest dissection. The treated liver lobes were harvested, and each segment was fixed using 10% buffered formalin, placed in tissue cassettes, and submitted to three independent pathology labs (Histo-Tec Laboratories, Hayward, CA Pathology Associates, Inc., Berkeley, CA, and Pathology Research Laboratory, Inc., South San Francisco, CA). All staining was performed on 5-μm-thick cross-sections cut through a plane perpendicular to the surface of the electrodes at the center of the treated region. Masson’s trichrome staining was used to delineate the margin between normal and ablated cells in tissue, and hematoxlin and eosin (H&E) staining was performed to study the morphology of the affected cells. The H&E stained section was first cut from the tissue block, and another parallel slice was separately taken for the Masson’s trichrome staining. In all cases the treatment was delivered across two electrodes separated by 2 mm, +/- 0.15mm with slight tissue compression (original size in the range of 4.5mm-6mm, +/- 0.75mm). The histological cross sections, however, show liver samples that returned to their original dimensions after the electrodes had been removed.

Study Results

The histological results obtained from applying the conventional electrolysis and the MREFP protocols to the liver tissue samples are shown in Figure 21 - Figure 26. These results are presented as a function of the charge delivered during the protocol. The figures presented follow a similar format: the top left image in each figure is a representative tissue sample stained by Masson’s trichrome, and the middle left image shows a cross-section of the tissue sample stained with H&E. The top, middle and bottom figures on the right present higher magnifications of H&E stained regions from areas on the top, middle, and bottom of the treated region, respectively. In viewing the results, we would like to point the reader’s attention to the most important measure of comparison, which is the extent of tissue ablation from the outer surface of the liver, as a function of total delivered charge. As outlined in the introductory section, we distinguished between “non-transverse” and “transverse” damage based on the partial or transverse nature of the lesion, respectively.

Liver Histology after Treatment by Conventional Electrolysis

Figure 21, Figure 22 and Figure 23 show the histology of liver samples treated by conventional electrolytic ablation protocols as a function of decreasing delivered charge dosage. These figures are presented to demonstrate an observable relationship between the delivered charge dosage and the degree of tissue damage penetration visible in the histology. Figure 21 presents a liver sample treated with a conventional
electrolysis protocol that involves applying a current of 8 mA for one minute, resulting in a total delivered charge per electrode surface area of 1.25 C/cm². Masson's trichrome is used to differentiate ablated and unaffected tissue after treatment. A rough outline of the area affected by the procedure was overlaid on the image to focus on the region of interest (part (a) of Figure 21). The most important observation is that the damage under these experimental conditions is “transverse”, i.e. the lesion vertically spans the entire liver sample from the top to bottom surface. H&E staining (part (b) of Figure 21) also presents evidence consistent with transverse damage, and clear delineation between treated and non-treated regions is observed.

Figure 22, presents a liver sample treated with an electrolysis protocol that involves applying a current of 4 mA for one minute, resulting in a total delivered charge per surface electrode area of 0.625 C/cm². The area affected by the procedure is outlined (part (a) of Figure 22), and it can be seen that for this electrolytic dosage the damage is “non-transverse”, i.e. the lesion does not span the sample between the electrodes. This is in contrast to the results exhibiting “transverse” damage observed in part (a) of Figure 21. As expected, the damaged area is adjacent to the sample surfaces that came in contact with the anode and cathode, while the center area of the sample appears normal. H&E staining shows consistent results: the lesion does not span the sample vertically, and once again, a clear delineation between the treated and non-treated regions is seen (part (b) of Figure 22). These results show that the extent of damage produced by 0.625 C/cm² (parts (a) and (b) of Figure 22) is partial, and is qualitatively less extensive than the damage produced by delivering a charge of 1.25 C/cm² (parts (a) and (b) of Figure 21).
Figure 21: Electrolysis with a delivered charge of 1.25 C/cm². The effect on the liver tissue is seen after applying an electrolytic protocol of 8 mA for 1 min. (a) Masson’s trichrome staining is used to differentiate the affected tissue from normal tissue, and this border is roughly marked. (b) H&E staining of the tissue is shown, and closer looks at the top (anode side), middle, and bottom (cathode side) sections are shown in (c), (d), and (e), respectively. L is used to indicate the presence of a lesion in the tissue, and N indicates normal, unaffected tissue.
Figure 22: Electrolysis with a delivered charge of 0.625 C/cm². The effect on the liver tissue is seen after applying an electrolytic protocol of 4 mA for 1 min. (a) Masson’s trichrome staining is used to differentiate the affected tissue from normal tissue, and this border is roughly marked. (b) H&E staining of the tissue is shown, and closer looks at the top (anode side), middle, and bottom (cathode side) sections are shown in (c), (d), and (e), respectively. L is used to indicate the presence of a lesion in the tissue, and N indicates normal, unaffected tissue.
The last electrolysis experimental group was treated by a current of 4 mA applied for 30 seconds, delivering a total charge per electrode surface area of 0.31 C/cm². The histological results of these ablation parameters are shown in Figure 23. As in Figure 22, the most important observation shown here is that the damage is “non-transverse”, i.e. the lesion does not span the liver sample (part (a) of Figure 23). In qualitative comparison with part (a) of Figure 22, it can be observed that the tissue region affected by this smaller charge dosage (Figure 23, 0.31 C/cm²) is much smaller than that affected by the larger charge dosage (Figure 22, 0.625 C/cm²) and substantially smaller than that affected by the largest charge dosage administered (Figure 21, 1.25 C/cm²). The treated area surrounds the liver surface that was in contact with the anode and cathode, while the central area of the sample appears normal. As also seen in Figure 21 and Figure 22, there is a clear delineation between the treated and non-treated regions (part (b) of Figure 23). The relationship between the decreased charge dosage and the decreased extent of the damage does not come as a surprise; while we acknowledge that a useful extension of this research would attempt to quantify this relationship, at this point, our goal was to test whether allocating resources to further, more detailed experimental explorations could be justified. Specifically we wanted to compare the results obtained for the electrolytic ablation to the results of MREFP stimulation presented below.
Figure 23: Electrolysis with a delivered charge of 0.31 C/cm². The effect on the liver tissue is seen after applying an electrolytic protocol of 4 mA for 30 seconds. (a) Masson’s trichrome staining is used to differentiate the affected tissue from normal tissue, and this border is roughly marked. (b) H&E staining of the tissue is shown, and closer looks at the top (anode side), middle, and bottom (cathode side) sections are shown in (c), (d), and (e), respectively. L is used to indicate the presence of a lesion in the tissue, and N indicates normal, unaffected tissue.

For all three electrolysis experimental groups, the treated region exhibits variable pale eosinophilia and widespread congestion as compared to the normal parenchyma.
The appearance of the lesion at the anodic side (top surface) is different from that at the cathodic side (bottom side surface of the liver). This is considered to be a hallmark of electrolytic ablation [16, 211]. The treated region exhibits an irregular shape with a greater volume affected near the tissue surfaces that were in contact with the electrodes, as illustrated in Figure 21 and Figure 22 for the higher levels of delivered charge. Furthermore, the affected area near the anode is larger than that near the cathode. This asymmetry is typical of electrolytic damage. A closer look shows that large blood vessels in the treated zone, appearing as white open areas in the figures, are mostly free from entrapped erythrocytes. The parenchyma in the treated regions exhibits variations in color, which seem to partially correspond to the lobular structure. Centrilobular regions are darker in most cases, while other areas in the treated region are paler in color when compared to the untreated tissue.

In some samples (e.g., part (b) of Figure 22), a band of condensed strongly eosinophilic tissue is separated from the rest of the liver by an edematous layer. It is believed here that this is a consequence of the electro-osmotic migration of water from the anode to the cathode and is caused by the volume confinement produced by the use of two plate electrodes. An edematous layer is observed to a varying degree in some samples from each experimental group, but does not appear to be related to the amount of charge delivered. It is likely that this is a consequence of the local presence of large blood vessels and the resulting flow pattern in the treated region.

Images (c), (d), and (e) of Figure 21, Figure 22, and Figure 23 present a closer look at the ablated regions on the anodic side, in the middle of the treated tissue, and on the cathodic side, respectively. The histology seen at the margin between treated and normal tissue near the anode side of the liver is typical for electrolysis. As seen in part (c) of Figure 21, a sharp border, several hepatocytes thick, exists between the lesion (left) and the surrounding normal liver (right). A mild congestion in the affected sinusoids delineates the treated lesion from the unaffected tissue. The cells are eosinophilic with a visible outline and a disrupted cytoplasm. The nucleus outline is preserved, though it appears slightly condensed. These histological features also appear in parts (c) of Figure 22 and Figure 23.

In part (d) of Figure 21, a mild congestion can be observed in the affected sinusoids towards the center of the liver. This congestion delineates the treated tissue from the surrounding normal tissue. The transition zone between the normal (right) and ablated (left) cells is much larger than seen near the anode, and pockets of cells with normal appearance are present between affected cells. The affected cells are eosinophilic and resemble the cells near the anode. Here, too, the nucleus outline is preserved, though slightly condensed. These characteristics are also seen in part (d) of Figure 22, where the border between the top ablated region and the center of the tissue is examined. In part (d) of Figure 23, for the lowest delivered electrolytic charge, hepatocytes towards the center of the liver between the two electrode surfaces are intact and normal. It is important to observe that the hepatocytes in the center of the liver between the electrodes in part (d) of Figure 21 are affected due to the transverse ablation, and the hepatocytes in the center region are normal in part (d) of Figure 23 due to the non-transverse ablation resulting in a non-transverse ablation zone.
The effect of the electrolytic ablation at the border of the treated area on the side of the liver in contact with the cathode is examined in part (e) of Figure 21, Figure 22 and Figure 23. Here, as near the anode, there is a sharp border, several hepatocytes thick, between the lesion and the surrounding normal tissue, and once again a mild congestion in the affected sinusoids delineates the treated lesion from the surrounding normal tissue. The treated cells near the cathode differ from those near the anode; the nucleus is pale and retained less stain than near the anode, likely due to a loss of chemical function. As a result of cell lysis, there is a disruption of the plasma membrane, homogenization of the cytoplasm, and loss of normal lobular structure. It can be seen here that the cells near the anode are more eosinophilic than near the cathode. A band of edematous tissue near the cathode side can be viewed in more detail in parts (e) of Figure 22 and Figure 23. Though this band is not present in part (e) of Figure 21 for the 1.25 C/cm² delivered charge, it should be emphasized that edematous tissue bands similar to what is seen in parts (e) of Figure 22 and Figure 23 were observed in some cases for the parameters of Figure 21. Figure 21 is used here to illustrate how blood flow and large blood vessels can affect the local outcome of electrolytic ablation procedures. A possible explanation to this observation might have to do with the different electrical properties of blood vessels relative to liver tissue. By being more conductive than their environment, blood vessels might create preferential paths for current flow which concentrates the electrolytic damage in their vicinity. This is an interesting phenomenon which deserves additional exploration and we intend to pursue it in our future work.

Liver Histology after Treatment by Multiple Reversible Electroporation Field Pulses

The effects of applying multiple reversible electroporation field pulses across the liver sample are depicted in Figure 24, Figure 25 and Figure 26. These results serve as a means to examine the general pattern of how tissue is affected by MREFP as a function of the total delivered charge dosage. Our goal was to compare the pattern observable under MREFP stimulation with the results from conventional electrolytic studies presented in Figure 21, Figure 22 and Figure 23.

