

Micro-devices for Investigating Pulsed Electric Fields-Mediated Therapies at Cellular and Tissue Level

Mohammad Bonakdar

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Mechanical Engineering

Rafael V. Davalos, Chair
Lisett R. Bickford
Yong W. Lee
Mark R. Paul
Scott S. Verbridge

2 May 2016
Blacksburg, VA

Keywords:
Blood-brain barrier
Electroporation
Microfluidics
Drug delivery
Electrical impedance spectroscopy

Copyright © Mohammad Bonakdar

Micro-devices for investigating pulsed electric fields-mediated therapies at cellular and tissue level

Mohammad Bonakdar

ABSTRACT

Recent attempts to investigate living systems from a biophysical point of view has opened new windows for development of new diagnostic methods and therapies. Pulsed electric fields (PEFs) are a new class of therapies that take advantage of biophysical properties and have proven to be effective in drug delivery and treating several disorders including tumors. While animal models are commonly being used for development of new therapies, the high cost and complexity of these models along with the difficulties to control the electric field in the animal tissue are some of the obstacles toward the development of PEFs-based therapies. Microengineered models of organs or Organs-on-Chip have been recently introduced to overcome the hurdles of animal models and provide a flexible and cost-effective platform for early investigation of a variety of new therapies. In this study microfluidic platforms with integrated micro-sensors were designed, fabricated and employed to study the consequences of PEFs at the cellular level. These platforms were specifically used to study the effects of PEFs on the permeabilization of the blood-brain barrier for enhanced drug delivery to the brain. Different techniques such as fluorescent microscopy and electrical impedance spectroscopy were used to monitor the response of the cell monolayers under investigation. Irreversible electroporation is a new focal ablation therapy based on PEFs that has enabled ablation of tumors in a non-thermal, minimally invasive procedure. Despite promising achievements and treatment of more than 5500 human patients by this technique, real-time monitoring of the treatment progress in terms of the size of the ablated region is still needed. To address that necessity we have developed micro-sensor arrays that can be implemented on the

ablation probe and give real-time feedback about the size of the ablated region by measuring the electrical impedance spectrum of the tissue.

Micro-devices for investigating pulsed electric fields-mediated therapies at cellular and tissue level

Mohammad Bonakdar

GENERAL AUDIENCE ABSTRACT

All biological systems ranging from cells to organs have distinctive physical properties that could be leveraged for a better understanding of their functions. New diagnostic and therapeutic techniques have been developed through this point of view. Electric fields have a great potential to manipulate biological systems at a cellular level. This has resulted in several new therapies incorporating pulsed electric fields for treating different diseases such as tumors and brain disorders. Animals are commonly used to try new therapies before they could be applied on human patients. However, the high cost and complexity of these animal trials hinders the treatment development process, which encourages development of substitute platforms that could be used instead of animals. The goal of this study was to develop artificial models of the organs and sensors using engineering techniques to test the effects of electric fields for several therapeutic purposes. Electric fields are able to improve the treatment of brain-related disorders, by enhancing drug delivery to the brain. In this study, by developing artificial models of the brain vasculature we were able to look into this phenomenon and optimize the electric fields for a more efficient therapy

.

Acknowledgments

Great achievements in life are never possible without the support from great people. Getting the PhD is a milestone in my life that was achieved mainly by consistent hard work but also facilitated by scientific and social support from my advisor, collaborators, many good colleagues and friends and financial support from several institutions. As a member of the Bioelectromechanical Systems Laboratory (BEMS) I was privileged to work under the supervision of Dr. Rafael Davalos, who as a great mentor guided me through this scientific journey by providing innovative ideas and solutions to every problem. By his personality, Dr. Davalos taught me how an ideal mentor looks like. Being part of his research group was a gifted opportunity that shaped the direction of my future career. I also want to thank my committee whose comments and feedbacks helped me improve the quality of this research.

At the beginning of my PhD study at Virginia Tech I was working with Dr. Daniel Inman and Dr. Gary Seidel for two years before I decided to change direction and switch to the new field. I highly appreciate their mentorship and support during that period and their help during my transition.

I learned a lot from my past and present lab-mates and took advantage of their help and collaboration during my research and experiments. Coming from a completely different background it was initially challenging to learn the basics and start the work. This was facilitated through the help of my former lab-mates especially Dr. Alireza Salmanzadeh, Dr. Mike Sano and Dr. Paulo Garcia. My special thank also goes to Dr. Jaka Cemazar who was always one of the first people in the lab with whom I discussed my challenges and always did his best to help me. I also want to especially thank Eduardo Latouche, my good friend and colleague, whose collaboration with the testing of the impedance sensor was critical for the development of this device. I

appreciate the help from Elisa Wasson during different stages of my experiments to develop and test the blood-brain barrier (BBB) platforms. I also thank Suyashree Bhonsle for her collaboration with our experiments on the perfused liver model and Dan Sweeney for his help regarding all electronic issues. The great professional and friendly environment that these guys provided in the lab was an important motivation for coming to work every day. The fabrication of all the micro-devices in this work was realized in the cleanroom of the ECE department of Virginia Tech. That could not be possible without the training and great technical support that I continuously received from the cleanroom manager, Donald Leber.

The interdisciplinary research mandated collaborations with a lot of people from inside and outside of my field which significantly enhanced my scientific vision. Among the many collaborators that I enjoyed working with, I want to especially thank Dr. Roop Mahajan, Dr. Scott Verbridge, Jill Ivey, Akanksha Kanitkar, Dr. Steve Poelzing, Sharon George, Dr. Rick Davis, Ami Jo, Dr. Rich Helm and Dr. Lucas Vu.

The financial support for my work was partially provided by the National Science Foundation, the Institute for Critical Technologies and Applied Sciences of Virginia Tech.

Apart from scientific aspects, I enjoyed and benefitted from being a part of the fantastic community of friends in Blacksburg. The great social environment and support that was provided by my good friends was essential for my productivity and survival away from my family for this long period of time. Among these friends I want to especially thank Dr. Masoud Agah, Dr. Leyla Nazhandali, Dr. Javad Torabinejad and Dr. Amin Karami.

None of my achievements would have been possible without all kinds of support that I received during all stages of my life from my parents. They did their best to provide me with the best level of education. Words cannot explain how much I love them and how much I have missed them.

And finally, I want to thank my beloved wife Dr. Reyhaneh Ojani, who stood next to me during the last six years and made my days beautiful and my nights peaceful. She was always my source of energy and confidence during hard times.

Dedication

Dedicated to

36,000 young students who along with other courageous men, women and soldiers dedicated their lives in defending my country during the 8-year invasion of Iran by the Iraqi army in 1980s; The men of dignity who presented me and my generation with peace and security with the price of abandoning their wishes and giving their lives; The ones whose incomplete study allowed to complete mine.

Attribution

Elisa Wasson has contributed in experiments, writing and performing the statistical analysis of experimental data for Chapter 2.

Eduardo Latouche has performed the finite element modeling for the electric field distribution, and performed some of the experiments in Chapter 5 and contributed in writing.

Suyashree Bhonsle and Charles Aardema has contributed in running the perfused organ models in Chapter 5.

Table of Content

Chapter 1: Introduction.....	1
1.1. The role of micro-devices for understanding biological systems	1
1.1. Organs on Chip.....	3
1.2. Blood-brain barrier and drug delivery to the brain	4
1.3. <i>In vitro</i> models of the BBB for transport studies	9
1.4. Electroporation	12
1.5. Electrical impedance spectroscopy for characterization of biological systems	15
Chapter 2: Blood-brain barrier-on-Chip: Electroporation of adhered brain endothelial cells toward controlled permeabilization of the BBB	18
2.1. Abstract	18
2.2. Device design and fabrication	19
2.2.1. Device design.....	19
2.2.2. Device fabrication.....	20
2.3. EF distribution inside the channel using FEA.....	22
2.4. Fluorescence calibration.....	22
2.5. Cell culture and characterization of the cell monolayer inside the chip	23
2.5.1. Cell culture.....	23
2.5.2. Immunofluorescence staining of tight junction proteins	25
2.5.3. Confocal microscopy	26

2.6.	Determination of the reversible and irreversible electroporation thresholds	28
2.7.	Uptake of large impermeable molecules due to PEFs.....	32
2.8.	Conclusions	35
Chapter 3:	Electrical impedance spectroscopy for monitoring the permeabilization of the BBB on Chip	37
3.1.	Abstract	37
3.2.	Device design and fabrication	37
3.2.1.	Micro-channel design.....	37
3.2.2.	Impedance sensor design and fabrication	38
3.3.	Cell culture on chip	42
3.4.	Electrical impedance spectroscopy	43
3.5.	Impedance sensor characterization.....	46
3.5.1.	Electrode material	47
3.5.2.	Effect of electrolyte conductivity.....	49
3.5.3.	Effect of redox probe concentrations	49
3.5.4.	Effect of fluid flow over the electrodes	52
3.6.	Impedance spectroscopy of endothelial cell monolayer on gold electrodes	55
3.6.1.	High density seeding.....	60
3.6.2.	Low density seeding	61
3.1.	Impedance spectroscopy of endothelial cell monolayer on ITO electrodes.....	64

3.2.	Osmotic disruption of the endothelial barrier measured visually and electrically.....	66
3.3.	PEFs-induced disruption of the endothelial barrier measured electrically	70
Chapter 4:	Laminated microfluidic platform for studying transport across the BBB	72
4.1.	Abstract	72
4.2.	Introduction	72
4.3.	Device fabrication	73
4.4.	Cell culture	74
4.5.	70kDa FITC-dextran as a tracer for permeability of BBB.....	76
4.6.	Permeability of the BBB-on-Chip.....	79
4.7.	Summary and Future work.....	81
Chapter 5:	Electrical impedance micro-sensor and its application in ablation monitoring during irreversible electroporation therapy	82
5.1.	Abstract	82
5.2.	Introduction	83
5.3.	Fabrication of the impedance micro-sensor array	87
5.4.	Different configurations of the impedance sensor	92
5.5.	Electrical impedance spectroscopy	95
5.6.	Electroporation setup.....	96
5.7.	Dynamic conductivity function and IRE threshold.....	97
5.8.	Finite element analysis	103

5.9. Lesion monitoring during IRE treatment in potato	106
5.10. Validation of a perfused organ model for verification of irreversible electroporation treatments	110
5.10.1. Organ preparations	110
5.10.2. Comparison of the ablation sizes in the perfused organ and in vivo	111
5.11. Lesion monitoring in liver	112
5.12. Conclusions	113
Chapter 6: Summary and future work	115
References:	118
Appendix A	134
Appendix B	138
Appendix C	145
Appendix D	149

Chapter 1: Introduction

1.1. The role of micro-devices for understanding biological systems

With the recent advancements in engineering knowledge and development of new tools and technologies for fabrication and observation at small scales, many of the technological barriers for manipulation, stimulation and observation of biological systems from organ to cellular level have been removed. The fast growing areas of Lab-on-Chip and Organs-on-Chip are the consequences of these technological achievements and are intended to enhance our understanding of living systems in general and to develop new therapies and diagnostic tests in particular. Taking advantage of the microfluidic platforms, the Organs-on-Chip models are developed to mimic human organs from certain aspects and provide useful information about their response and functionality in response to specific treatments and therapies. These models have been able to resolve several challenges pertaining to the common use of animal models for the treatment development, including ethical issues and their high cost. Animal studies are an inevitable part of the long process for the development of any kind of drug or therapy. No therapy could be even tested on human patients without a record of promising results in preclinical studies on animals. However, in the early stages of treatment development it is worthy to reduce the number of sacrificed animals whenever possible. Reducing the cost of experiments in the early stages also provides more flexibility and allows for more exploratory studies for finding the best treatment. The Organs-on-Chip models also provide more control over the experimental conditions due to their simplicity compared to the complexity of the real organ. By leveraging the capabilities of

microfluidic systems it is possible to create microenvironments with highly controlled chemical, physical and mechanical characteristics as required by the specific experiments.