Figure 24 presents a sample treated with a typical reversible electroporation protocol of 8 pulses of 100 microseconds in length at a frequency of 1 Hz, resulting in a total applied charge per unit area of 0.0001 C/cm². Trichrome staining shows tissue damage near the points of contact with the electrodes (part (a) of Figure 24), and the ablation results observed in part (a) of Figure 24 are consistent with pervious observations by Marshall's group [93, 210, 211]. The region of tissue ablation has been roughly outlined, and it can be seen that the treated area surrounds the liver surface that was in contact with the anode and cathode, while the center area of the liver sample appears normal. H&E staining presents consistent findings - lesions can be seen near the surfaces in contact with the electrodes (part (b) of Figure 24), and a clear delineation is observed between treated and non-treated areas. This figure clearly
shows that the damage is local and the affected area does not span the entire liver sample.

Figure 24: MREP with a delivered charge of 0.0001 C/cm². The effect on the liver tissue is seen after applying an electroporation protocol of 8 pulses of 100 microseconds at a 1 Hz frequency. (a) Masson’s trichrome staining is used to differentiate the affected tissue from normal tissue, and this border is roughly marked. (b) H&E staining of the tissue is shown, and closer looks at the top (anode side), middle, and bottom (cathode side) sections are shown in (c), (d), and (e), respectively. L is used to indicate the presence of a lesion in the tissue, and N indicates normal, unaffected tissue.
Figure 25 presents histology of a liver sample treated with a MREFP sequence of 99 pulses of 100 microseconds long applied at a 1 Hz frequency to deliver a total charge dose of 0.0012 C/cm². To compare ablated volumes between different sets of parameters, the area of the tissue affected by the procedure has been outlined (Figure 6a). It can be seen here that the affected area surrounds the liver surfaces that were in contact with the anode and cathode, while the bulk area of the liver sample appears normal. H&E staining also shows that the damage is non-transverse, i.e. the lesion does not span the liver sample vertically. As before, a clear delineation is seen between treated and non-treated areas (part (b) of Figure 25).
Figure 25: MREP with a delivered charge of 0.0012 C/cm². The effect on the liver tissue is seen after applying an electroporation protocol of 99 pulses of 100 microseconds at a 1 Hz frequency. (a) Masson’s trichrome staining is used to differentiate the affected tissue from normal tissue, and this border is roughly marked. (b) H&E staining of the tissue is shown, and closer looks at the top (anode side), middle, and bottom (cathode side) sections are shown in (c), (d), and (e), respectively. L is used to indicate the presence of a lesion in the tissue, and N indicates normal, unaffected tissue.
The final set of experimental parameters in this study consisted of MREFP sequence of 99 pulses, at 1 ms pulse length, and an applied frequency of 1 Hz, resulting in a total charge dose of 0.0124 C/cm². This experimental group produced transverse damage, i.e. a lesion that penetrates transversely through the liver from the top surface to the bottom surface, as outlined in part (a) of Figure 26. H&E staining is consistent with transverse damage as it also shows that the lesion vertically spans the liver sample, and a clear delineation between treated and non-treated areas is observed (part (b) of Figure 26).
Figure 26: MREP with a delivered charge of 0.0124 C/cm². The effect on the liver tissue is seen after applying an electroporation protocol of 99 pulses of 1 ms at a 1 Hz frequency. (a) Masson’s trichrome staining is used to differentiate the affected tissue from normal tissue, and this border is roughly marked. (b) H&E staining of the tissue is shown, and closer looks at the top (anode side), middle, and bottom (cathode side) sections are shown in (c), (d), and (e), respectively. L is used to indicate the presence of a lesion in the tissue, and N indicates normal, unaffected tissue.

In Figure 24-Figure 26, the treated region exhibits variable pale eosinophilia and widespread congestion as compared to the normal parenchyma, similar to the tissue
treated with conventional electrolysis in Figure 21, Figure 22 and Figure 23. The lesion shape is irregular and, for higher levels of delivered charge (Figure 25 and Figure 26), the volume of ablated tissue is larger near the anodic surface (top side) in comparison to the cathodic surface (bottom side of liver). The ablated regions are further examined on the anodic side (part (c) of Figure 24-Figure 26), in the middle of the treated tissue (part (d)), and on the cathodic side (part (e)). Typical electrolysis histology can be seen at the margin between treated and normal liver on the side of the liver that was in contact with the anode (parts (c) of Figure 24, Figure 25 and Figure 26). There is a sharp border, several hepatocytes thick, between the lesion and the surrounding normal liver. The cells are eosinophilic with a visible outline and a disrupted cytoplasm, and the nucleus outline is preserved, though slightly condensed.

In part (d) of Figure 24, the region towards the center of the tissue consists of hepatocytes that are intact and normal, further indicating that the ablation was non-transverse, i.e. the lesion did not penetrate through the entire sample thickness. Figure 6d focuses on the border between the lesion and the normal tissue in the center of the sample. Here, the appearance is very similar to what was seen in the tissue close to the anodic side, with congestion in the affected sinusoids delineating the treated tissue (top) from the surrounding normal tissue (bottom). The affected cells are eosinophilic and resemble the cells near the anode. In Figure 26, for a delivered charge of 0.0124 C/cm², the treated region towards the center of the liver (part (d) of Figure 26) mimics the electrolytic ablation results, displaying a mild congestion of the affected sinusoids, and the presence of eosinophilic cells in the treated region resembles this near the anode. Here, there is a larger transition between normal (right) and ablated (left) cells with pockets of cells appearing normal observable between regions of affected cells.

A closer look at the border of the treated area on the side of the liver in contact with the cathode (part (e) of Figure 24) reveals a sharp transition zone, several hepatocytes thick, between the lesion and the surrounding normal tissue. The treated cells near the cathode differ from those near the anode, as shown in parts (e) of Figure 24, Figure 25 and Figure 26. The nucleus is pale and retains less stain than near the anode, likely due to a loss of chemical function. As a result of cell lysis, there is a disruption of the plasma membrane, homogenization of the cytoplasm, and loss of normal lobular structure. In comparison, the cells near the anode are more eosinophilic than near the cathode.

Discussion

This study examines the histology of tissue in which charge is delivered by multiple reversible electroporation field pulses (MREFP) in comparison to the histology of tissue when charge is delivered via conventional electrolysis protocols. The study of conventional electrolytic ablation uses a set of parameters comparable to that typically used in literature. The results for the experiments in which a charge of 1.25 C/cm² was delivered with conventional electrolysis are shown in Figure 21 and are consistent with other studies on the histology of electrolysis of the liver [16, 211]. Specifically, the entire ablated tissue was sharply demarcated from the normal liver tissue, with visible
congestion in the sinusoids at the margin of the lesion. The appearance of the treated tissue near the anode, the cathode, and in the core of the treated tissue is markedly different. Near the cathode, there is colliquative necrosis and the nuclei are not stained, the cell membrane is not detected, and there is homogenization of the cytoplasm and loss of lobular structure. Near the anode, the damage is coagulative, the cytoplasm is eosinophilic, and the nuclei morphology is visible. The core of the ablated zone, between the cathode and the anode is eosinophilic. The dissimilar appearance of the tissue is caused by different products of electrolysis near the anode and the cathode, resulting in different ablative reactions. Near the anode, the pH is low, and many of the toxic chemical species are related to the various pH-dependent components of Cl. Near the cathode, on the other hand, the pH is high, and the toxicity is primarily caused by various components of OH⁻ [16].

Studies have shown that the tissue ablation produced by conventional electrolytic ablation protocols is dependent on the amount of charge delivered. The ablated regions occur near the electrode for lower charges and are not transverse and increase outward from the electrode with an increase in the delivered charge, to become transverse. The dependency of the extent of ablation on the charge dosage is shown in Figure 21, Figure 22 and Figure 23. The observed decrease in the degree of ablation with a decreasing total delivered charge dosage is consistent with that reported in the literature, and these histological results are also consistent with many other studies on electrolysis [16, 38, 211]. The experimental results shown in Figure 21, Figure 22 and Figure 23 serve to illustrate a general pattern of how the degree of tissue damage caused by conventional electrolytic ablation protocols is a function of the charge delivered. The affected region propagates from the electrodes towards the core of the tissue, and the extent of tissue ablation is directly related to amount of charge delivered. For lower doses of charge the ablation is non-transverse, i.e. localized to the electrode surfaces, and as the charge dosage is increased, so, too, does the extent of the damage. Above a certain threshold, the damage becomes transverse, i.e. the lesion penetrates through the entire thickness of the sample, as seen in Figure 21. Furthermore, the appearance of the treated tissue near the anode is different from that near the cathode. For the higher levels of delivered charge, the treated volume near the anode is larger than that seen near the cathode. These characteristics appear to be the hallmarks of conventional electrolytic ablation.

For the experimental protocols that applied MREFP, the first key observation is that reversible electroporation pulses can indeed cause tissue ablation. Furthermore, the resulting pattern of tissue ablation is very similar to that obtained by conventional electrolysis. Results from an eight pulse reversible electroporation protocol (250 V/cm, 100 microsecond pulses at a frequency of 1 Hz) typical to reversible electroporation treatments [203, 204, 212, 214] are shown in Figure 24. The results in Figure 24 show that the morphology of the affected regions of the tissue is similar to that obtained with electrolysis. This is consistent with the findings of Marshall’s group, who have shown and predicted that in conventional reversible electroporation electrolysis should occur [93, 209, 210]. As is typical for electrolytic ablation, the histology near the anode and cathode are different, and the tissue damage is localized near the electrodes.
Before we introduce the observation differentiating MREFP ablation from traditional electrolysis ablation, we would like to direct the reader’s attention to Figure 27 which synthesizes the Masson trichrome staining results from the six representative experiments. This figure is introduced to aid the comparison between the two modalities and introduces no new information; in fact, we obtained Figure 27 by grouping the first (a) images of Figure 21-Figure 26. The left column aggregates images of conventional electrolysis treated samples by decreasing charge dosage, and the right column aggregates images of MREFP treated samples by decreasing charge dosage.

![Figure 27: Aggregated Masson trichrome staining results from 6 representative experiments. (a) Electrolysis 1.25 C/cm² (b) Electrolysis 0.625 C/cm² (c) Electrolysis 0.31 C/cm² (d) MREFP 0.0124 C/cm² (e) MREFP 0.0012 C/cm² (f) MREFP 0.0001 C/cm²](image-url)
The second key observation following from the MREFP experimental series can be seen in Figure 26: the affected tissue retained the characteristics seen in conventional electrolytic ablation. The damage is **transverse**, i.e. the lesion penetrates through the liver, and the appearance of the treated lesion at the anode is different from that at the cathode. The MREFP results could be conveniently compared to conventional electrolysis results using Figure 27 as an aid, in order to highlight a very important observation from this study: when the damage was applied using the MREFP modality, relative to conventional electrolytic ablation, a much lower charge dosage was required to cause a comparable damage. In part (d) of Figure 27, a **transverse** liver sample damage is achieved with a MREFP by delivering a charge of 0.0124 C/cm². In comparison, in part (c) of Figure 27 the damage is only partial, although the conventional electrolysis delivered 25 times more charge per unit area of electrode (0.312 C/cm²). In summary, the histological results in Figure 24-Figure 26 show that the appearance of the treated tissue near the anode is different than that near the cathode, consistent with electrolytic ablation.

The key finding of this study can be emphasized further when comparing the ablated volume obtained using MREFP with a total delivered charge of 0.0124 C/cm² (part (d) of Figure 27) to the volume ablated after applying a total charge of 1.25 C/cm² through conventional electrolysis (part (a) of Figure 27). The degree of damage caused by the two methods is comparable, qualitatively referred to as **transverse** in our notation, but it is noteworthy that MREFP applies between one and two orders of magnitude less charge.

The results presented here show that tissue treated by MREFP exhibits some degree of similarity to a damage pattern associated with electrolytic ablation. Specifically, the extent of the affected zone increases with the number of pulses delivered and the pulse length i.e. the total charge delivered, and the ablated region starts close to the electrodes and moves outward into the tissue. This is typical of a diffusion driven effect. This pattern is different from ablation induced by electroporation, which is generated when an electric field is produced between the electrodes. Because the electric field is essentially produced instantaneously and uniformly near the centerline between the electrodes, the affected region around the centerline between the electrodes has a homogenous appearance [206]. Therefore, nature of tissue damage caused by MREFP is different from that caused by irreversible electroporation. To further differentiate with electroporation, MREFP results in a treated volume which is preferentially larger at the anode, compared to the cathode. All these features appear to be the hallmarks of conventional electrolytic ablation as presented in Figure 21-Figure 23 and discussed above. The results of this study suggest that when repeated reversible electroporation field pulses are delivered to tissue, the mechanism of cell death appears to be consistent with the mechanism of electrolytic ablation. We believe that this study produces further support to the findings of Marshall’s group that electrolysis can occur in conventional reversible electroporation, proving lethal to the electroporated cells [93, 209, 210]. The toxicity of the electrolytic reactions at the electrodes has also been well-known in the food industry. A recent comprehensive review on this issue can be found in [215].
Due to the different damage mechanisms between electroporation-based approaches and conventional electrolysis, we have found that the quantity allowing a meaningful comparison between these two electro-treatment modalities is the total charge dosage delivered. We have designed our study to evaluate the qualitative degree of damage as a function of total charge dosage delivered. As shown in Figure 22 and Figure 23, a conventional electrolysis delivered dose of 0.312 C/cm² and 0.624 C/cm², respectively, causes only non-transverse damage. In contrast, delivering a dose of 0.0124 C/cm² using 1ms pulses with MREFP produces a transverse damage to the tissue i.e. a lesion that penetrates through the liver thickness (Figure 26). The time scale for diffusion is comparable. In addition, after the total charge was delivered, each animal was observed for half an hour while under anesthesia, allowing the tissues to be exposed to the electrolytic components for a similar period of time for both the conventional electrolysis and MREFP procedures. Therefore, it is apparent that other factors, beyond the total delivered charge, influence the differences observed between results obtained with MREFP, in comparison with conventional electrolysis. Reversible electroporation results in the permeabilization of the cell membrane, and this effect should occur uniformly between the two parallel electrodes used in this study. In comparison to the time scale of diffusion by electrolysis, the permeabilization of the cell membrane by reversible electroporation is an instantaneous phenomenon. Therefore, this study suggests that reversibly electroporated cells are affected by much lower doses of electrolytic products than intact cells, and thus, MREFP can be used as a more effective electro-ablation technique than conventional electrolysis.

The extent of ablation can be compared to that obtained using conventional electrolytic parameters that delivered an intermediate charge dosage of 0.625 C/cm² (parts (a) and (b) of Figure 22) and small charge dosage of 0.31 C/cm² (parts (a) and (b) of Figure 23). This observation is one of the key findings consistent with our key hypothesis: the histology data indicates that the extent of affected area produced by delivering a total charge dosage of 0.0012 C/cm² by MREFP (parts (a) and (b) of Figure 25) is comparable to that obtained by applying a much higher total charge by conventional electrolysis. Although at this point the data is qualitative, the difference in the charge delivered is 2 orders of magnitude.

While this remains to be proven, a reasonable explanation for the observed phenomena of increased damage under MREFP is the interaction between the permeabilization of the cell membrane and the products of electrolysis. Leveraging this interaction presents the potential to induce cell death using a lower charge dosage in a permeabilized cell, in comparison to a cell with an intact membrane.

A possible application of the findings in this study is a method of tissue ablation that uses reversible electroporation fields to permeabilize the cell membrane to products of electrolysis and induce thereby cell death. This study explored a protocol in which the reversible electroporation field pulses also produce products of electrolysis at the electrodes. However, other uses of this concept are possible. For instance, conventional electrolysis could be done first and then reversible electroporation pulses could be delivered to enhance cell death in the region in which the cell membrane has
permeabilized and to facilitate the penetration of the products of electrolysis into the cells.

The use of MREFP in tissue ablation has also a potential application in extending the ablated tissue volume without increasing the voltage on the electrodes. When irreversible electroporation field pulses are applied between electrodes, the tissue ablation protocol is designed to produce irreversible electroporation fields in a certain volume of tissue adjacent to the electrodes. However, due to the nature of the electric field distribution, a region exists outside the irreversible electric field zone in which the field is lower, i.e. typical of reversible electroporation protocols. The simple change in protocol of producing multiple pulses will generate damage in the region exterior to the irreversible electroporated region through the MREFP phenomena. Obviously, the multiple pulses need to be designed in such a way that the electrolytic products will diffuse to the regions in which reversible electroporation fields form. Nevertheless, this could be a simple method to extend the tissue ablation affected volume during irreversible electroporation.
2. Original Study: Preventing Electrolysis - AC Coupled Electroporation

Experimental Context

When a biological cell is exposed to a high electric field, nano-scale defects, termed in the literature as pores, occur in its membrane leading to dramatic qualitative changes in the cell's membrane permeability [78, 216]. This biophysical phenomenon has become an important tool in various domains of modern science, medicine and biotechnology [217, 218]. Depending on the strength of the electric field, the cell either reseals the pores and returns to its normal activity, or sustains a sufficient shock that would lead it to death. When the effect is reversible, and the cell can recover from the induced “interference”, the electroporation is referred to as reversible [74, 217]. When the damage is too great and the cell is not able to return to its normal function, the phenomenon is naturally called irreversible electroporation [74, 78].

It is widely accepted that electroporation depends on several electric parameters, such as electric field strength, polarity, and time of application. With respect to electric field strength, when all the other parameters are kept constant, electroporation is a threshold phenomenon, i.e. an electric field of a magnitude $|\vec{E}| < |\vec{E}_r|$ would not cause electroporation, a field of a magnitude $|\vec{E}_r| < |\vec{E}| < |\vec{E}_i|$ would cause reversible electroporation, and a field of larger magnitude $|\vec{E}| > |\vec{E}_i|$, would lead the cell into irreversible electroporation. In life-science research, reversible electroporation is leveraging the increase in the cell membrane's permeability to introduce molecules, drugs and genes into biological cells [217]. The application of electroporation in the irreversible mode has recently gained attention in medicine as a potentially useful tissue ablation method [74, 88]. In the food-sterilization field, electroporation has been primarily used in the irreversible mode. Pulsed-Electric-Fields (PEF), as the phenomenon is known in this domain, have been studied for several decades as a useful method to kill bacteria and other pathogens naturally proliferating in foods [219].

Most of the research in the field of electroporation, both in life-sciences and food-sterilization domains has been focused on DC electric fields [220]. In such a setting, a typical way to induce electroporation would include bounding the treated medium with a parallel plate configuration of electrodes with DC voltage pulses applied to them. While making the instrumentation simple, the DC-based method of electroporation introduces various electrolytic processes taking place at the electrode-electrolyte boundary [28, 92]. These electrolytic effects include electrochemical reaction with the electrodes and the formation of gases next to the surface of the electrodes. When exposed to high electric fields, these gases become ionized, which leads to arcing, generation of high local temperatures, and the resulting strong pressure waves. Even in the absence of arcing, the electrolytic processes occurring at the electrolyte-electrode boundary lead to various phenomena, which affect the process of electroporation. These include release of metal ions in the solution and formation of various ionic chemical species that affect cells and electrodes. This problem has not been addressed in the context of biological applications of electroporation, because typically, the protocol involving electroporation is on the order of milliseconds during which the pulses are administered. During this time, the electrolytic effects are not prominent and are normally ignored and not
reported. However, there is no doubt that everyone doing experimental work on electroporation must have noticed at least occasional arcing.

Advances in micro-electromechanical technology have made it possible to design a single cell micro-electroporation chip [114]. This has paved the way to the flow-through, single cell, microelectroporation chip [130]. Microelectroporation is an important component in many lab on a chip devices [131]. Attempts to further miniaturize electroporation have led to devices that electroporate only a part of the cell's membrane [132], and to nano-electroporation [128]. Literature survey indicates a growing interest in micro-electroporation, and a thorough review of flow-through micro and nano/electroporation devices can be found in [133]. However, as the size of the effective electroporation volume reduces, geometric considerations begin to play an important role. The effective electroporation volume decreases proportionally to $s^3$, whereas the active surface area approximately decreases proportionally to $s^2$; where $s$ is a typical dimension for electroporation. This leads to increased dominance of the mass transport phenomena prompted by electrolysis, since the relative concentrations of the reaction products are higher in smaller scale electroporation chambers. The presence of arcing and electrolytic reactions introduces undesirable effects, which often lead to structural damages of electroporation electrodes as illustrated in Figure 28. We have observed this damage when applying a series of 10 100-microsecond pulses at 5000 V/cm on a sample containing 0.9% NaCl solution. We have used planar interdigital electrodes fabricated using a standard PCB process with 150 um spaces between the anode and the cathode. The applied voltage was 75V. Furthermore, while in micro-electroporation, arcing can usually be avoided due to reduced voltages and flow through induced convection, the formation of gas bubbles can lead to interrupted flow in micro-channels, limiting the utility of flow-through electroporation devices [86].

![Figure 28: Damaged electrodes used in DC electroporation. Damage was caused by arcing.](image-url)
Using AC fields seems like a natural attempt to be taken to address the undesired effects caused by DC electric fields and indeed several recent works report efforts in this direction. In a recent work from our group, bipolar pulses have been used to reduce the effects of electrolysis in a micro-channel, in particular the formation of gas bubbles [86]. However, in that work, the electrodes were still galvanically coupled to the electroporated medium, which introduces products of electrolytic reactions into the sample. Also, the authors of the study report, that in order to avoid the electrolytic effects, the bipolar pulse frequency has to be within a limited range. This can be explained by noting that for a slow enough frequency, the square pulses administered still induce short-term electrolysis, which might potentially lead to undesired bubble formation. Another work reported a design of a microfluidic based AC electroporation device [221] using gold-deposited electrodes, which, it was suggested, are less likely to suffer from electrolysis induced electrode erosion. The authors were able to reduce the required AC voltage by fabricating microelectrodes within close proximity to each other (30[um]). However, in our experiments, we find that galvanically coupled gold micro-electrodes are affected by electrolysis (Figure 28).

In this study, we propose to address the issue of electrolysis in micro and nano electroporation chips, through elimination of the electrolytic process by capacitively coupling the electroporation electrodes to the medium, across a dielectric layer. To the best of our knowledge, there are only two other reported attempts to use capacitively coupled electroporation [222], [223]; in a context completely different from ours. The approach we propose in this paper is based on a recent work performed in our group, which introduced the concept of Singularity Induced Electroporation (SIE) [128]. In the SIE approach, a specific configuration of planar electrodes eliminates the need in high-voltage electroporators, and can operate from a battery power supply. While our proposed device architecture requires a higher voltage level than currently available from a standard battery, we build upon previous results reported in [128, 224] as we employ a similar geometric configuration of electrodes, but introduce a novel element: a thin dielectric layer which isolates the electrodes from the electrolyte and capacitively couples AC produced electric fields across the layer.