Looking at the biological systems and their disorders from a physical point of view and considering the biophysical phenomena that takes places during normal and abnormal functioning of these systems, has resulted in development of both new diagnostic tools and therapies for a variety of different diseases. There has been a special interest in pursuing this path for better understanding and devising novel treatments for cancer [1]. Pulsed electric fields (PEFs) have proven to be one of the physical methods with a great therapeutic potential including drug delivery and tumor ablation. This method was found to be useful in enhancement of drug delivery to the brain by temporary disruption of the blood-brain barrier (BBB). However, it still needs to be studied in depth and characterized in order to unveil its complete therapeutic potential. The cellular response to PEFs which constitutes the overall tissue-wide response is still not fully understood. On the other hand, accurate control over the extent of electric field is not possible *in vivo* due to the complex structure and inhomogeneity of organs. Considering all the challenges regarding the use of animal models for this purpose, the Organ-on-Chip models can provide a suitable platform for studying the effects of PEFs-based therapies. The underlying hypothesis for this dissertation is that micro-systems can be used to actively monitor and deepen our understanding of the response of tissues to electric fields. In Chapter 2, we developed a microfluidic platform and used that to investigate permeabilization of adhered brain endothelial cells and gain information about the possibility of transcellular transport across the BBB in response to PEFs. This model is enhanced in Chapter 4 in which the transport across the BBB model could be measured in response to PEFs. The development of miniaturized biosensors has also enabled monitoring a wide range of physical and biological properties of organs at the tissue and cellular level. Biosensors could be embedded

in the Organ-on-Chip platforms for a more accurate and quantitative monitoring at the cellular level. In Chapter 3 we presented microfluidic models of the BBB with embedded impedance sensors and discussed the application of electrical impedance spectroscopy to monitor permeabilization of the BBB in a microfluidic setting. Chapter 4 presents a laminated microfluidic platform for a more explicit measurement of BBB permeabilization. At the tissue level, biosensors could be used in monitoring the health and functionality of the organs especially after transplantation. The biosensors could also be used during different therapies to take advantage of the undergoing biophysical phenomena and monitor the progress of the treatment. This capability is highlighted when dealing with electric field-based therapies in which little to no information is available from the inside of the tissue. In Chapter 5 we have addressed this challenge and discussed the development of an impedance micro-sensor array that could be used for real-time monitoring of ablations by irreversible electroporation, which is a kind of PEFs-mediated therapy. Based on the developed platforms and the obtained results, some possible future works are discussed in Chapter 6.

1.1. Organs on Chip

Microfluidics provide an alternative platform to standard *in vitro* systems for analyzing cells and tissues in a more physiologically relevant environment [2, 3]. Organs-on-chip platforms utilize the advantages of microfluidics in order to recreate *in vivo* conditions of the body in an easily testable *in vitro* setting. With micro-scale flow channels, microfluidic devices are able to resemble *in vivo* conditions for cell and tissue analysis. A general overview of the microfluidics device specifications for different cell cultures is provided in [4]. The main parts of a microfluidic cell culture device could be the cell culture area and the media channels. In some cases membranes [4-6] and electrodes [6, 7] are also incorporated into the device. Two major ways of media circulation

inside the microfluidic device are direct flow and perfusion. In the direct flow mode media flows over the cells, applying shear force to the cells, which may be detrimental for some cell types including hepatocytes [8]. In the perfusion mode media flows in a separate channel and diffuses into the cell culture area through a perfusion barrier or a membrane [5, 9]. A great overview of recent achievements in this field has been provided by several reviews [10, 11]. These efforts have so far yielded the development of microfluidic models of liver [12, 13], lung [14, 15], heart [16], intestine [17], brain [18], kidney [19], blood vessels [20, 21], and tumors [22].

1.2. Blood-brain barrier and drug delivery to the brain

Many potential therapeutics for disorders of the central nervous system (CNS), which are proven to be effective *in vitro*, have limited to no effect *in vivo*. This is due to their large molecular weight, water solubility and charge, which render it difficult for them to successfully cross the blood-brain barrier (BBB). Only small and lipid-soluble molecules are able to passively cross the BBB [23]. However, most drugs used to treat CNS disorders are large water-soluble molecules, therefore making the BBB a significant hindrance in treating patients. In the United States alone, 80 million people are suffering from CNS disorders such as brain cancer, Alzheimer's and Parkinson's disease [23]. Despite the high need for development of effective CNS drugs, only a small portion of newly discovered therapeutics are clinically relevant and have the ability to overcome the BBB. First discovered by Paul Ehrlich in 1885 [24], the BBB is comprised of a layer of highly restrictive cells that line the walls of brain microvessels. Different vascular and parenchymal cell types contribute to the formation of this barrier including endothelial cells, astrocytes [25], pericytes [26], neurons, the extracellular matrix and the basement membrane [27, 28]. An *in vitro* study showed that about 75-80% of the contribution to barrier function is made by the brain capillary endothelial cells lining the luminal surface of the microvessels [29]. These endothelial cells form

intercellular tight junctions (TJs) which limit the transport of hydrophilic molecules larger than 500 Da [30]. Therefore, the biggest obstacle in delivering an effective treatment to brain tissue is penetrating this endothelial cell layer.

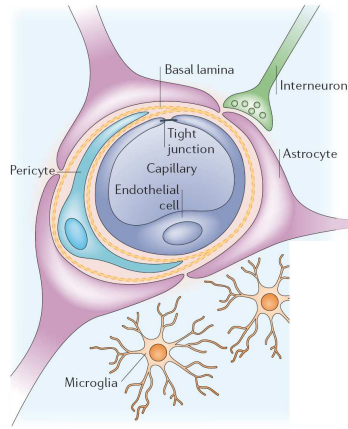


Figure 1-1. Schematic representation of the cross section of brain microvasculature. (Picture taken from [25] with permission)

The structure and molecular basis of the TJs are described in several reviews [31, 32]. TJs exist in all barrier systems which include epithelial and endothelial barriers. However, the cerebral TJs differ from epithelial and peripheral endothelial cells in terms of morphology and constituent proteins, which causes the unique barrier property of the BBB [31]. Several transmembrane and cytoplasmic proteins have been detected in the TJ complex such as Occludin [33], Claudin-1, -2 [34], ZO-1 [35], ZO-2 [36], ZO-3 [37], 7H6 [38], Cingulin [39] as well as junctional adhesion molecules (JAM) [40]. Figure 1-2 shows the molecular structure of the TJs at the BBB.

At the TJs the connection between the cells is maintained by strands of Claudins and Occludin proteins and JAMs. These molecules are anchored from both sides to the actin filaments in the cytoplasm of the adjacent cells through the ZO-1 submembraneous TJ-associated protein [41]. It should be noted that although ZO-1 is an integral part of the TJ structure, it has been observed in

other cells that do not form TJs [42]. The expression level and localization of the ZO-1 protein also does not correlate with the integrity of the TJs [43].

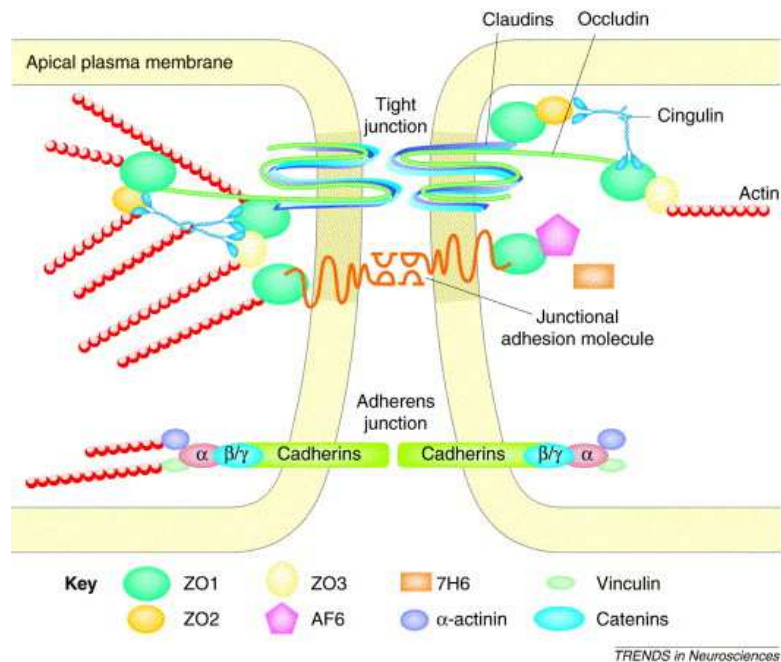


Figure 1-2. Proposed interactions of the major proteins associated with tight junctions (TJs) at the blood–brain barrier (BBB). (Picture taken from [44] with permission)

Normal and disrupted transport across this layer may occur through two pathways. The transcellular pathway, which is through the cell membrane and cytosol, and the paracellular pathway which is located at the cell-cell TJs. The paracellular pathway works on the basis of passive diffusion for hydrophilic molecules. Several different mechanisms contribute to the transport through the transcellular pathway as shown in Figure 1-3.

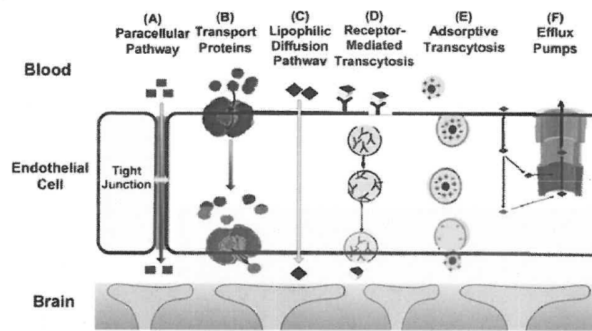


Figure 1-3. Illustration of different pathways across the BBB (picture taken from [45] with permission).

Facilitating drug transport through the BBB has been performed by either chemical modification of the drug for better penetration or temporary disruption of the BBB. The former technique includes rendering the drug molecules lipophilic or amending them with carriers that transport them through the BBB [46-48]. As another alternative to avoid the BBB, the drug is directly injected into the targeted region of the brain [49]. However, that requires opening the skull and is limited by slow diffusion of drug into the brain.

Several methods have been developed to temporarily bypass or permeabilize the BBB through paracellular or transcellular pathways. These methods include focused ultrasound [50-52], osmotic disruption [49, 53], drug delivery vehicles [47, 54] and pulsed electric fields (PEFs) [55, 56] including electroporation [57, 58]. The ultrasound technique works in conjunction with a contrast agent which is injected into the blood stream. The contrast agent contains preformed microbubbles which explode and release high energy in response to ultrasound radiation [59]. Despite showing the capability to non-invasively and reversibly disrupt the BBB, focused ultrasound is limited by the small coverage area the focal beam can target leading to extended treatment times for large tissue volumes [60]. The ultrasound wave is also significantly attenuated by the bone tissue [50]. Osmotic and pharmacological disruption using agents such as mannitol or bradykinin have also

been studied as a way to transiently open the BBB but do so in a systemic manner which may lead to adverse effects such as headache, nausea, and abnormal neuron function [51, 61]. In the osmotic disruption, injection of a hypertonic solution such as Mannitol causes vasodilation and shrinkage of cerebrovascular endothelial cells which results in the widening of the intercellular space and increased permeability across the BBB [62].