The goal of this study is to advance the concept of AC-based, capacitively coupled, micro and nano-electroporation by developing a set of analytical design tools. Using these tools, we propose to characterize the electroporation process and generate physical insight into the effects of the various design parameters. Our work is mathematical and employs numerical simulations and closed-form analytical tools.

In this work, we present a theoretical analysis of a micro-electroporation system in which capacitively coupled electrodes are separated from the electroporated solution.
by a dielectric. The purpose of this work is to serve as a theoretical basis for further experimental exploration, as well as a design tool for performance optimization. We present a mathematical model supported by in silico experimental results, and outline an experimental design, which draws intuition and design guidelines from the findings of the in silico simulations.

Methods and Materials

Our mathematical model consists of a 2D cross-sectional geometry with two planar metal electrodes placed on an insulating substrate as depicted schematically in Figure 29. One of the electrodes is grounded and the other one is connected to an AC voltage source.

The electrodes are coated by a thin layer of insulating material, which replaces the galvanic coupling with capacitive link. On top of the insulating material, a bulk volume of solution is placed. A simulation of the electroporation process requires solving the field equation. Since our model contains both conductive (electrolyte filled solution) and dielectric materials (insulating coating), both displacement and conduction current exist. The main equation solved is current conservation:

\[ \nabla \cdot \mathbf{j} = 0, \]  

(55)
where $\nabla \cdot ()$ is the divergence operator, and $\vec{J}$ stands for the local current density vector. The current density has a conductive component and a displacement component, and it is given by:

$$\vec{J} = (\sigma + \varepsilon_0 \varepsilon_r \frac{\partial}{\partial t}) \vec{E}. \tag{56}$$

$\vec{E}$ represents the local electric field, $\sigma$ is the conductivity, $\varepsilon_0$ is the vacuum permittivity and $\varepsilon_r$ is the relative permittivity of the material. The electric field is linked to the potential field by the relationship:

$$\vec{E} = -\nabla U. \tag{57}$$

The field equation is solved for the geometrical configuration of Figure 29, subject to Dirichlet boundary condition (sinusoidal voltage), imposed at the electroporation electrodes. The remainder outer surface of the domain was insulated.

Solving the Laplace equation makes it possible to estimate the associated Joule heating $P$, which is the heat generation rate per unit volume, caused by the electrical field:

$$P = \sigma |\nabla \phi|^2. \tag{58}$$

This term is then added to the Heat Equation to represent the amount of heat generated during the electroporation procedure:

$$\nabla \cdot (k \nabla T) + P = \rho C_p \frac{\partial T}{\partial t}. \tag{59}$$

In equation (59), $k$ is the thermal conductivity of the solution, $T$ is the temperature, $\rho$ is the solution density and $C_p$ is the heat capacity of the tissue. The intent of the thermal analysis is to estimate the temperature changes occurring during the electroporation. In a typical application, when electroporation is used for its non-thermal effects, the protocol is often optimized to operate in non-thermal regime [212, 225].

The geometric and electrical parameters characterizing such a configuration are summarized in Table 5. Our analysis is based on a Finite Element Model (FEM) implemented in Comsol Multiphysics 4.3b. Our solution was run on a triangular mesh with boundary layer mesh close to the expected high potential gradient domains. As a means of quality control, the mesh size was reduced to verify that no artifacts are introduced into the solution due to sampling issues. The difference between the solutions was within the relative tolerance specified to the numerical solver (1e-4).

**Study Results and Discussion**

**Experimental Validation**

The goal of our study is to characterize the concept of AC capacitively coupled micro and nano-electroporation. Numerical simulation and closed-form analytical
solutions facilitate achieving this goal economically. However, to gain confidence in the mathematical analysis, we will first verify the mathematical solution with an experiment. To test the experimental validity of our model, we have developed a prototype for an insulator coated electrode using Pyralux AC 091200EV (DuPont, USA) flexible circuit material consisting of 12.5 [um] thick Kapton sheet with a 9[um] layer of electrodeposited copper on one side. The 12.5 [um] Kapton layer models the insulation layer we envision in the AC coupled device (Figure 29). The inter-digital electrode was patterned using standard photolithography methods and sealed at the exposed (copper) side by placing an adhesive-based, 200 [um]-thick Kapton layer on top of the electrode. The thick insulator layer on the top of the electrodes models the substrate (Figure 29) and its high impedance causes most of the displacement current to flow through the thinner Kapton layer of 12.5 [um]. Although our prototypical device uses a larger scale geometry, our motivation behind fabricating it was to confirm the basic assumptions of our model and we demonstrate this confirmation below by comparing the measured impedance with the one predicted by the mathematical model. Part (a) of Figure 30 shows a schematic drawing of an inter-digital electrode, and part (b) of Figure 30 shows a top view of a fabricated electrode. For the experiment, we immersed the insulated electrode into a sodium chloride solution (0.08 g/l), designed to have a conductivity of 0.1 [S/m] [226].

![Figure 30: Inter-digital electrodes (a) schematic, (b) fabricated Electrode](image)

We then characterized the performance of the electrode using impedance analyzer (Agilent 4294H) measuring the impedance between the two electrodes in the [40Hz-1MHz] frequency range. We have compared the data obtained from a simulated model of equations (55)-(57) with the parameter values of Table 5. To obtain the simulated current through the device, we have used the Electrical Circuit module of Comsol, and added a small current sense resistor in series with the electroporation device depicted in Figure 30. The magnitude of the impedance was obtained from Ohm’s law relationship: \(|V| = |Z| |I|\). The simulated impedance data was compared to the experimentally measured impedance profile, shown in Figure 31. It can be seen that the experimental measurements compare well to the estimates provided by the mathematical model.
Numerical Experiments

Figure 32-Figure 35 were obtained from numerical experiments solving equations (55)-(57) with parameter values from Table 6. The results are presented to provide insight on the effect of a dielectric insulation layer in a capacitively coupled micro-electroporation system.

Figure 31: Experimental vs. simulated impedance magnitude data.

Figure 32: Phase shift in the electrolyte solution.
Figure 32 illustrates the phase shift between the stimulation voltage and the induced voltage in the electrolyte, caused by the dielectric nature of the insulator coating. The presented field magnitudes and the stimulation voltage were normalized for visualization purposes. The electric field in the insulating layer (red curve) is in phase with the stimulating voltage, whereas the electric field in the electrolyte solution (blue curve) is approximately 90 degrees out of phase with the stimulating voltage. The exact phase difference depends on the conductivity on the solution (data not shown).

A representative potential profile along a vertical axis (shown green in Figure 29) is presented in Figure 33. Part (a) of Figure 33 shows the potential profile when the stimulation voltage is at its peak: The entire voltage drops on the insulator, and there is no potential drop (i.e. no field) inside the electrolyte solution. Part (b) of Figure 33 shows the potential profile when the stimulation voltage is at its zero: at this point, a voltage drop inside the electrolyte is induced by the lagging-behind displacement current.

![Figure 33: Potential profile (a) stimulation peak (b) stimulation zero.](image)

Part (a) of Figure 34 shows a map of the peak field magnitude of a representative configuration. Part (b) of Figure 34 shows a field profile taken inside the electroporated solution adjacent to the insulating surface. The measured slice is shown as a blue, horizontal arrow on Figure 29. A vertical step $\Delta h$ equal to 10% of the insulator thickness $h_i$ was used as the height above the insulating surface for measuring the profile.
For comparison, we have simulated a configuration without the insulating layer. Part (a) of Figure 35 shows a map of the peak field magnitude of a comparable configuration. Part (b) of Figure 35 shows a field profile taken inside the electroporated solution at the same location relative to the electrodes as in Figure 32. Figure 35 indicates that while there is little qualitative difference between the two configurations, the quantitative difference is large: the field is 2-3 orders of magnitude higher without the insulator. This is clearly a drawback of the capacitative coupling mode, as a much higher external voltage is required in order to provide the same electric field in the medium.

Having said that, we present a sensitivity study in a later section, which provides an insight on the effect of different system parameters as well as concrete design guidelines for mitigating the effect.

**Lumped-element model**

The design of AC capacitively coupled micro and nano-electroporation systems may benefit from a simplified, lumped-element circuit model that can be used to get a first order approximation of the effects of various electric parameters. Such a design is presented in Figure 36. The insulating layers were modeled as two capacitors $C_1$ and $C_2$. 
while the electrolyte was modeled as a linear resistor $R_{solution}$. Parallel plate capacitor model was used to estimate the capacitance. Conductance was then estimated using the systems geometry and the electric properties of the materials:

$$C = \frac{\varepsilon_r \varepsilon_0 A_c}{d}, \quad G = \frac{\sigma A_G}{l}. \quad (60)$$

In equation (60), $C$ stands for capacitance, $\varepsilon_r$ is the relative permittivity of the material, $\varepsilon_0$ is the dielectric permittivity of free space, $A_c$ is the plate area and $d$ is the distance between the plates. $G$ is the conductance, $\sigma$ represents the material conductivity, $A_G$ represents the cross-sectional area of the conductor and $l$ represents the length of the conductor. Since the geometry of our model does not map directly into the formulations of equation (60), we have used these estimates: $A_c = w_e \times 1, d = h_t, A_G = w_e \times 1, l = 2d_e$. See Table 6 for numerical values specification. Using the nominal configuration, the capacitance and the conductance are estimated and the current through the system is estimated using Ohm’s law, and taking into account the electrical impedance of the equivalent circuit:

$$I = \frac{V}{SF \left( Z_{equivalent} \right)} = \frac{V}{\frac{SF}{wC_1} + SF \cdot R_{solution} + \frac{SF}{wC_2}}. \quad (61)$$

A typical approach allowing accounting for a complex geometry in terms of a more simplified geometry, uses a Shape Factor (SF) that correlates between the two configurations. The SF is calculated from the exact solution, by comparing the actual current calculated in a certain complex geometrical configuration with calculations in expression (61), and evaluating the SF yielding identity of both sides. Because the equations are linear, the SF value remains constant when linear scaling of dimensions are used and does not change much in the neighborhood of the estimated geometry. In
our case, the SF used was 193.53. Figure 37 shows the relative impedance of the capacitative layer vs. the resistive impedance of the electrolyte solution.

![Figure 37: Relative Impedance Magnitude.](image)

The geometric and electrical parameters were set to their nominal values listed in Table 6, and only the insulator thickness was varied. It can be seen from this figure that for the taken parametric configuration, an insulating layer thicker than 100nm causes most of the voltage drop to occur across the capacitative link.

**Design Considerations**

Following are studies dealing with various design considerations of importance in an AC capacitively coupled micro and nano-electroporation system.