In addition, researchers have shown that immunoliposomes, endogenous peptides or other vehicles successfully carry therapeutic drugs across the BBB [63, 64]. Once there, many are unable to reach the concentration necessary for effective treatment. Despite all the achievements and capabilities of the aforementioned methods for disruption of the BBB, they still have some drawbacks and limitations that encourage the development of other alternative techniques.

PEFs have shown promise in treating neurological and psychiatric disorders. PEFs are short, intense electrical pulses which have been used in deep brain stimulation (DBS) [65, 66], electrochemotherapy (ECT) [67, 68] and irreversible electroporation (IRE) [69-71] for tumor ablation. PEFs have the potential to enhance the permeability of either of the transcellular or paracellular pathways of the BBB. In the paracellular pathway, TJs significantly restrict transport in between cells due to their secure connection to the cytoskeleton. Due to the fact that PEFs disrupt the cell membrane, and therefore the cytoskeleton, they have the potential to disrupt the TJs as well [55]. Since electroporation causes transient pores to form in the membrane, it may be possible to enhance transport through the transcellular pathway, which has rarely been studied in the literature.

It has been shown that PEFs disrupt the cytoskeletal organization and result in delocalization of junction-specific proteins such as VE-cadherin, which weakens the cell-cell integrity [72] and enhances the paracellular permeability. In a recent study Lopez-Quintero *et al.* showed that PEFs

of low amplitude (2.5V/cm) and high frequency (200Hz) like the ones used in deep brain stimulation (DBS), can cause the increased permeability of the BBB [73]. Since due to the low amplitude these pulses may not induce electroporation, it is postulated that the permeabilization happens only through the paracellular pathway. The disruption of the TJs and increased permeability of the BBB has also been reported after exposure to different types of electromagnetic fields [74] including continuous wave electromagnetic fields [75], intense electromagnetic pulses [76, 77] and microwave radiation [78] .

Garcia *et al.* investigated the effects of low intensity EF on BBB transport in an *in vivo* study. They found that the threshold for BBB disruption is between 400 V/cm and 600 V/cm for ninety 50 μ s pulses delivered at 1 Hz [57]. In another *in vivo* study Arena *et al.* studied the effect of bipolar sub-microsecond pulses on BBB permeability and found that BBB disruption occurs at much lower thresholds of 250 V/cm without any muscle contraction [79]. Hjouj *et al.* studied reversible and irreversible electroporation thresholds of the BBB in rats and monitored the disruption by MRI [58]. They found that reversible disruption occurred between EF intensities of 330 V/cm and 500 V/cm using a total number of fifty to ninety, 50-70 μ s pulses at a frequency of 4 Hz, resulting in BBB disruption volumes larger than tissue damage volumes.

1.3. *In vitro* models of the BBB for transport studies

Animal studies have an essential role in the process for development of new therapies. However, their high cost and complication, hinders the process of treatment characterization. It has also been shown that about 50% of results obtained from animal models cannot be translated into correct human responses [80]. So far the majority of the studies on the BBB permeability has been conducted on animals. *In vivo* experiments on BBB permeabilization, such as the ones mentioned above, provide mostly qualitative results with limited flexibility over controlling the experimental

conditions and monitoring the outcomes. Therefore *in vitro* models are necessary for obtaining more quantitative information, although their physiological relevance is always a matter of debate. *In vitro* models can provide more freedom in exploring different parameters and facilitate monitoring the response at the cellular level without the complications of animal studies [81].

Taking advantage of different cell culture techniques, *in vitro* models have been previously developed to simulate the permeability of the BBB to different substances [82, 83] as well as investigate the effect of different stimuli such as chemicals, [84] pressure shock [85], radiation [86] and electromagnetic fields [77, 87, 88]. These models exploit monolayer cultures of cerebral endothelial cells as the main constituent of the BBB in different platforms such as transwells [89-91], microfabricated membranes [92], tube [93], collagen matrix [94] and microfluidic channels [6, 95-97] and monitor their integrity and permeability using tracers [98], transendothelial electrical resistance (TEER) [6, 89] or electrical impedance spectroscopy (EIS) [96, 99].

Early *in vitro* models of the BBB have used transwell inserts for culturing the endothelial cells and studying the transport across them. Using the transwell platform some researchers have also made artificial models of BBB with no cells by incorporating a lipid bilayer as the cell representative [100]. The static models of the BBB using transwells have incorporated cultures of endothelial cells alone or co-cultured with astrocytes and pericytes. These models commonly use 10 μm thick polystyrene or polycarbonate membranes with 400 nm pores. The endothelial cells are usually cultured on the top (luminal) surface of the membrane, while other cells such as astrocytes and pericytes are cultured on the bottom (basolateral) side of the membrane. Colgan *et al.* studied a co-culture of brain endothelial cells with astrocytes on opposite sides of the transwell membrane and investigated the effect of serum on the permeability of the barrier [101]. The transwell platform

also allows triple culture of different cell types in which the third cell type is cultured on the bottom of the well. An example is the triple culture of endothelial cells, astrocytes and pericytes [90].

Despite the significant achievements in understanding of the BBB by static transwell models, the static models are not physiologically relevant due to the lack of flow induced shear stress which has an important role in development of BBB characteristics [102-105]. Several investigators have developed dynamic models of the BBB by culturing brain endothelial cells inside microfluidic channels [6, 96, 97, 106] and investigating permeability of the BBB to different therapeutics.

Booth *et al.* [6] developed a dual layer microfluidic device with a perforated membrane in between. b.End3 cells (rat brain endothelial) and C8D1A (astrocytes) are cultured on opposite sides of the membrane. They monitored the permeability of the BBB model by measuring the transmembrane electrical resistance and permeability assays of some chemicals. They also investigated the permeability of molecules with different molecular weights across the BBB stimulated by different pH environments and chemicals. A similar work is done by Griep *et al.* [96] using human brain endothelial cells. They modulated the permeability of the BBB mechanically and biochemically by exposing it to flow induced shear force and TNF- α , respectively.

While these *in vivo* and *in vitro* studies have provided insight into the permeability of the BBB in response to PEFs and other therapeutics, no distinction has been made between the paracellular and transcellular pathways. Although both of these pathways are essential in transporting molecules across the BBB, each of them allows for quite specific, distinct molecules to cross depending on their size and hydrophilic or hydrophobic nature. Therefore the ability to target and controllably permeabilize each independently may allow for better delivery of drugs.

We have developed several microfluidic models of the BBB to study the effect of PEFs on the permeabilization of the BBB from different perspectives. In Chapter 2 we show a microfluidic

platform that enables us to find the reversible and irreversible electroporation thresholds for adhered brain endothelial cells. The results from these experiments give insight into transcellular permeabilization of the BBB. In Chapter 3 microfluidic devices with integrated impedance sensors are used to monitor the permeabilization of the BBB model, in a more real-time and quantitative manner. In Chapter 4 another microfluidic model of the BBB is discussed in which transport across the cell monolayer could be measured using fluorescent tracers. This platform could be used to investigate both paracellular and transcellular permeabilization of the BBB.

1.4. Electroporation

Electroporation is the phenomenon in which the cell membrane permeability increases due to the formation of nano-scale pores which is induced by exposure to short, high amplitude PEFs [107]. Depending on the pulse parameters and duration of the treatment, this process could be either reversible or irreversible. When reversible, the cell membrane recovers and the cell survives. Reversible electroporation has been used for gene and drug delivery. In the irreversible electroporation, membrane permeabilization is permanent and the process results in cell lysis. Irreversible electroporation (IRE) has been used for decades for *in vitro* studies and especially for killing bacteria. However, it was not until the early 2000 that it was implemented as a tissue ablative technique [108] and has so far been implemented to cure over 5500 human patients [109]. Unlike energy-based ablation therapies such as radiofrequency ablation (RFA) [110], ultrasound [111], cryoablation [112] and microwave ablation [113], IRE kills cells within the target tissue with a primary non-thermal mechanism that helps preserve the underlying structure and the blood vessels. Since its invention in 2005 [108] as a tissue ablation modality, IRE has gained increased attention with many *in vitro*, *ex vivo*, animal and clinical studies being performed each year. IRE was first implemented in human patients to treat renal tumors [114]. Further clinical studies on

human patients include liver [115-117], pancreas [118-120] and lung [121]. According to a comprehensive review of clinical trials of IRE by Scheffer *et al.* [122], the majority of the clinical trials have been performed on human liver tissue. When using the alternative RFA technique for liver tumors, the adjacent structures including gallbladder, bowel and bile ducts are prone to thermal damage. That makes IRE favorable for treating liver tumors with minimal to no peripheral damage.

During the IRE procedures, the electric pulses are delivered through a combination of electrodes inserted within the region of interest. Pretreatment planning is necessary based on the electrode configuration and the applied electric potential to estimate the electric field distribution within the tissue and find the volume of ablation. Mathematical [123] and finite element modeling [124, 125] of the electric field distribution could be performed for this purpose .

It is always desired to ablate as much of the tumor as possible while minimizing damage to the surrounding healthy tissue. Monitoring the lesion size is also possible during and after completion of the treatment using a variety of techniques such as ultrasound imaging, MRI [126], and electrical impedance spectroscopy [127].

A multitude of *in vitro* electroporation studies exist to help find the mechanism of action of electroporation at a cellular level. From studies on isolated cells [128], cell suspensions [129] and artificial membranes [130] it is known that electroporation happens when the transmembrane potential of the lipid bilayer reaches a certain threshold. Based on the electroporation assessment method the threshold value is reported in the literature in the range of 200 mV-1000 V [131]. Before electroporation happens, the induced transmembrane potential ΔV_m is proportional to the applied electric field and varies across the surface of the cell. For a spherical cell in suspension as shown in Figure 1-2, ΔV_m is expressed according to the Schwan's equation [132]:

$$\Delta V_m = \frac{3}{2} |\vec{E}_{ext}| r \cos(\theta) \quad (1-1)$$

Equation (1-1) has the interesting consequence that electroporation does not happen uniformly across the cell membrane. Instead, areas facing the electrodes will get electroporated faster. This fact has been experimentally observed in cells [133]. Electroporation also happens asymmetrically across the cell due to the resting transmembrane potential, which exists because of the accumulation of negative ions inside and positive ions outside of the cell. Therefore the side facing the anode reaches the critical ΔV_m faster than the other side. The asymmetric electroporation has been studied in several experimental works [134].