**Dielectric breakdown considerations**

One of the key design constraints of the capacitively coupled electrodes is the dielectric breakdown threshold of the insulating layer. Dielectric films tend to exhibit greater dielectric strength than thicker samples of the same material. For instance, the dielectric strength of silicon dioxide films of a few hundred [nm] to a few [um][μm] thick is approximately \(0.5 \frac{GV}{m}\). The dielectric breakdown voltage determines the frequency of the stimulation and the range of insulator thickness values which can be used under idealized conditions. In reality, materials exhibit defects which lower their breakdown voltage [227], and the exact values will have to be determined experimentally. In this work we take the dielectric breakdown into consideration during the parametric sensitivity analysis in a later section.
Dosage Considerations

Multiple models for optimizing electroporation protocols were reported [212, 225, 228], however for the most part they deal with DC pulses and do not address AC high-frequency stimulation. Considering electroporation as a threshold phenomenon, the following model could be used to estimate the time that the sample is exposed to the supra-threshold e-field magnitude. In the equation outlined below, $t_{\text{exposure}}$ represents the time that the sample is exposed to supra-threshold e-field, $f$ represents the stimulation frequency, $E_{\text{peak}}$ is the peak magnitude of the electric field and $E_{\text{threshold}}$ is the ratio of the peak e-field magnitude that leads to electroporation. $N_{\text{cycles}}$ stands for the number of stimulation cycles. The detailed derivation of equation (62) is presented in the appendix.

$$t_{\text{exposure}}(f, E_{\text{peak}}, E_{\text{threshold}}) = 4 \cdot \frac{1}{f} \cdot N_{\text{cycles}} \cdot \left[ \frac{\pi - \arcsin \left( \frac{E_{\text{threshold}}}{E_{\text{peak}}} \right)}{2\pi} \right].$$ (62)

Figure 38 shows a single period of a sinusoidal E-Field with supra-threshold regions marked for a threshold of 0.6. A typical electroporation protocol [229] uses a sequence of 10 pulses of 100 [us] with 1 second interval between adjacent pulses. We have used the proposed mathematical model to develop a comparable AC stimulation protocol. First, the peak e-field was estimated in the sample of interest using the transient solution of the field equation. Since we assume capacitive coupling, the peak e-field magnitude depends on the geometry of the problem and the frequency of stimulation. Once a threshold field magnitude is determined depending on the stimulation protocol [230], the required number of stimulation periods can be found.
Thermal Analysis

One of the prominent features of classical electroporation, and the reason for its growing popularity in life science research and medicine, is the relative insignificance of thermal effects. In a typical setting, the DC pulses normally used have to be short enough and far apart from each other to avoid causing a significant change in the temperature of the electroporated medium. Joule-heating induced thermal effects in capacitively coupled electroporation were estimated by solving the heat equation in the entire domain. To reduce computational cost, the overall current was estimated without solving the heat equation and then it was prescribed as a transient boundary condition to a simplified problem, which did not include the insulating layer. The mesh without the insulating layer was significantly simpler (1650 elements compared to 219508 elements with a 250nm thick insulating layer), yet physically equivalent in terms of the heat generated in the electrolyte. Depending on the thickness of the insulator and the stimulation frequency, the impedance of the capacitative interface changes relative to the resistive electrolyte. This affects the relative voltage drop inside the bulk of the electrolyte solution, affecting the field and therefore, the Joule heating of the solution. A conservative estimate (assuming all the voltage dropping inside the bulk solution) is presented in Figure 39. Figure 39 was obtained from the solution of equations (55)-(57) with the nominal parameter values from Table 6, which simulate the Joule heating of the solution. Under this configuration, a rate of temperature raise is approximately $0.15^\circ C/\text{Cycle}$. This estimate of the thermal effect of electroporation can be incorporated into protocol design.

![Figure 38: Supra-Threshold E-Field Regions.](image-url)
Field Decay Profile

Field penetration depth is an important design metric for practical applications of electroporation to bulk biological solutions. Simulation results indicate that the field strength decays exponentially with the distance from surface of the dielectric coating. The main parameter affecting the decay rate is the solution conductivity which determines the current density and therefore the field in the solution. Figure 40 was obtained from the solution of equations (55)-(57) with parameter values taken from Table 6. Figure 40 shows characteristic decay profiles for several representative conductivities: $0.01\frac{S}{m}$ and $0.001\frac{S}{m}$. The field strength and the distance from the reference point were normalized for easier visualization. As expected, the more conductive is the solution, the faster is the decay of the e-field.

![Figure 39: Maximum Temperature.](image)
Sensitivity Analysis

When studying electroporation, the electric field magnitude in the sample is one of the key parameters that characterize the nature of the effect. To test the effect of different electric and geometric parameters on the field strength, we have conducted several parametric studies. Parts (a)-(e) of Figure 41 present the variation in peak magnitude of electric field as a function of different system parameters swept over a typical experimental range. The nominal parameter values used for these simulations are summarized in Table 6. The equations (55)-(57) were solved while having all the parameters fixed at the values given by Table 6, besides the parameter for which the sensitivity is being analyzed.

Part (a) of Figure 41 presents the sensitivity of the peak electric field magnitude to the thickness of the insulating layer. The green horizontal line indicates the breakdown threshold field for the insulator, taken from [227]. All the simulation parameters were taken from Table 6 besides the stimulation voltage. For this simulation, the applied voltage was chosen $V_{stim} = 500V$ (Amplitude) in order to demonstrate the effect of the breakdown voltage threshold. The insulator thickness varied between 250 [nm] and 50 [um]. The red straight line in a log-log plot indicates exponential dependence of the peak electric-field magnitude on the thickness of the insulating layer. The overlaying red lines with different markers indicate small sensitivity of the peak insulator e-field on the stimulation frequency. This can be explained by noting the phase shift between the e-field inside the solution and the insulator. When the field inside the insulator is at its peak, the voltage drop inside the solution is almost zero due to the lagging, therefore, the stimulation frequency has almost no effect. Part (a) of Figure 41 shows that a
prescribed stimulation voltage, determines a minimum thickness of the insulator. For the simulation parameters used, the minimal insulator thickness was found to be 500 [nm].

The blue lines in part (a) of Figure 41 show the sensitivity of the peak e-field inside the solution to variation in frequency and insulator thickness. It can be observed that higher frequency leads to a higher peak field in the solution as predicted from the simple lumped-element model. The peak field values were measured at a distance of $\Delta h$ above the insulator: $\Delta h$ was chosen as 10% of the insulator thickness $h_i$. Simulation data presented in part (a) of Figure 41 indicates that increasing the frequency of the stimulation would increase the peak e-field strength in the solution. While our simulation accounts for the large scale physical phenomena occurring in the electroporation chamber, biological aspects related to the electrical properties of the cell’s membrane are not accounted for.

Part (b) of Figure 41 shows the dependence of peak e-field in the electroporated solution on the stimulation frequency. To generate this figure, equations (55)-(57) were solved using parameter values listed in Table 6, while varying the frequency in the 10[Hz]-10[MHz] range. A straight line in log-log plot indicates an exponential dependence which is consistent with the lumped circuit model, i.e. equation 60.

While the absolute thickness of the insulator determines the electric field magnitude, the lateral dimensions, such as the electrode width and the inter-electrode gap, vary only relatively to the thickness of the insulator. The sensitivity of the peak e-field inside the electroporation medium to the ratio of inter-electrode gap and insulator thickness is presented in part (c) of Figure 41. To generate this figure, equations (55)-(57) were solved using parameter values listed in Table 6, while varying the ratio of inter-electrode gap to insulator thickness in the 1-10 range. The simulation data suggests that minimizing the gap between the electrodes would lead to increased e-field in the solution. This could be used as a design guideline, i.e. having multiple thin planar electrodes separated by a thin gap.

Part (d) of Figure 41 shows the dependence of peak e-field in the electroporation medium on the dielectric constant of the insulating material. To generate this figure, equations (55)-(57) were solved using parameter values listed in Table 6, while varying the dielectric constant of the insulator in the range 2-6. The simulation data indicates that an insulator with a higher dielectric constant would lead to a higher effective e-field. This finding is consistent with the lumped-element model prediction, since the dielectric constant of the insulator increases the capacitance, which in turn leads to a high voltage drop on the resistive solution. It also provides a guideline in the choice of insulating material: while we typically used $SiO_2$ as our nominal insulator with a dielectric constant of 3.9, other insulating materials have dielectric constants 1-2 orders of magnitude higher, e.g. $TiO_2$ with a constant in the range of 86-173 [231].
Part (e) of Figure 41 shows the dependence of peak e-field in both the electroporation medium and the insulating layer on the conductivity of the electroporation medium. To generate this figure, equations (55)-(57) were solved using parameter values listed in Table 6, while varying the solution conductivity in the range of $[0.001 \, \text{S/m}, 1 \, \text{S/m}]$. The red line, corresponding to the peak e-field magnitude inside the insulator is almost flat for the entire range of conductivities tested. The small decline in the peak e-field inside the insulator corresponds to a more “even” distribution of the voltage drop between the chamber components, when the electroporated medium becomes less conductive, i.e. more polarizable. The straight blue line corresponding to the peak e-field inside the solution corresponds to an exponential dependence. This indicates that generating a high electric field inside a highly conductive medium is a challenge. This behavior is familiar to researchers experimenting with electroporation of strong ionic solutions, and is sometimes addressed by removing ionic contents from the electroporation medium for better results.

![Figure 41: Sensitivity analysis of peak e-field in solution (blue) and insulating layer (red) as a function of varying parameters (a) Insulator Thickness (b) AC Frequency (c) Inter-electrode gap/Insulator Thickness ratio (d) Dielectric Constant (e) Solution Conductivity](image-url)
In this section we illustrate a typical design process for an AC coupled micro-electroporation system. Assuming that our target parameters are a threshold e-field of $300 \ [V/cm]$, commonly reported for reversible electroporation, a solution with conductivity of $0.1 \ [S/m]$, and a typical protocol of 8 pulses, each pulse lasting 100 microseconds. Given these input parameters, and assuming our fabrication process uses SiO$_2$ for coating, we can employ the model in order to determine the required insulator thickness, the stimulation frequency and amplitude, and the number of AC cycles. Using a frequency of $100 kHz$, we fall within the classical sub-MHz region and also reduce the need in a specialized high frequency power supply. Our simulation results, using equations (55)-(57) using the design parameters in Table 6, show that using a 100V (peak) stimulation on an electrode separated from the solution by a 500 [nm] layer of SiO$_2$, would result in a peak e-field magnitude of $320 \ [V/cm]$ penetrating several micrometers deep into the solution before dropping to sub-threshold level. Using 0.75 as the $E_{effective}/E_{peak}$ ratio, the number of cycles yielding a comparable AC stimulation protocol is 22, which leads to peak increase in temperature of at most $3.3^\circ C$ per single stimulation AC burst. While these parameters will require experimental validation and adjustment for unaccounted factors, such as material non-idealities, convective cooling, fabrication non-idealities, we see our contribution in a theoretically-based analytic tool that can help focus and troubleshoot future experimental efforts.

We see the main limitation of micro/nano-electroporation in general, and our work in particular, in the relatively low ratio of active/total volume which we define as the fraction of the volume where super-threshold magnitude of electric field is achieved. The precise numeric value of this ratio depends on the threshold, the field and the overall size of the device, however it is by all means much lower than traditional electroporation devices. We humbly acknowledge that this work is a first of its kind and it was developed as a first step towards exploring electroporation through AC fields. We set as a goal to test the basic feasibility using numerical tools before additional efforts are allocated to experimental efforts. We envision ameliorating the active volume limitation by addressing the problem in two directions: first, the geometry of the device itself – by stacking a series of thin devices close to each other we could further increase the active volume to non-active volume ratio in the aggregated device. An alternative geometry might include a flow-through architecture where the liquid to be sterilized is passed through a single focus point where electroporation occurs. While this might not increase the active volume, this approach would lead to a more reliable sterilization process – by definition, all liquid will have to pass through the “active region”. The second direction to work around the limitations would involve mixing: we envision a mechanical mixing system, which circulates the liquid in the sterilization chamber which increases the probability that any micro-organism present in the solution would be sooner or later exposed to the super-threshold electric field. The focus of this work was on the feasibility of generating electroporation level fields without galvanic coupling, and no
performance optimization attempts were made. We intend to address design optimization in future work.