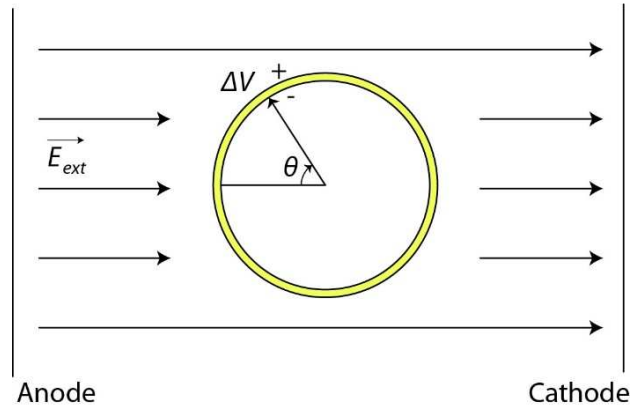


Figure 1-4. Schematic of the experimental setup

It should be noted that Eq. (1-1) is only valid for the special case of spherical cells. Some correction factors have been introduced for some special non-spherical cases [135]. In reality the cells in a tissue have more complicated geometries for which an analytical expression does not exist anymore. In these cases either finite element analysis [136] or experiments are needed to find the correct relation. In Chapter 2 we have shown how a microfluidic platform was used to find the thresholds for reversible and irreversible electroporation of adhered brain endothelial cells.

Cell membrane conductivity increases after electroporation. The intact cell membrane which is a lipid bilayer, is insulative. However, after electroporation electrical current can pass through the induced pores, which makes the membrane more conductive. Several studies have shown the effect of electroporation on the passive electrical properties of single cells [137-139], cell suspension [140-142], adherent cell cultures [143, 144] and living tissues [145]. We used this effect of electroporation to devise a method and device to monitor the progress of IRE ablation therapy in tissues which is discussed in Chapter 5.

The field of IRE therapy is growing fast with new pulsing protocols [146], electrode designs [147, 148] and chemical agents [149] being proposed to enhance the treatment. The advantages of a next generation IRE system called High-Frequency IRE have also been studied in the literature. These include the ability to administer IRE therapy without the use of neuroblocking agents to mitigate muscle contractions during the procedure [150] and creation of a more uniform electric field distribution by reducing the impedance barrier posed by low conductivity tissue [151].

1.5. Electrical impedance spectroscopy for characterization of biological systems

Electrical impedance spectroscopy (EIS) is a non-invasive powerful method for characterizing electrical properties of materials and their interfaces. Depending on the targeted system, the electrical properties give insight into many inherent physical, chemical and even biological properties that could not otherwise be measured. EIS works on the basis of stimulating the system with a voltage (or current) and observing the response as a resulting current (or voltage). The stimulation could be one of the following three forms: A step function, a white noise, or a single frequency signal [152].

EIS has been traditionally used in electrochemical systems for applications such as corrosion monitoring [153], coating evaluation [154], battery health monitoring [155] and semiconductor

characterization. However in recent years, this method is gaining applications in the characterization of biological systems at the cellular and tissue level as a minimally invasive method. EIS also has a wide range of applications in the food industry mainly for quality control of food products such as meat [156, 157] and fruit [158]. In biological studies EIS has applications in tissue engineering, cell culture, monitoring tissue viability and physiological properties. In medicine EIS is gaining applications in disease diagnosis. At the cell level EIS has been used to monitor the electrical properties of single cells [159] and cell cultures in 2D and 3D [160]. Electric cell-substrate impedance spectroscopy (ECIS) is a popular technique for monitoring the growth, motion, integrity and permeability of 2D cell cultures [161, 162]. In this technique the cells are cultured over the surface of co-planar electrodes and their properties such as viability and integrity are reflected in the acquired impedance spectrum. This technique has been widely used to monitor the behavior of endothelial cells *in vitro* in different conditions [152, 163-165]. Integration of ECIS into microfluidic devices has provided new opportunities for analyzing cells [166]. At the tissue level EIS has been used to determine the physiological state of the tissue in different conditions. In tissues EIS contains information about the individual cells as well as the extracellular matrix and the intercellular junctions [167]. The effect of drug toxicity and ischemic conditions on the state of different organs such as liver [167], heart [168] and skeletal muscle [169] has been investigated by this technique.

Equivalent circuit models of biological systems

In all of the EIS applications the electrical impedance between the electrodes is measured in a wide frequency range and subsequently an equivalent electrical circuit model of the cell/tissue is fitted to the data. The fitting provides key parameters such as conductivity and capacitance of cell membrane, conductivity of extracellular space, gap junctional resistance and even information

about the intracellular organelles. Figure 1-5 and Figure 1-6 show some equivalent circuit models for cell monolayers and tissue, respectively.

In medicine EIS has been used to detect cancer, based on the characteristic differences between the impedance of the healthy and cancerous tissue [170, 171]. EIS has also been used to monitor the effect of different therapies such as IRE [127] and photodynamic therapy [172] on the target tissue. In Chapter 3 EIS is used to monitor the permeabilization of endothelial cell barrier by integration of impedance micro-sensors into a microfluidic platform. In Chapter 5 EIS is used to monitor the progress of tissue ablation during IRE.

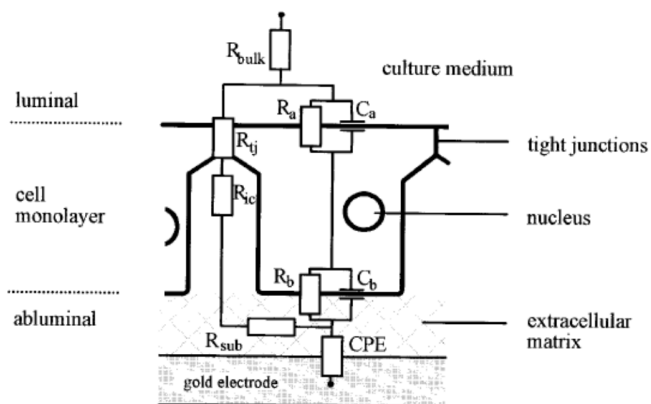


Figure 1-5. Equivalent circuit model of endothelial cell monolayer (picture taken from [173] with permission)

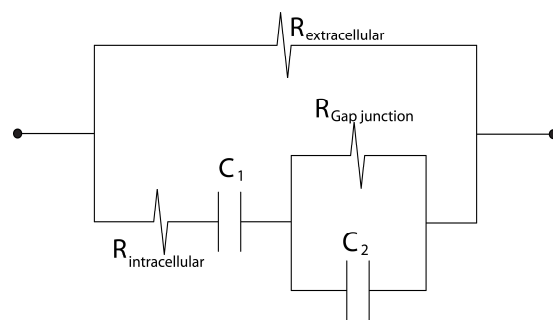


Figure 1-6. Equivalent circuit model of tissue with intercellular gap junctions [167]

Chapter 2: Blood-brain barrier-on-Chip: Electroporation of adhered brain endothelial cells toward controlled permeabilization of the BBB

Elisa Wasson has contributed in running experiments and analyzing data for this chapter. This chapter was published as an article in the *Biophysical Journal* as ref [174] and is reprinted here under permission from the journal.

2.1. Abstract

The blood-brain barrier (BBB), mainly comprised of brain microvascular endothelial cells, poses an obstacle to drug delivery to the brain. Controlled permeabilization of the constituent brain endothelial cells can result in overcoming this barrier and increasing transcellular transport across the BBB. Electroporation is a biophysical phenomenon that has shown potential in permeabilizing and overcoming this barrier. In this study we developed a microengineered *in vitro* model to characterize the permeabilization of adhered brain endothelial cells to large molecules in response to applied pulsed electric fields (PEFs). We found the distribution of affected cells by reversible and irreversible electroporation and quantified the uptaken amount of naturally impermeable molecules into the cells as a result of applied pulse magnitude and number of pulses. We achieved $81 \pm 1.7\%$ (N=6) electroporated cells with $17 \pm 8\%$ (N=5) cell death using an electric field magnitude of about 580 V/cm and 10 pulses. Our results provide the proper range for applied electric field (EF) intensity and number of pulses for safe permeabilization without significantly compromising cell viability. Our results demonstrate that it is possible to permeabilize the endothelial cells of the BBB in a controlled manner therefore lending to the feasibility of using PEFs to increase drug transport across the BBB through the transcellular pathway.

2.2. Device design and fabrication

2.2.1. Device design

The microfluidic device is comprised of dual tapered channels 30 mm long with varying width ranging from 1000 μm at the ends to 300 μm at the center. Designing the device in this manner creates a gradient of EF magnitudes. According to Ohm's law the EF magnitude inside the channel is governed by the following equation:

$$E(x) = \frac{1}{\sigma} J(x) = \frac{I}{\sigma A(x)} \quad (2-1)$$

where σ is the conductivity of the medium, J is the current density, x is the length along the channel and A is the cross sectional area of the channel. It is desired to have a linear gradient of EF along the length of the channel. According to Eq. (2-1) the magnitude of the EF and the cross section of the channel are inversely proportional. Hence, in order to get a linear $E(x)$, the cross sectional area of the channel needs to be inversely proportional to the length along the channel, x .

That requires the channel width profile to be a section of the curve defined by $w(x) = \frac{C}{x}$ curve, where C is a constant and the endpoints of the section are the values of the desired channel widths which are 1000 and 300. These endpoints and the constant C are determined by satisfying the following boundary conditions:

$$\begin{cases} w(x) = \frac{C}{x} \\ w(a) = 1000 \\ w(a+15000) = 300 \end{cases} \quad (2-2)$$

where a is the x -axis value of the curve that gives us a width of 1000. Solving the above equations simultaneously gives the numeric values for a and C . To have a symmetric channel geometry, two of the above sections were attached end to end as shown in Figure 2-1a.

Due to the device design, the cells in the middle of the channel experience an EF magnitude 3.3 times greater than the cells at the ends. Designing the device in this manner ensured that each part of the channel shows the cell behavior in response to a specific EF magnitude and allows for testing multiple conditions in one experiment. The symmetric, dual taper channel has an advantage over a single tapered channel for transitioning the high EF region from the channel end to its middle where there is no disruption of the induced EF due to the ports. Moreover, the symmetry of the channel mandates a symmetric response from the cells in the channel after exposure to PEFs, which may serve as a verification tool for each experiment.

Finite element analysis (FEA) was used to verify the magnitude of the EF at each point along the channel. The analysis was performed in COMSOL Multiphysics 4.4 in a 2D model using the AC/DC module. The channel geometry was designed in AutoCAD 2014 and exported into the COMSOL software. All the boundaries of the channel were assumed to be insulating. The ground potential of 0V was applied to one end of the channel and the charged potential corresponding to the pulse magnitude was applied to the other end.

2.2.2. Device fabrication

Photolithography and replication molding were used to fabricate the microfluidic channel in polydimethylsiloxane (PDMS). A photolithography mask was designed in AutoCAD software and printed on a transparency sheet (Cad Art Services, Portland, OR). A four inch silicon (Si) wafer was cleaned and silanized using 1,1,1,3,3,3-hexamethyldisilazane (ACROS Organics) to enhance photoresist adhesion, followed by spin coating with AZ9260 photoresist (AZ Electronics). Photolithography was performed by exposing the wafer to UV light through the mask (Karl Suss MA6 mask aligner, Garching, Germany). After exposure the photoresist was developed in

AZ400K developer (AZ Electronics). The wafer was etched to a depth of 90 μm using deep reactive ion etching (DRIE) (Alcatel, Annecy, France).

As cheaper alternative technique for mold fabrication, the structural photoresist SU-8 (Microchem) could be used, which eliminates the subsequent step to etch the Si wafer. This method is useful in circumstances where either DRIE is not available or the high cost of DRIE operation needs to be avoided. This method is fast and especially useful when trying a new design. However, the obtained mold is not durable and usually after a few trials the adhered photoresist delaminates. The fabrication steps to yield features of 100 μm thickness are summarized in Appendix A.