Interim Conclusion

The goal of our study was to lay ground for experimental exploration of capacitively coupled AC electroporation. We accomplished our goal by developing a set of analytic and numerical tools, which provide physical insight about the importance of different parameters, and equip us with practical design guidelines. Due to the large parametric space available for exploration, we find simulation tools especially useful for focusing experimental efforts.

Based on the simulation results, we have found that due to the dielectric nature of the insulating layer, the displacement current dominates, hence, it is 90 degrees out-of-phase with the stimulation voltage. In the electrolyte solution, however, the conduction current dominates over the displacement current. Since it is the conduction current that induces the potential over the electrolyte, it should be expected that the voltage in the electrolyte will be out-of-phase with the applied stimulation voltage.

Sensitivity analysis reveals that the key parameters affecting the peak magnitude of the e-field include the thickness of the insulating dielectric coating the electrodes, and the frequency of the administered AC stimulation. This result confirms the intuition provided by the crude, lumped-circuit model, where a thinner dielectric corresponds to a larger capacitance. Larger capacitance, coupled with higher stimulation frequency, leads to increased voltage drop on the electrolyte solution, yielding higher e-field. Similar argument can explain the increase in peak electric field as the dielectric constant of the insulator increases. While increasing the stimulation frequency increases the e-field in the electroporation sample, when going beyond the 1MHz range, one might need to consider cell membrane effects. For a typical cell, the specific membrane capacitance is such that starting at 1MHz, the membrane increasingly behaves as a short-circuiting element. This affects the way that the voltage is distributed on the different components of a single cell. This combination of constraints guides us to focus the experimental range of frequencies for exploration in the hundreds of kHz range.

In addition to revealing key parameters, the results of our numerical experiments indicate that the peak values of the electric field occur next to the edges of the electrodes. This suggests that having multiple, laterally narrow electrodes might be preferable in terms of optimizing the supra-threshold region.

A thermal analysis taking into account the Joule effects produced by the electric field indicates that the temperature changes in the case of capacitive coupling are relatively low for a typical configuration. For other geometries and/or electric parameters, the thermal analysis provides a modeling tool, which can be used to design and optimize electroporation protocols.

In summary, our in silico simulations suggest that capacitively coupled electrodes might be a feasible approach for in vitro electroporation of biological solutions. AC insulated electroporation might eliminate the effects of electrolysis, which reduce experimental efficiency and impede technological advances. In our future work, we intend to expand the experimental effort and perform electroporation experiments using AC coupled electrodes.
### Study Appendix

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<td>100[V]</td>
</tr>
<tr>
<td>$h_s$</td>
<td>Substrate Thickness</td>
<td>200[um]</td>
</tr>
<tr>
<td>$h_{sol}$</td>
<td>Solution Height</td>
<td>2000[um]</td>
</tr>
</tbody>
</table>

**Table 5:** Model electric parameters used for experimental validation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$h_e$</td>
<td>Electrode Thickness</td>
<td>50[um]</td>
</tr>
<tr>
<td>$w_e$</td>
<td>Electrode Width</td>
<td>500[um]</td>
</tr>
<tr>
<td>$h_i$</td>
<td>Insulator Coating Thickness</td>
<td>250[nm]</td>
</tr>
<tr>
<td>$d_e$</td>
<td>Inter-electrode Gap Distance</td>
<td>500[um]</td>
</tr>
<tr>
<td>$\varepsilon_i$</td>
<td>Insulator Dielectric Constant</td>
<td>3.9, $SiO_2$ from [234]</td>
</tr>
<tr>
<td>$\varepsilon_s$</td>
<td>Solution Dielectric Constant</td>
<td>79.5 from [233]</td>
</tr>
<tr>
<td>$\sigma_i$</td>
<td>Insulator Conductivity</td>
<td>1e-14 from [234]</td>
</tr>
<tr>
<td>$\sigma_s$</td>
<td>Solution Conductivity</td>
<td>0.1[S/m]</td>
</tr>
<tr>
<td>$V_{stim}$</td>
<td>Stimulation Voltage</td>
<td>100[V]</td>
</tr>
<tr>
<td>$f_{stim}$</td>
<td>Stimulation Frequency</td>
<td>$10^6$ [Hz]</td>
</tr>
<tr>
<td>$C_p$</td>
<td>Specific Heat Capacity</td>
<td>4.186 [J/g °C] from [235]</td>
</tr>
<tr>
<td></td>
<td>Thermal Conductivity</td>
<td>0.58 [W m$^{-1}$ K$^{-1}$] from [236]</td>
</tr>
<tr>
<td>$V_{bd}$</td>
<td>Breakdown Voltage</td>
<td>5[MV/cm] from [227]</td>
</tr>
<tr>
<td>$h_s$</td>
<td>Substrate Thickness</td>
<td>500[um]</td>
</tr>
<tr>
<td>$h_{sol}$</td>
<td>Solution Height</td>
<td>1000[um]</td>
</tr>
</tbody>
</table>

**Table 6:** Model electric and thermal parameters used for numerical experiments.
A possible model for estimating the time that the sample is exposed to the supra-threshold e-field magnitude is presented below. If we define $t_{\text{exposure}}$ as the time that the sample is exposed to supra-threshold e-field, $f$ represents the AC stimulation frequency, $E_{\text{peak}}$ is the peak magnitude of the electric field and $E_{\text{threshold}}$ is the ratio of the peak e-field magnitude that leads to electroporation. To clarify, if $E_{\text{peak}}$ is 300 V/cm and we assume that electroporation occurs at 240 V/cm, then $E_{\text{threshold}}$ would be chosen as 240/300 = 0.8. $N_{\text{cycles}}$ stands for the number of stimulation cycles.

Focusing on a single quarter of a cycle of the AC sinusoid, $\arcsin\left(\frac{E_{\text{threshold}}}{E_{\text{peak}}}\right)$ gives us the angle in radians which corresponds to a sub-threshold field. By subtracting this angle from $\frac{\pi}{2}$, we get the complimentary angle, corresponding to supra-threshold field. Dividing by the length of the cycle in radians and multiplying by 4 (to compensate for initially taking only a quarter), we get the relative proportion of the cycle in which the field is above the specified threshold. By factoring in the period given by $T_{\text{period}} = \frac{1}{f}$ and scaling by the number of cycles, we get the total time that the sample has been exposed to a supra-threshold electric field. To summarize:

$$t_{\text{exposure}}(f, E_{\text{peak}}, E_{\text{threshold}}) = 4 \cdot \frac{1}{f} \cdot N_{\text{cycles}} \cdot \frac{\frac{\pi}{2} - \arcsin\left(\frac{E_{\text{threshold}}}{E_{\text{peak}}}\right)}{2\pi}.$$
Chapter 7: Final Thoughts
Summary of Conducted Work

This dissertation presents a systematic attempt to gain insight into the fundamental elements of tissue electrolysis processes. Although electrolysis is used as a tissue ablation mechanism in a clinical setting, the available tools to monitor and study this process are limited, and this hinders the progress of the research in the field. This work is a step taken into a new direction, which involves developing new tools for electrolysis-focused research.

Chapter 3 establishes an experimental model, which mathematically describes the underlying electrochemistry through a set of differential equations. The mathematical model is validated using pH sensitive dyes on an agar gel and is used as an experimental platform in later studies.

Chapter 4 focuses on using EIT to monitor the ongoing process of electrolysis in a tissue. We use the gel model established earlier as a proxy for tissue. This part of the dissertation shows that it is feasible, under some conditions, to use EIT as a tool for imaging electrolysis. This is a contribution to the arsenal available to the clinical physician/researcher interested in using electrolysis as an ablation modality.

Chapter 5 focuses on MRI as an imaging modality for the process of electrolysis. Using the earlier established experimental model, MRI imaging results are compared to pH sensitive dyes. This study demonstrates the potential value of MRI in the arsenal of tools available to electrolysis practitioner.

Chapter 6 focuses on applications related to electrolysis: the first application combines two modes of electro-stimulation, electrolysis with electroporation, in order to deliver more damage than classic electroporation using much less charge. The second application demonstrates a potential design for electroporation devices which avoid electrolysis by insulating the electrodes from the sample and using an AC electroporation stimulation.
Limitations and future work directions

Tissue electrolysis is largely an unchartered research territory and a great deal of work remains. While this dissertation has taken several tiny steps of exploratory nature into this direction, the following immediate questions should be answered in order to get a better sense of the scope in which electrolysis can be useful:

1. The connection between conductivity and pH, characterization of the range where EIT can be useful for electrolysis monitoring: to further characterize the utility of EIT in the context of monitoring local pH changes, additional work needs to be done to quantify the relationship between conductivity and pH for a set of tissues.

2. Numerical characterization of MRI sensitivity to pH changes: for MRI, as well as for EIT, the utility of the tool would be characterized by the useful range of pH changes that can be monitored, and this range depends on the tissue type itself due to the minimal observable contrast reliably picked-up by MRI. Additional work needs to be performed to characterize the parametric space.

3. Animal models: *in vitro* and *in vivo* tissue models: the limitation of the currently employed tissue model is its simplicity – further validation work needs to be done to confirm the feasibility of the methods in a series of real biological tissues e.g. liver, muscle, cardiac tissue.

4. Hybrid protocol design and optimization: for a combined protocol including both electrolysis and electroporation, a set of new models and tools needs to be designed to allow for an effective protocol design and treatment planning.

Some potentially interesting, more theoretical study could expand the limit of available knowledge by exploring the question around the asymmetric nature of electrolysis (cathode vs. anode): due to the differential nature of the electrochemical reactions occurring at the anodic and cathodic sites, the effect of electrolysis varies at these two locations. A better fundamental understanding of the key differences would help utilize these differences for novel and potentially more efficient applications.
Appendix

Original Study: EIT Imaging of Single Cell Electroporation

Study Context

Electroporation (EP), a method for permeabilization of the cell membrane using high strength electric field pulses, is an important tool in today’s biotechnology. It is thought that as a result of the applied electric field, nano-scale defects (pores) form in the membrane of the cell [78]. When the pores reseal and the cell recovers, the phenomenon is known as reversible electroporation (RE). RE is typically used for introducing molecules through the cellular membrane, gene electro-transfer and electro-fusion [75]. When the cell does not recover the permeabilization, it dies [77], and the process is referred to as irreversible electroporation (IRE). IRE is used in tissue ablation, as well as in water and food sterilization [74]. While numerous research and clinical applications of electroporation are commonly used [75], and there is a qualitative understanding of electroporation [75, 78, 237], fundamental understanding of the biophysical mechanisms of electroporation is relatively incomplete. Theoretical models developed over the years, e.g. [104, 106, 238-240], attempt to describe the formation of defects across the cell membrane, its permeabilization and the processes involved in electroporation. However, the short time scale and small length scale of the process make experimental studies challenging. Single cell experimental studies of the electroporation process typically use chemical markers [241] and optical [81, 108] tools to monitor the changes occurring in the cell. Perhaps the most commonly used experimental technique involves optical detection of a fluorescent dye that enters the cell through the permeabilized cell membrane, e.g. [237]. However, the mass transport process across the membrane depends on the dye size in relation to the pore size. It has been recently shown that smaller dye molecules could detect cell membrane permeabilization that is not observed with larger dyes [109]. This issue has come to the forefront with advances in nanosecond pulse cell permeabilization technology [110].