The fabricated mold was then silanized using trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma Aldrich, St. Louis, MO) under vacuum for one hour as a mold release. PDMS (Sylgard 184, Dow Corning) was prepared by mixing the prepolymer and curing agent with a 10:1 weight ratio. The mixture was then degassed and poured onto the Si wafer. The PDMS was cured at 100°C for one hour. After curing, the inlets and outlets were punched and the PDMS device was bonded to glass slides using air plasma (Harrick Plasma, Ithaca, NY). Figure 2-1 shows a schematic of the channel along with the final device.

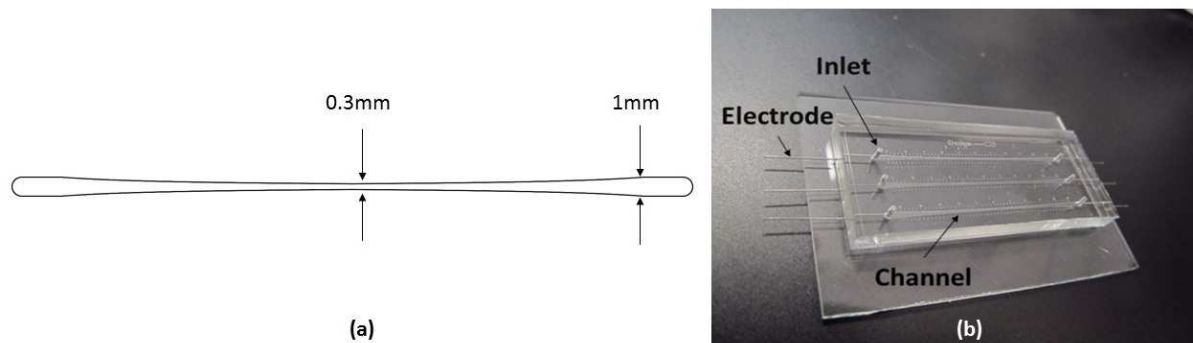


Figure 2-1. (a) Schematic of dual tapered microfluidic cell culture channel, (b) Microfluidic device comprised of three separate dual tapered channels.

2.3. EF distribution inside the channel using FEA

Figure 2-2 shows the EF distribution in the channel and the EF magnitude along the centerline of the channel for an applied potential of 1500 V. As it is shown in the figure, the design of the channel provided a linear variation in the EF magnitude from 213 V/cm at the ends to 714 V/cm at the center of the channel. It should also be noted that the EF magnitude did not change significantly along the width of the channel.

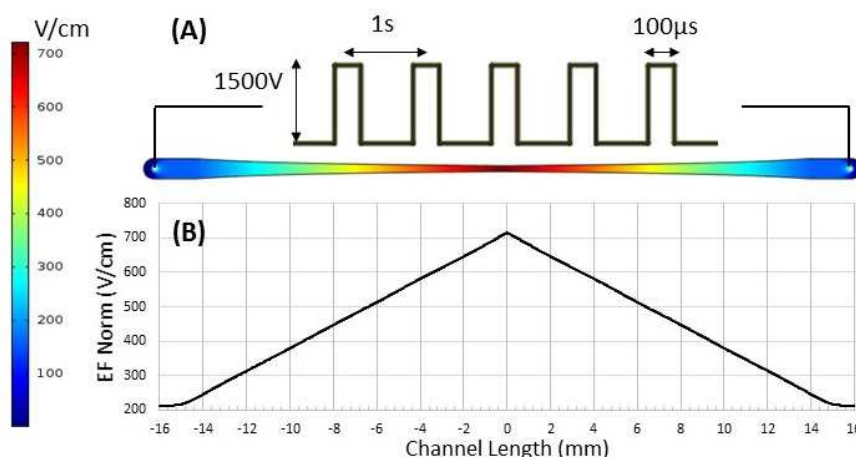


Figure 2-2. EF distribution in the channel as a result of applied potential at the channel ends (A) EF contours in the channel and (B) EF magnitude along the channel centerline

2.4. Fluorescence calibration

To relate the fluorescence of the images to the concentration of the uptaken dextran molecules, a calibration curve was developed. This was done by using microfluidic channels of different heights in order to simulate the height of the adhered cells found using confocal microscopy. The channels were filled with solutions of different concentrations of 4 kDa FITC-dextran in PBS and imaged using fluorescent microscope. The purpose of imaging was to record the intensity of fluorescent light emitted from the fluid inside the channel for each of the configurations and find a correlation between the intensity and the concentration. The fluorescence intensity was then obtained using ImageJ. Figure 2-3 shows the fluorescent intensity as a function of the concentration of 4 kDa

FITC-dextran inside channels of different heights. Our observations using confocal microscopy showed that after adhesion and spreading, the thickness of the cell monolayer varied between 2 μm (at the cell edges) to around 7 μm (above the nuclei). Hence, four different heights around this range were selected for obtaining the calibration curves. It was found that the intensity varied linearly with respect to both the concentration and the height. Linear regression was used to find an empirical relation giving the fluorescence intensity as a function of FITC-dextran concentration and height. The linear dependence of intensity on channel height was used in the following section to obtain the profile of adhered endothelial cells according to the fluorescence of absorbed dextran.

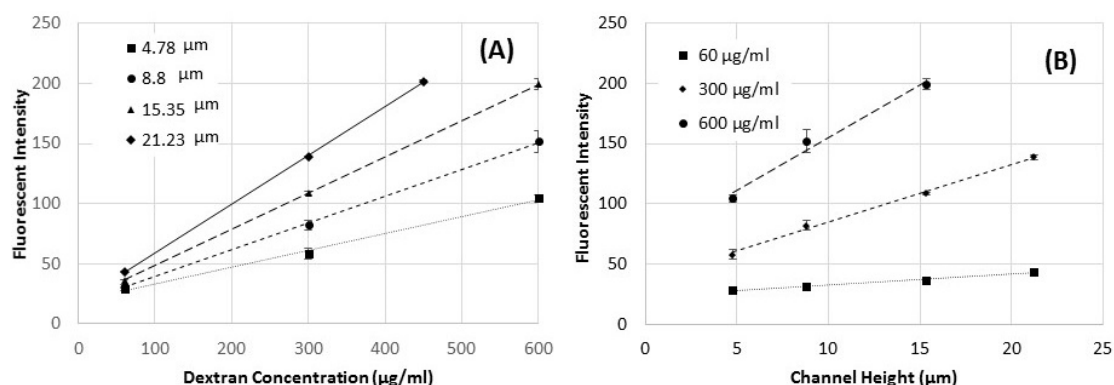


Figure 2-3. Calibration curves for fluorescent intensity of 4 kDa dextran in the microchannel (A) for different heights (B) for different concentrations

These calibration curves will be used in the subsequent sections to find the concentration of uptaken dextran molecules into the cells as well as finding the profile of the adhered endothelial cells.

2.5. Cell culture and characterization of the cell monolayer inside the chip

2.5.1. Cell culture

Previous *in vitro* models of the BBB have been developed from a variety of different primary cells and immortalized cell lines [175]. Selection of the proper cell type for a physiologically relevant

model of the BBB is an active line of research especially among biologists. However, the focus of this work is on development of the platform for cell permeabilization. Adjustments of the biological aspects including the cell type is left for future studies.

In this study we used the mouse brain endothelial cell line, bEnd.3 (ATCC, Manassas, VA), which has been used in previous *in vitro* BBB models and has shown to be adequate for basic transport studies [6, 176-179]. The bEnd.3 cells were cultured in T-75 flasks at 37 °C and 5% CO₂ and maintained in complete growth media consisting of DMEM (ATCC) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% (v/v) penicillin-streptomycin (Life Technologies). The cells were routinely passaged at 70-90% confluence. In order to prepare the microfluidic device for cell seeding, the PDMS channels were first sterilized with ethanol. To promote cell adhesion and proliferation, the channel was treated with 50 µg/ml human fibronectin (Trevigen, Gaithersburg, MD) for one hour in an incubator. It should be noted that several different types of fibronectin including mouse fibronectin were examined and despite the interspecies differences it was found that the human fibronectin was more effective for cell adherence to the channel. The Complete growth media was then introduced into the device and incubated for two hours. Endothelial cells were collected by washing and detaching with trypsin. The trypsin was then neutralized by the addition of media (serum neutralizes trypsin) and centrifuged for five minutes at 120 × g. The trypsin solution was removed and fresh media was added to obtain a concentration of approximately 10 million cells/ml. The cells were then introduced into the device through manual injection using a syringe and tubing until an even distribution was achieved in the channel. The device was incubated at 37 °C for two hours allowing the cells to fully attach. Then, complete media was provided to the channel using media-filled pipet tips at the inlet and outlet. The devices were incubated for two days at 37 °C and 5% CO₂

until the cells were confluent in the channel. Figure 2-4 shows the confluent endothelial cell layer inside the channel at second day after seeding.

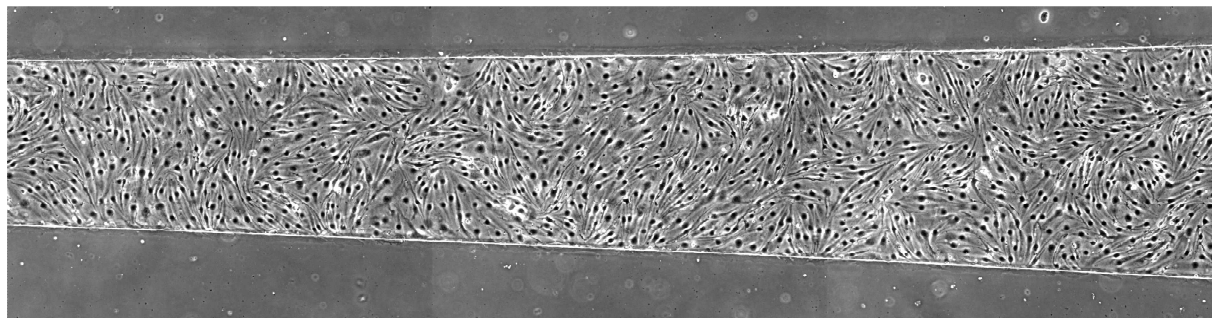


Figure 2-4. Confluent monolayer of bEnd3 cells inside the tapered channel at day 2. Picture taken using phase contrast microscope at 5x magnification.

2.5.2. Immunofluorescence staining of tight junction proteins

In order to show the integrity of the endothelial cell monolayer in the microchannel, immunofluorescence staining was performed to visualize TJs before pulsing. Among different TJ proteins, zonula occludens 1 (ZO-1) was chosen as the target protein because it serves as a bridge which links all of the TJ proteins (occludens, claudins, JAM) to the cytoskeleton of the cell. Cells were fixed in the microfluidic channel using 4% (v/v) paraformaldehyde (Boston Bioproducts, Ashland, MA) for one hour at 37 °C. Cells were then rinsed with PBS for 15 minutes and incubated in permeabilization buffer consisting of 0.1% TritonX-100 (Life Technologies) and 2% bovine serum albumin (Sigma Aldrich) in PBS for 30 minutes. This was used for permeabilization of the cells and to prevent non-specific protein binding. Subsequently, cells were washed for 15 minutes in PBS followed by 2 hour incubation at 37 °C with primary anti ZO-1 monoclonal antibody (Cat#: MABT339, Millipore) diluted in the permeabilization buffer. After rinsing with PBS for 15 minutes, cells were incubated with secondary antibody, goat anti-mouse IgG FITC conjugated (Cat#: AP181F, Millipore), for 1 hour at 37 °C. Finally, the channels were rinsed with a solution of NucBlue (Life Technologies) in PBS and imaged using fluorescence microscopy. TJ staining

protocol is summarized in Appendix A. Figure 2-5 shows the localization of the tight junction protein ZO-1 at the boundaries of endothelial cells.

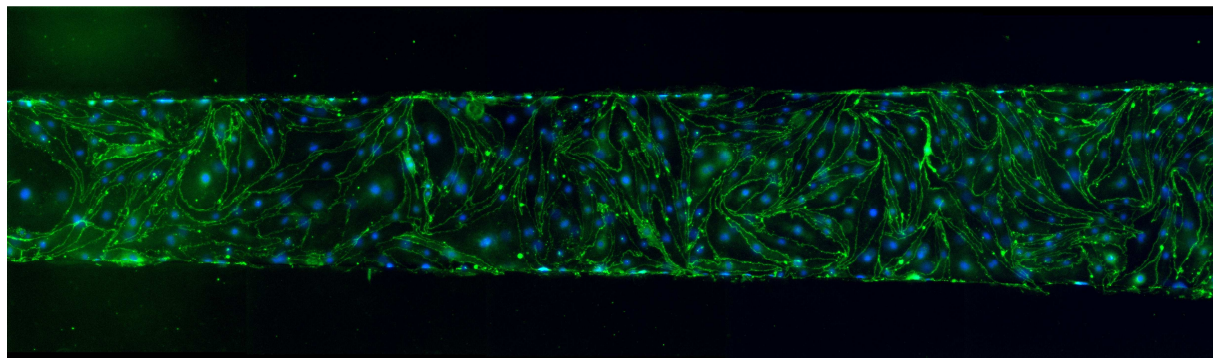


Figure 2-5. Immunofluorescence staining of TJ protein ZO-1. Cell nuclei are counterstained with NucBlue.

2.5.3. Confocal microscopy

Confocal microscopy was performed to obtain the average height of the cell monolayer. Prior to imaging the cells were stained with rhodamine phalloidin and DAPI (Invitrogen) which stain F-actin filaments and nuclei, respectively (see Appendix A for more details on staining). Following staining, the cells in the microchannel were imaged using ZEISS LSM880 confocal microscope (ZEISS, Germany) with 40X objective. The pinhole aperture for confocal fluorescence was adjusted to produce a Z resolution of approximately 1 μm . Images were collected in steps of 0.1 μm in the Z direction. ZEN Black software (ZEISS, Germany) was used to analyze the sections and build the 3D image. Figure 2-6 shows cross sectional images of the cell monolayer at different heights from the channel base, as well as several side views of the cells along different transverse sections. The maximum height of the cells was located above the cell nuclei with an average value of $7.0 \pm 1.4 \mu\text{m}$. The rest of the cell body possessed a maximum height of less than 4 μm . The staining technique, clearly visualized the boundary of the nucleus, but not the boundary of the cell body as shown in the side views of Figure 2-6. Despite a sharply contrasted nucleus, the rest of the

cell body was blurred. Hence another technique using the fluorescent intensity of uptaken FITC-dextran was used to find the outer profile of adhered cells.

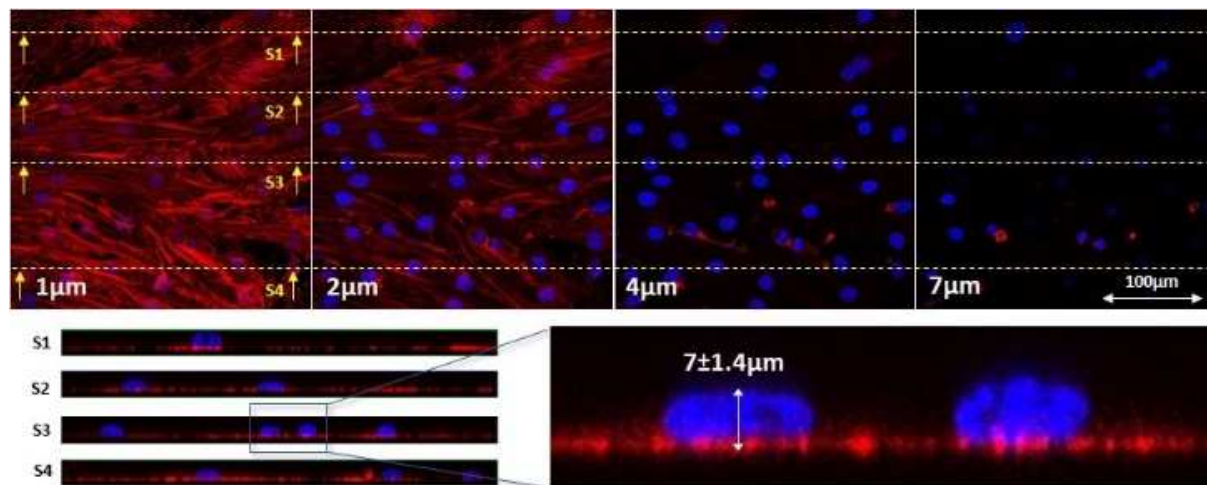


Figure 2-6. Confocal images of the endothelial cell monolayer. Top: Cross sectional images of the bEnd.3 cell monolayer in the microchannel taken at different heights Bottom: Side views show the relative height of the nuclei (blue) compared to the rest of the cell body (red)

Based on the size of the cells and the diffusivity of the dextran molecules inside the cytosol, it is possible to assume a uniform distribution of absorbed dextran in the cell body after a few minutes. Therefore, the fluorescent intensity at each point becomes linearly proportional to the height of the adhered cell, as shown in the calibration section (Figure 2-3B). We used this fact in addition to the absolute height of the cells above the nuclei-according to the confocal imaging-to find the height distribution of the adhered cells. Figure 2-7 shows the adhered bEnd.3 cells in the microchannel and the outer profile along their longitudinal directions. Using this method, the average height of the endothelial cell monolayer was calculated to be $3.15 \mu\text{m}$ ($N=30$).

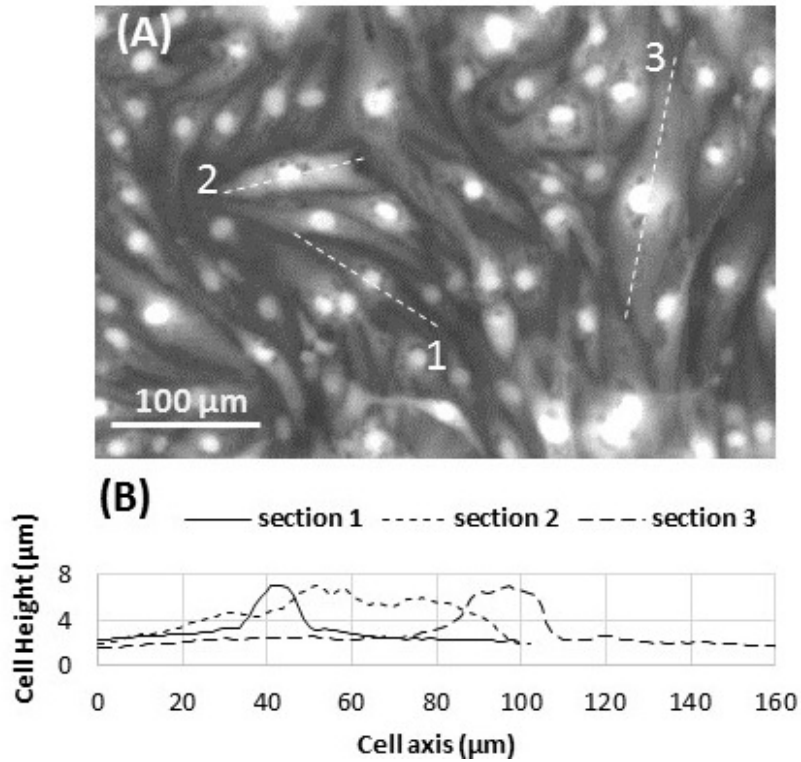


Figure 2-7. Finding the height profile of cells from fluorescent image (A) Fluorescence image of adhered bEnd3 cells with absorbed FITC-dextran (B) Height profile of the adhered cells

2.6. Determination of the reversible and irreversible electroporation thresholds

When using PEFs to permeabilize the endothelial cells of the BBB for drug transport, it is desirable to avoid irreversible effects on the cell membrane, which can result in permanent damage and leakage of the blood vessels. Hence, it is necessary to know the IRE threshold and the margin for reversible electroporation. The IRE threshold is a function of applied number of pulses. A treatment comprised of more pulses needs a lower EF magnitude to induce IRE. Figure 2-8 shows the distribution of dead cells inside the channel for different number of pulses. As it is shown, by increasing the number of pulses, more cells in the lower EF region (wider section of the channel) were irreversibly electroporated. Expanding upon this, Figure 2-9 shows the percentage of dead cells along the channel for different number of pulses and pulse magnitudes.



Figure 2-8. IRE-treated cells for different number of pulses. (See Fig. S1 for high resolution color image)

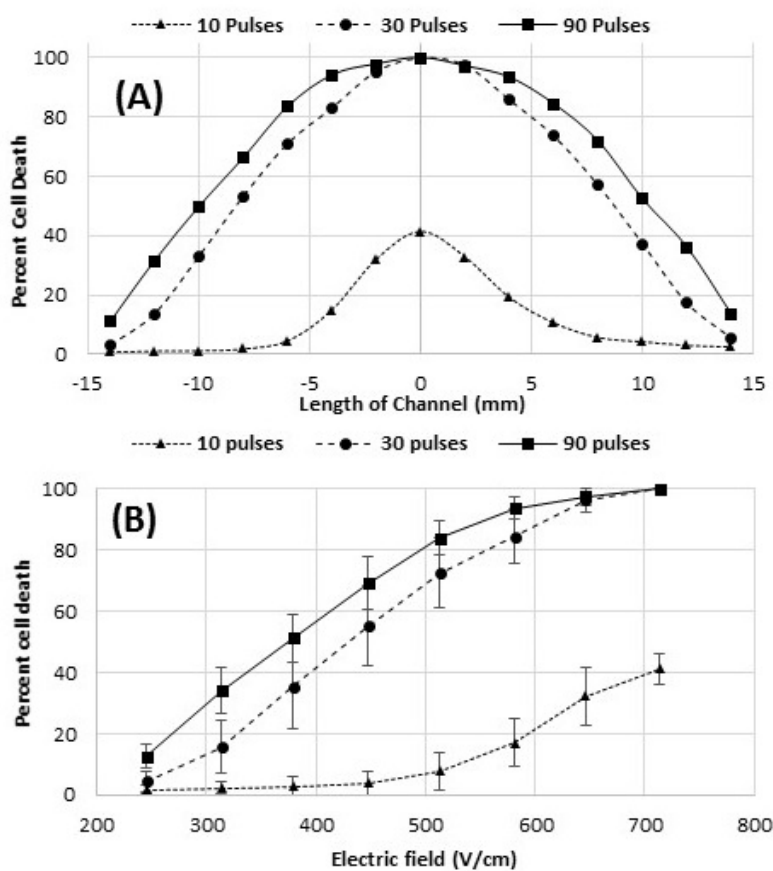


Figure 2-9. Distribution of dead cell population after EP. (A) Dead cell population along the channel for different number of pulses (B) Dead cell population as a function of PEF magnitude for different number of pulses.