Ions, naturally present in all biological media, are much smaller than any of the dyes currently used. The transport of ions across the cell membrane is a good indicator of the membrane’s permeabilization. The relative small size of ions compared to the dyes has given rise to an approach in studying electroporation based on detecting ionic currents through electroporated biological media. At the macroscopic scale, the observation that electrical properties of cells change with electroporation, have led us to propose first theoretically [242] and then prove experimentally [121], that electroporation in living tissue can be monitored in real time with Electrical Impedance Tomography (EIT). Electrical impedance tomography is used in a variety of scientific fields, from geology to semiconductor characterization [243], to medical imaging. EIT produces an image of the electrical properties of the examined media. In a typical EIT application, electrodes are placed around the volume of interest and small, sinusoidal currents are injected into the tissue, while voltages are measured on its boundary. Using the finite element method, the complex impedance of the analyzed domain is modeled, and a
solution for the most likely impedance configuration that fits the measurements is obtained [135]. Our group has developed a variety of EIT techniques to monitor minimally invasive surgery procedures such as electroporation and cryosurgery. In a recent work from our group [121], multi-frequency EIT has been applied for monitoring irreversible electroporation in tissue.

At the microscopic scale, the observation that the cell’s electrical properties change with electroporation, has been observed and utilized by us for the single cell micro-electroporation technology [114]. However, while this tool allowed the observation of the dynamics in the cell’s electrical properties during electroporation, it provided only bulk information without any details on the geometrical configuration of the electroporated surface. Moreover, the micro-electroporation technology used a geometry that is different from the one used in conventional biotechnology applications. It has now occurred to us, that the EIT technology, which has been successfully applied to imaging electroporation in tissue, could be potentially applied to a single cell model system, to yield a new research tool for studying electroporation with high spatial and temporal resolution. A major advantage over our previous micro-electroporation technology [114] is that EIT is inherently producing a 3-D image and therefore, we should be able to obtain geometrical details on the process of electroporation at the single cell level. Furthermore, EIT data acquisition is very fast compared to other imaging modalities and depending on the stimulation frequency, and the number of electrodes, an image dataset acquisition time can be reduced to microseconds. While the temporal resolution of the envisioned technology is too low to detect the evolution of the pores in the cell membrane during nanosecond or single microsecond type pulses, we anticipate being able to monitor the dynamics of electroporation during longer than microsecond pulses, and between the pulses. In addition, EIT should allow visualizing and monitoring of the pore resealing process, since this process is at a time scale much larger than the time resolution of EIT. In the past, EIT has been used to monitor the growth of a large mold cell [244]. Electrical Impedance Topography (rather than Tomography) was developed to characterize the local capacitance of the cell membrane of a single xenopus oocyte by Dharia, et al. [245]. Microelectrode arrays are commonly used for research on single neurons e.g. by Spira and Hai [246]. However, to the best of our knowledge, there is no previous study on using EIT to study electroporation at the single cell level. Therefore, the goal of this study is to use mathematical modeling to explore the feasibility of the use of EIT to monitor electroporation at the single cell level.
Methods and Materials

In Silico Model

The Electroporation/EIT scheme was tested *in silico*, on an idealized model of a round homogeneous cell of radius $R_{\text{cell}}$, positioned concentric in a saline (conductivity $\sigma_{\text{saline}}$) filled round measurement chamber of radius $R_{\text{chamber}}$ shown in Figure 42. The cell’s cytosol is modeled to have a uniform internal conductivity $\sigma_{\text{cytosol}}$. To add some complexity, a small round (radius $R_{\text{organelle}}$) off-center inhomogeneity with a conductivity $\sigma_{\text{organelle}}$, acting as a very simplistic model for a nucleus or an organelle inside a cell (the addition of the inhomogeneity has no physical significance). A number, $N_e$, of round point electrodes of radius $r_e$, surrounded the measurement chamber. An opposite pair of electrodes was used as the electroporation electrodes (marked as $V+$ and $V-$), to initiate the electroporation process. We used this configuration for testing the concept, since it represents ideal conditions for imaging electroporation with EIT and therefore, it can serve as an upper limit to study the resolution of EIT imaging of electroporation. The parameters examined in this analysis were the ratio $R_{\text{cell}}/R_{\text{chamber}}$ and the number of electrodes $N_e$. The material properties were not varied in this study.
**Analysis**

The mathematical analysis consists of three simulations: A) simulation of the process of electroporation in a cell using the model described above, B) simulation of the EIT data acquisition during single cell electroporation, and C) EIT image reconstruction from the data acquired in previous step. Following are the mathematical formulations used in the analysis.

**A) Simulation of the Process of Electroporation in a Cell**

A simulation of the process of electroporation in a single cell requires the simultaneous solution of two equations. One is the field equation. Since our model contains both conductive (cytosol, extracellular fluid) and dielectric materials (cell membrane), both displacement and conduction current exist. The main equation solved is current conservation:

\[
\nabla \cdot \vec{J} = 0,
\]

Where \(\nabla \cdot ()\) is the divergence operator, and \(\vec{J}\) stands for the local current density vector. The current density has a conductive and a displacement components and is given by:

\[
\vec{J} = (\sigma + \varepsilon_0 \varepsilon_r \frac{\partial}{\partial t} \vec{E}),
\]

where \(\vec{E}\) represents the local electric field, \(\sigma\) is the conductivity, \(\varepsilon_0\) is the vacuum permittivity and \(\varepsilon_r\) is the relative permittivity of the material. The electric field is linked to the potential field by the relationship:

\[
\vec{E} = -\nabla U.
\]

The field equation was solved for the geometrical configuration of Figure 42, subject to Dirichlet boundary condition (voltage), imposed at the electroporation electrodes. The remainder outer surface of the domain was insulated.

For the electroporation model, we employ the single-round-cell macro-model proposed by Krassowska and Filev [105]. Their model uses a set of ordinary differential equations to model the membrane local pore density evolution, from information on the electric fields at the cell membrane (see additional details in supplementary material). The equation used by this model is:

\[
\frac{dN(\theta, t)}{dt} = \alpha e^{\left(\frac{v_m}{V_{ep}}\right)^2} \left(1 - \frac{N}{N_{eq}(V_m)}\right)
\]

where \(\alpha\) is the pore creation rate coefficient \((10^9 m^{-2}s)\), \(V_{ep}\) is the characteristic electroporation threshold voltage (taken as 0.258V). \(N_0\) is the equilibrium pore density for the membrane at \(V_m = 0\) \((1.5 \times 10^9 m^{-2})\) and \(N_{eq}\) is the equilibrium pore density for a given voltage \(V_m\), and is given by \(N_{eq}(V_m) = N_0 e^{q \left(\frac{v_m}{V_{ep}}\right)^2}\). \(q\) is an electroporation constant \((q = 2.46)\). To get the local pore density, equation (4) is solved with the initial condition \(N(\theta, 0) = 0\) (no pores).
The combination of these two equations is non-linear since the electric field affects the formation of pores, which in turn affects the electrical properties of the analyzed domain and thereby the electric field. To solve these two equations, we assumed a linearized Euler forward integration scheme in time. To simulate the electroporation dynamics, we have used the COMSOL MultiPhysics (v4.3) Finite Element software package to solve the field equation in the entire domain, and generate the information on the electric fields at the cell membrane, required for solving the Krassowska and Filev (KF) model pore evolution equation. Cell membrane conductivity was modeled using the pore dynamics evolution obtained from a simplified version of the KF model [105]. Our solution was run on a mesh consisting of 10200 elements. We have reduced the mesh size to verify that no artifacts are introduced into the solution due to sampling issues. The difference between the solutions was within the tolerance specified to the numerical solver (1e-4). The electroporation pulse was introduced as a Dirichlet boundary condition through the electroporation electrodes. The applied voltage was chosen as 1V, consistent with previous experimental studies [113].

**B) Simulation of the EIT Data Acquisition During Single Cell Electroporation**

In an EIT implementation electrodes are placed around the volume of interest and very small, sub-sensory, sinusoidal currents are injected into the tissue, while voltages on the tissue boundary are measured. This information is then used to produce a map of the electrical impedance in the analyzed domain. To simulate the data acquisition process during single cell electroporation with an *in silico* experiment, we have solved the Laplace equation for our geometry (Figure 42) , with boundary conditions determined by a sinusoidal current injection patterns:

\[
\nabla \cdot (\sigma(x,y,z) \nabla U(x,y,z)) = 0,
\]

where \( U(x,y,z) \) is the electric potential sampled at a given point in our domain, and \( \sigma(x,y,z) \) is the conductivity at a given point. The analysis in part A, above, was used to provide information on the conductivity of the analyzed domain, including the cell membrane, at the instant the simulated data for EIT reconstruction was acquired. Equation (5) was solved using a finite elements scheme implemented in COMSOL MultiPhysics (v 4.3). For the EIT imaging data acquisition simulation purposes, we have used an adjacent electrodes current stimulation pattern [139], with amplitude 500\( \mu \)A. In this simulation of data acquisition, data was obtained at several points in time during electroporation: before the onset of the electroporation pulse, during the pulse and after the pulse.

**C) EIT Image Reconstruction From Acquired Data**

An EIT system attempts to reconstruct a conductivity map of a domain of interest from a set of known, injected, current-measured resulting voltages, typically at the
boundary of the geometric domain. In this in silico study, we have replaced measured currents and voltages with calculated currents and voltages from simulation B) above.

In a typical EIT reconstruction algorithm, a map of impedance is guessed and the voltages resulting from injected currents calculated using Equation (5). These voltages are compared to the measured voltage and used as feedback for an iterative scheme to modify the guessed map of impedance implemented, until the calculated and measured voltages converge within a certain tolerance. Here, a modified Newton-Raphson (NR) method was used for reconstructing the image from the input data due to its excellent convergence properties [247]. This method attempts to iteratively minimize a cost function representing the overall voltage measurement discrepancy between the input (measured) voltages and the reconstruction algorithm’s internal model. The Jacobian needed for the NR method was calculated using a sensitivity matrix approach [248]. Total Variation (TV) regularization was used to overcome the ill conditioning of the Jacobian matrix [249]. While a different reconstruction mechanism, such as e.g. front tracking, could potentially yield better reconstruction quality in terms of contrast, our goal in this work was a preliminary qualitative assessment of the capabilities of conventional EIT-based approach to image single cell electroporation.

**Performance Evaluation**

To evaluate the performance of our EIT system, we have used several figures of merit: 1) Blur Radius (BR) index, which is a general measure of resolution [250] and 2) porated sector size (PSS), which is a quantitative index we have developed considering the specifics of our model and application. The porated sector size $\alpha_p(\theta, n)$ takes two parameters: the reference angle $\theta$, and a relative change percentage $n$. For the geometry presented in Figure 42, the cathode corresponds to $\theta = 0^\circ$ and the anode corresponds to $\theta = 180^\circ$. $\alpha_p$ returns the relative arc length in which the conductivity

![Figure 43: Porated sector size (a) ideal input, (b) EIT reconstruction.](image)
changed more than $n$ percent relative to the pre-stimulation condition. For example, in part (a) of Figure 43, assuming the cathode is on the right, we would get: $\alpha_p(0^\circ, 10) = 0.125$, and $\alpha_p(180^\circ, 10) = 0.25$. In a similar fashion, we define a metric $\beta_p(\theta, n)$ which acts on the result of EIT reconstruction (shown in part (b) of Figure 43). To quantify the quality of EIT reconstruction, we introduce a figure of merit $\gamma_p(\theta, n)$ defined as:

$$\gamma_p(\theta, n) = 1 - \left| \frac{\alpha_p(\theta, n) - \beta_p(\theta, n)}{\alpha_p(\theta, n)} \right|.$$

This quantity can be thought of as a measure of angular match, or relative error, between the (ideal) input and the reconstructed result.