Several factors such as cell orientation cell size and distribution cause individual cells in the same proximity to be affected differently by the pulses of the same magnitude. Our experiments showed

that the cells which are oriented parallel to the electric field get electroporated at a lower electric field than those that are perpendicular to the field. This results in a distribution of live/dead cells along the channel instead of a sharp delineation between live and dead cells. The percentage of dead cells differed significantly among the examined treatment parameters (ANOVA, $p < 0.0001$). Post-hoc comparisons indicated that the dead cell percentage for 10 pulses was significantly different from 30 and 90 pulses at higher EF magnitudes whereas lower EF magnitudes (245-314 V/cm) did not show a significant difference. These results can be seen in Figure 2-9, which shows that for a wide range of EF magnitude the induced cell death for 10 pulses (0-40%) is much lower than 30 and 90 pulses (5-100%). This finding is consistent with the fact that usually 8-10 pulses are applied for drug delivery during electrochemotherapy. For 10 pulses almost no cell death is observed for EF lower than 400 V/cm. It can also be seen that 30 and 90 pulses at higher EF magnitudes (646-714 V/cm) and low EF magnitudes (245 V/cm) did not result in a significantly different percentage of cell death whereas an EF magnitude of 314 V/cm for 30 and 90 pulses resulted in significantly different percentages. Previous *in vivo* studies show that an EF magnitude of 500 V/cm and 90 pulses induce IRE[58]. Our results show that the same pulsing results in 85% cell death.

Figure 2-10 shows the distribution of electroporated cells visualized by uptake of PI. The percentage of electroporated cells differed significantly among the examined treatment parameters (ANOVA, $p < 0.0001$). Post-hoc comparisons indicated that the electroporated cell percentage for 10 pulses was significantly different from 30 and 90 pulses at mid-range EF magnitudes (314 – 513 V/cm) whereas low and high EF magnitudes (245, 646-714 V/cm) did not show a significant difference. Differences between 30 and 90 pulses were not statistically significant for equivalent EF therefore indicating that 30 pulses delivers a comparable amount of PI into the cell as 90 pulses.

The electroporated cells spanned a wider range of EF compared to the irreversibly electroporated cells. This is due to the fact that the cells were in contact with the PI molecules during treatment, therefore as soon as the pores opened, the molecules permeated the cells. On the other hand in the case of IRE, only the pores which were not recovered after one hour allowed the transport of PI molecules into the cell.

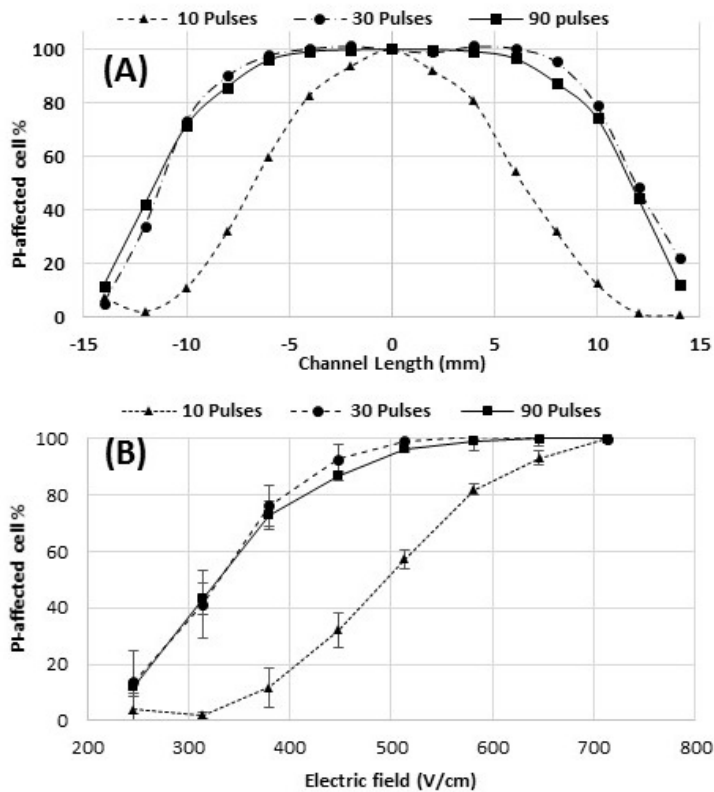


Figure 2-10. (A) Distribution of PI-permeated cells after EP (A) PI-permeated cell population along the channel for different number of pulses (B) PI-permeated cell population as a function of PEF magnitude

By comparing Figure 2-9 and Figure 2-10 one can find the proper number and magnitude of pulses for cell electroporation with minimal cell death. This comparison is made in Figure 2-11 for the specific case of 10 and 90 pulses which are typically used for electrochemotherapy and tumor ablation treatments, respectively. This comparison demonstrates how different EF magnitudes and

pulse numbers can electroporate a population of cells while keeping them viable. It is shown that for 10 pulses there is a drastic difference between the onset of electroporation and the onset of IRE. For example 10 pulses at 513 V/cm electroporated about 60% of the cells while causing only 10% cell death. On the other hand for the case of 90 pulses, less than 20% of the cells that were electroporated remained alive. These results explain the reason 8-10 pulses are typically delivered during electrochemotherapy treatments. It should be noted that PI becomes fluorescent once it binds to DNA after entering the cell. Since PI is not intrinsically fluorescent, the emitted light is restricted to the cell nuclei and does not reflect the quantitative amount of PI molecules present in the cell body. In order to study this uptake phenomenon in a more quantitative manner, FITC-dextran was investigated.

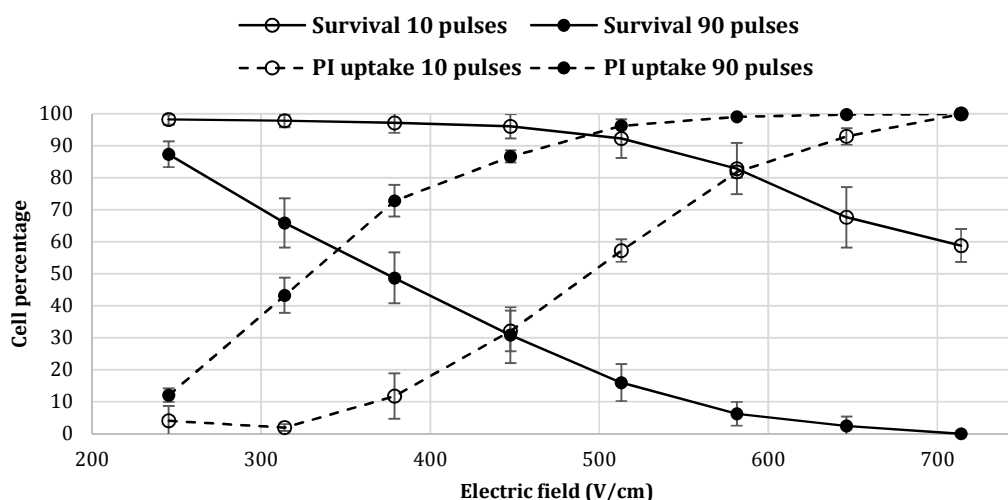


Figure 2-11. Comparison of IRE and PI uptake for 10 and 90 pulses

2.7. Uptake of large impermeable molecules due to PEFs

4 kDa FITC-dextran is intrinsically fluorescent and naturally impermeable to the cells due to its large size and hydrophilic nature. However, this substance can penetrate the cell membrane through induced pores formed by electroporation. Figure 2-12 shows the uptake of 4kDa FITC

dextran after electroporation. The percentage of affected cells differed significantly among the examined treatment parameters (ANOVA, $p < 0.0001$). Post-hoc comparisons indicated that the uptake of FITC-dextran into cells for 10 pulses was significantly different from 30 and 90 pulses at high EF magnitudes (646-714 V/cm) whereas low EF magnitudes for 30 pulses (245-314 V/cm) and mid-range EF for 90 pulses (447-513 V/cm) did not show a significant difference. Differences between 30 and 90 pulses were statistically significant for mid-range EF magnitudes (447-581 V/cm). Figure 2-13 indicates that uptake of dextran into the cells was more effective for lower EF magnitudes for 30 and 90 pulses which initially may seem counterintuitive. For cells which were reversibly electroporated, the molecules became trapped inside the cells after the pores were recovered. For the cells which were irreversibly electroporated, it is possible that the membrane remained leaky and could not retain the absorbed molecules for the duration of the experiment. Therefore, we hypothesize that dextran molecules escaped the cells during the washing period, reducing the emitted fluorescent light from those regions of the channel. This behavior was not observed for the uptake of PI (Figure 2-10), since PI binds to the DNA as soon as uptake occurs and cannot exit the cell. Figure 2-13A shows the fluorescent intensity of absorbed 4kDa FITC dextran along the channel for different number of pulses. Knowing the calibration curve for fluorescent intensity and the cell monolayer thickness, it was possible to find the approximate concentration of the accumulated dextran inside the cells. Figure 2-13B shows dextran concentration vs. EF magnitude for different number of pulses. Depending on the applied number of pulses, different ranges of EF magnitude gave maximum uptake of the FITC dextran molecules. For the case of 10 pulses, generally, the higher the EF magnitude, the higher the amount of uptake. This is due to the dominant occurrence of reversible electroporation instead of IRE even at the highest EF magnitude of 714 V/cm. In other words, despite some cells dying by increasing the EF

magnitude, other cells uptake enough molecules that the overall uptake by the monolayer is seen as increasing. However, that was not the case as the number of applied pulses were increased. Increasing the number of pulses resulted in more cell death at the higher EF zone.



Figure 2-12. Uptake of 4kDa FITC dextran after application of different number of pulses

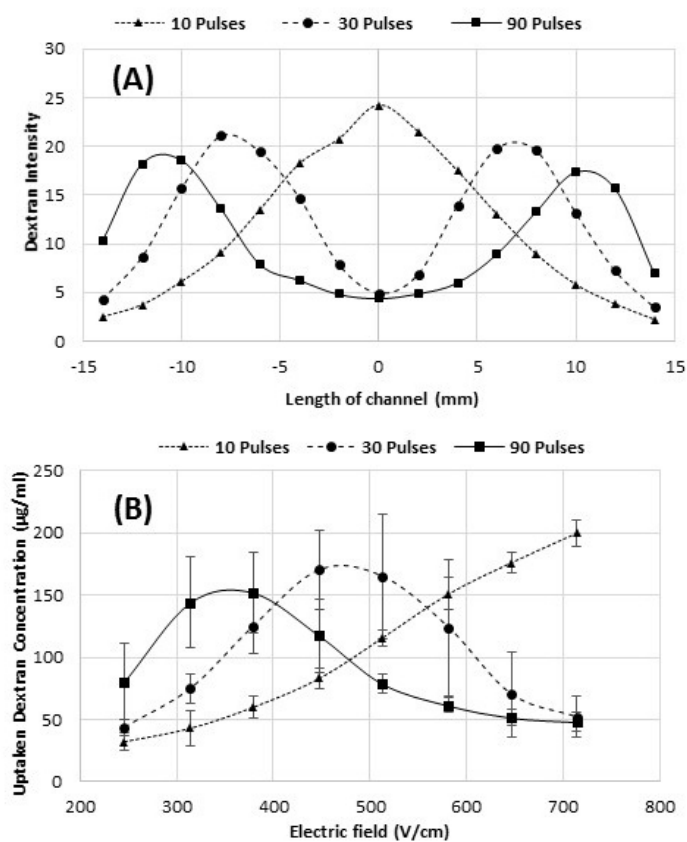


Figure 2-13. Dextran uptake during EP (A) Fluorescent intensity along the channel for different number of pulses. (B) Dextran concentration inside cell after pulsing vs. EF for different number of pulses. The dextran concentration in the pulsing medium was 10mg/ml. (Refer to the ESI for high resolution image of the transfected cells within the channel.)