**In Vitro Experimental Study**

We anticipate that the feasibility of the proposed EIT imaging approach for single cell electroporation will be tested with oocytes of *Xenopus Laevis*. A typical size for such an oocyte is 1-1.2mm in diameter. As a preliminary step to the experiments using living cells, we have developed an experimental model using glass beads of various sizes. This allowed us to characterize the performance of the system in somewhat idealized conditions, reducing the uncertainties associated with living cells, such as shape and dynamic processes, that might affect the measurements.

Using a standard printed circuit technology (PCB) we have manufactured a circular chamber with 32 electrodes surrounding it, illustrated in Figure 44. The electrodes were placed in the board as vias, which connect the two surfaces of the board, and a center hole was drilled in the center exposing half of each via to the elements. A centrifuge test-tube was cut in half and the capped part was glued to the PCB using hot-glue gun. A glass slide was attached to the bottom part of the PCB using epoxy resin. We have used the protocol reported in [251] to clean the electrodes before measurements could be taken.
Study Results and Discussion

Numerical Experiments

When studying electroporation, transmembrane voltage is one of the key parameters. Using the model described above, we present the transmembrane potential resulting from an external voltage step. We have used the geometry shown in Figure 42, and a voltage stimulus of 1V was applied between the electroporation electrodes. Part (a) of Figure 45 presents the transient response of the cell membrane to the electroporation pulse. In this figure, three regions can be distinguished: 1) Initial membrane charging, 2) The onset of electroporation and 3) Relaxation to resting potential. This graph can also be used to demonstrate the limitation of existing methods of monitoring electroporation. While the onset of electroporation can be inferred from the abrupt changes in the measured electrical signals, these signals carry no spatial information, i.e. the spatial extent of electroporation remains unknown. On the other hand, optical methods which are typically used to estimate the spatial effects of electroporation operate at a much slower time-scale, limited by transmembrane mass transport processes. The high temporal resolution of EIT approach might potentially be utilized to study fast time-scale phenomena.

Part (b) of Figure 45 shows the spatial and temporal evolution of pores on the cellular membrane. The different lines correspond to different time points after the administration of the pulse, and the horizontal axis corresponds to the angular position on the membrane’s surface, indicated by angle $\alpha$ in Figure 42 (only top half-cell is shown). The figure provides additional argument for the importance of spatial information in the context of electroporation: while pore density is high at the “poles”, i.e. regions close to the pulse administering electrodes, it is low at the intermediate regions. The poles correspond to angles 0 and $\pi$ (radians) in Figure 42.

Part (c) of Figure 45 shows the membrane conductivity estimated from the model at a typical point during electroporation, e.g. in the middle of section (2) of part (a) of Figure 45. The “heat-map” visualizes the conductivity of a section close to the anode. Warmer colors correspond to more conductive regions. Consistent with other theoretical models and experimental findings, the model predicts larger conductivity increases closer to the stimulation electrodes, and smaller changes away from them.

To visualize the onset of electroporation in the cell, three EIT acquisitions were performed at different time points of the process. Figure 46 shows the ideal membrane conductivity and the result of EIT reconstruction before, during (at 10% of the maximum conductivity) and immediately after the administration of the electroporation pulse. The white region corresponds to the EIT chamber and the round shape in the middle corresponds to the imaged spherical cell. Changes in the conductivity of the membrane can be observed and correlated with the changes in the ideal conductivity, which was provided as an input to the model.
can be observed and correlated with the changes in the ideal conductivity, which was provided as an input to the model.

Before the administration of EP pulse, the cell membrane is intact and it therefore has very low electric conductivity for low frequency ionic currents injected by EIT electrodes. Consistent with this assumption, the reconstructed simulation image in part (d) of Figure 46 shows only the silhouette of the cell, defined by the membrane. Part (e) of Figure 46 shows an image reconstructed from EIT measurements during the administration of the electroporation pulse. To produce this image, we chose a time...
point that corresponds to 10% of the peak conductivity reached at the membrane. Once the pulse has been applied between the electroporation electrodes, pores start to form on the cell’s membrane. The increased permeability leads to increase in the conductivity of the membrane and some of the ionic current injected by the EIT electrodes can go through the cell’s cytosol inducing changes in the voltages on the boundary electrodes. These changes are captured and reconstructed by the differential EIT algorithm. Since at this point, the conductivity of the membrane is still relatively low, only a small part of the current goes through the cell, and as a result, the image of the cytosol is vague. The image shown in part (f) of Figure 46 shows the result of EIT reconstruction after the onset of electroporation, coinciding with peak conductance of the cell’s membrane. The top and bottom sections of the cell membrane and the simulated organelle can be seen due to their different conductivity from the surrounding extracellular fluid. Since the conductivity of the medium is assumed to remain constant, we see no changes (white color) around the cell. We have estimated the quality of EIT reconstruction using the PSS quality metric introduced earlier. We have obtained \( \gamma_p(0^\circ, 10) = 0.848 \), and, \( \gamma_p(180^\circ, 10) = 0.756 \). Since \( \gamma_p = 1 \) indicates perfect match between the model and the reconstruction result, we see that the EIT reconstruction process introduces some error into the system, which can be expected due to the ill-posed nature of the EIT reconstruction problem. It is interesting to note that the electroporation process is not symmetrical as can be seen from in parts (c) and (f) of Figure 46. Our simulation results (not shown) indicate that this effect is due to the finite non-zero resting transmembrane potential. This result is consistent with reported experimental results [252].

These local changes in conductivity are caused by increased permeability of the cell membrane as the result of the EP pulse. The warmer colors correspond to increased conductivity regions as indicated by the color bar.

**Sensitivity Analysis**

Figure 47 shows the sensitivity of EIT reconstruction for different simulated organelle sizes. The simulations were performed during the EP pulse when the pore density is maximal. The color indicates the change in conductivity relative to homogenous reference image. Warmer colors stand for positive conductivity change and cooler colors for negative conductivity changes. To evaluate the sensitivity of the micro-EIT system, EIT reconstruction for various \( \frac{Cell\ Size}{Organelle\ Size} \) ratios and \( \frac{Chamber\ Size}{Cell\ Size} \) ratios was performed. The simulation results indicate the upper limit for reconstruction ability in ideal, noise-free conditions. As anticipated, the smaller organelles become increasingly harder to resolve. Parts (d) and (e) of Figure 47 show the EIT reconstruction for various \( \frac{Chamber\ Size}{Cell\ Size} \) (cc) ratios. In the described, idealized conditions, an organelle smaller than 40 times the cell, cannot be resolved. Another
design guideline indicated by the simulation is that the measurement chamber should be as close in size to the measured cell to maximize the image quality.

The effect of increasing the number of electrodes is reported in parts (a)-(d) of Figure 48. Varying the number of electrodes shows a behavior consistent with the law of diminishing returns: Parts (a)-(c) of Figure 48 show the result of EIT reconstruction of a simulated round inhomogeneity using a varying number of electrodes. Part (d) of Figure 48 shows that for the tested feature size, the increase from 8 to 16 electrodes significantly improves the quality of the resulting image, however the additional increase to 32 electrodes contributes marginally. Having noise present in the system is anticipated to skew this characteristic quantitatively, but qualitatively the behavior is reported to follow the pattern we have seen in our simulations [251]. This can be used as a guideline for designing an experimental system.

Figure 47: Sensitivity of EIT reconstruction (a-c) Varying cell/organelle (co) ratio (a) co ratio=40, (b) co ratio=20, (c) co ratio=10. (d-e) Varying chamber/cell (cc) ratio. (d) cc ratio=1.6 with organelle visible (outlined) inside the cell, (e) cc ratio=3, organelle cannot be distinguished.

Figure 48: Sensitivity of image quality to number of electrodes. Round phantom reconstructed with (a) 8 electrodes, (b) 16 electrodes, (c) 32 electrodes, (d) Blur Radius (BR) vs. #Electrodes.

Noise Analysis

To investigate the sensitivity of our reconstruction scheme to measurement noise, we have measured the average blur radius (BR) of a circular phantom at different levels of white Gaussian noise added to the measurements. As expected, results shown in Figure 49 indicate that as the level of noise increases, the blur of the system, as well as the additional “spot” artifacts, become more dominant and lead to decreased image quality.
Glass-Bead Model Experiments

To test our data acquisition system at the scale of interest (several mm), we have fabricated a data acquisition chamber using standard printed circuit board technology. We have used an EIT data acquisition system described in [121]. The SNR measured with our system was 42dB. We performed an EIT acquisition of a 2.5mm glass bead embedded in a 1.5 mM NaCl solution. Part (b) of Figure 50 shows the results of simulation based on EIT model based on an input of conductivity map shown in part (a) of Figure 50. Part (c) of Figure 50 shows an optical image captured by a digital camera showing an image of the real chamber with the glass bead surrounded by a dotted red line. Parts (d)-(e) of Figure 50 show the output of the EIT reconstruction based on real voltage measurements performed in our system. We have used current injections of 350uA amplitude at a frequency of 5khz. Each measurement took less than 20ms. Parts (d) and (e) of Figure 50 show reconstruction of different bead sizes.

Our experimental model system employing several glass beads in a saline solution, shows results consistent with numerical experiments, encouraging our further explorations with living single cell organisms of similar scale. It should be noted that while the idealized EIT image (Part (b) of Figure 50) shows increased blurring compared to the corresponding input, the EIT image reconstructed from real voltage measurements shows a smaller radius (2.2mm < 2.5mm) than the corresponding phantom (part (c) of Figure 50 vs. part (d) of Figure 50). We attribute this discrepancy to
the inherent 3D nature of the model: since our electrodes have finite thickness (~0.8mm), some of the stimulation current can flow above and below the imaged glass bead phantom, which makes it appear smaller in the EIT image than it is in reality. We intend to explore a 3D EIT reconstruction in our future work.

Since the spatial resolution of EIT is low compared to traditional medical imaging modalities [251], expecting this approach to enable resolving fine details, such as individual pores, would be too optimistic. However, when EIT is used as an electroporation visualization tool, its advantage is temporal resolution. Compared to alternative, optical methods, which are limited by dye size and the rate of passive diffusion, EIT temporal response is much faster, limited by the stimulation frequency and the number of electrodes. In a practical setting this implies temporal resolution on the order of milliseconds. On the other hand, compared to electrophysiological methods, such as patch-clamp based techniques, or micro-electroporation chambers, EIT based approach has the advantage of rich spatial information. For comparison, patch-clamp focuses on a specific region of the membrane, and micro-electroporation technique typically measures only bulk voltage-current dependence. We believe that by using an EIT based visualization, the benefits of both approaches could be enjoyed.

The presented preliminary mathematical model and simulation results of a single cell undergoing electroporation, coupled with preliminary experimental results, suggests that EIT might be a viable method to monitor the spatial and temporal dynamics of the process. This study was a preliminary inquiry into the conceptual feasibility of imaging the electroporation process using EIT. Based on the promising results of in silico simulations, confirmed by a glass bead model experimental data, we intend to test our system on living single cell organisms.
References


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