It should be noted that in the case of passive diffusion across the intact cell membrane, the transcellular pathway is restricted to lipophilic molecules. However electroporation can induce hydrophilic pores into the membrane, which may facilitate the transport of hydrophilic molecules such as dextran in this case. In the above experiments the rationale for choosing PI (668 Da) and FITC-dextran (4 kDa) as the target molecules was their resemblance in size to several drugs, which are currently being administered to patients with brain diseases and tumors for which penetration of the BBB remains a challenge. These drugs include Bleomycin 1415 Da, Doxorubicin 543 Da, Amphotericin B, 923 Da, and Paclitaxel, 853 Da. Being slightly larger than these drugs, the results for the uptake of 4kDa FITC-dextran into brain endothelial cells gives an upper limit for the uptake of these drugs into the BBB by applying the proper PEFs without causing any permanent damage to the BBB.

In addition to reversible electroporation which opens the transcellular pathway for transfer of substances, PEFs may also disrupt the TJs between the adjacent cells which opens the paracellular pathway [180]. The current study was specifically aimed at finding the thresholds for reversible electroporation and cellular uptake of different sized molecules, which could yield relevant information about the possibility of transcellular transport across the BBB. Studying paracellular transport requires access to both luminal and abluminal sides of the BBB which is left for future studies.

2.8. Conclusions

In this study we quantified the uptaken amount of molecules into adhered endothelial cells as a function of EF magnitude and the number of pulses by using emitted fluorescent light from the electroporated cells. To the best of our knowledge it is the first time that such analysis is performed for the electroporated cells. We implemented a tapered microfluidic channel to apply a gradient of

EF on adhered brain endothelial cells and visualized electroporation phenomenon by tracking the uptake of different naturally impermeable molecules into the cells. The tapered design allowed for testing of multiple conditions in one experiment, therefore making it a useful platform to test drug delivery using PEFs. Using this platform, we were able to show the difference between reversible and irreversible electroporation for different pulse numbers and a wide range of EF magnitudes. The majority of the cells which were electroporated with 10 pulses, recovered, however electroporation with 30 and 90 pulses was mostly irreversible. Results for 30 and 90 pulses were similar for the entire range of EF magnitude, although 90 pulses caused more cell death. These results provide the proper range of applied EF magnitude and number of pulses for safe permeabilization without significantly compromising cell viability. Our results demonstrate that it is possible to permeabilize the endothelial cells of the BBB in a controlled manner therefore lending to the feasibility of using PEFs to increase drug transport across the BBB through the transcellular pathway.

Chapter 3: Electrical impedance spectroscopy for monitoring the permeabilization of the BBB on Chip

3.1. Abstract

Electrical impedance spectroscopy (EIS) was utilized to provide a real-time and more quantitative method for monitoring the permeabilization process in the endothelial layer of the BBB. A microfluidic platform with embedded impedance sensors was designed. The impedance sensors were characterized in terms of different environmental conditions. The endothelial cells were grown on the impedance sensors inside the microfluidic device and their integrity was monitored electrically. Finally, the impedance measurement was used to monitor the permeabilization of the endothelial layer in response to stimulations by osmotic agent and pulsed electric fields.

3.2. Device design and fabrication

The platform for our BBB model is comprised of two main compartments: a microfluidic channel and an electrode array for electrical impedance sensing. The former is fabricated in polydimethylsiloxane (PDMS) by photolithography and replication molding and the latter is fabricated with either gold or indium-tin oxide (ITO) on glass by physical vapor deposition and chemical etching. The Fabrication process is illustrated in Figure 3-4.

3.2.1. Micro-channel design

The cell culture channel was designed in two different configurations: straight and tapered (see Figure 3-1). The straight channel was $L=3$ cm long, $h=100$ μm high, and $w=1\text{mm}$ wide. Upon application of electric potentials (V) to the ends of the channel, a uniform electric field was created along the channel as determined by the equation, $E = V / L$. The tapered channel was designed based on the design presented in chapter 2. However, here an asymmetric version was used in

order to get the electric field gradient in a longer distance. The width of the channel at the wider section is 1mm which narrows down to 300 μm according to the equation provided in section 2.2.1. To provide the cells with a continuous, slow, gravity induced flow of culture medium after the seeding and during the growth phase, the cell culture channel is amended by a high hydraulic resistance micro-channel. The hydraulic resistance of a rectangular cross sectional channel with length L_R , width w_R and height h is (see Appendix B)

$$R_R = \frac{12\eta L_R}{1 - 0.63(h/w_R)} \frac{1}{h^3 w_R} \quad (3-1)$$

where η is the fluid viscosity. The resistive annex is designed to give a hydraulic resistance of 1×10^{13} (Pa.s/m³), which roughly allows 500 μl of media stored initially in a pipet tip at the inlet to flow out in 12 hours. That time is enough to keep the fluid running into the chip overnight, before it could be recharged in the next morning.

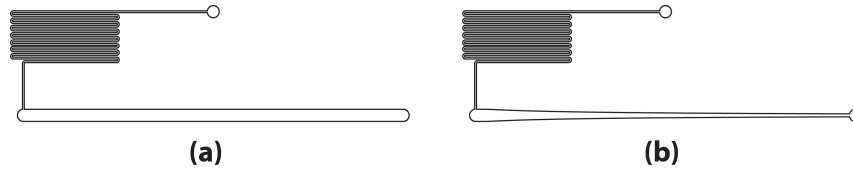


Figure 3-1. Cell culture channels to be used along with the impedance sensors (a) straight channel for uniform electric field (b) tapered channel for linear gradient of electric field

3.2.2. Impedance sensor design and fabrication

A variety of different geometries were tested for the impedance sensors to enable sensitive measurements of the cells. The ideal configuration to measure the impedance of an object is to place flat plate impedance electrodes at opposite sides of the object. In that case due to the induced uniform electric field, all points within the object will have the same contribution to the impedance measurement. However, this approach needs access to the opposite sides of the sample which may not be possible in all experimental configurations such as microfluidic platforms. Due to the

fabrication technique, we only have access to one side of the microfluidic channel which faces the glass substrate. Therefore the only option to have the impedance sensors is to fabricate them on the glass, facing the microfluidic channel. Following this technique we will end up having impedance sensors with both poles on the same plane, or simply coplanar electrodes.

To estimate the sensitivity distribution of an electrode setup, one can look at the electric field distribution generated by the same electrodes as obtained from FEM and shown in Figure 3-2.

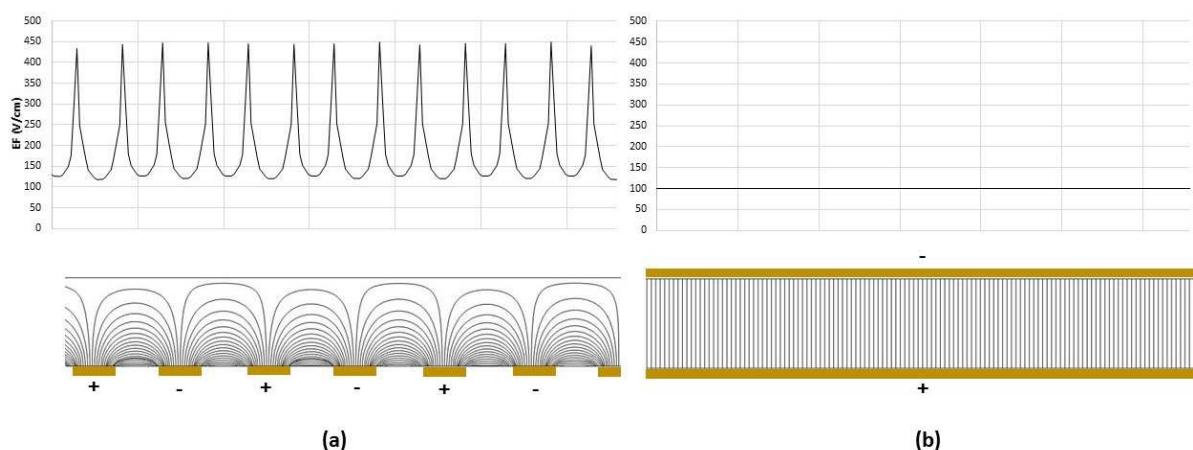


Figure 3-2. Electric field distribution produced by (a) interdigitated electrodes (b) parallel plate electrodes in a micro-channel. For both configurations, the plots show the electric field magnitude along a line 3 μm above the bottom surface of the channel. This distance was chosen based on the average thickness of the bEnd3 cell monolayer as determined in Chapter 2. The channel height is 100 μm and a potential difference of 1V is applied to the electrode pairs.

In case of parallel plate electrodes, all points between the two plates experience the same electric field magnitude. That is translated into equal sensitivity of the points in case of impedance sensing. However, in case of coplanar electrodes, the edge of the electrodes experience a higher electric field compared to the center of the electrodes, which means when the same configuration is used for impedance measurement, the impedance is more sensitive to the electrode edges than the electrode centers. Therefore, to increase the sensitivity of the impedance sensors, an interdigitated electrode array is used. To get multiple measurements along the channel, five pairs of interdigitated

electrodes, each containing 35 fingers are embedded on the chip. Figure 3-3 shows a schematic of the impedance chip.

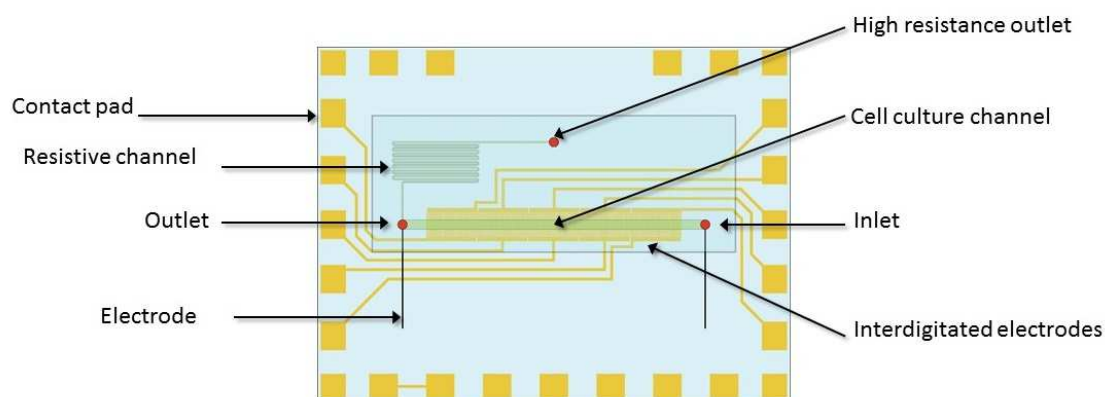


Figure 3-3. Schematic of the microfluidic device with impedance sensors.

For sensor material both gold and ITO were used in separate devices to compare the advantages and disadvantages of each for cell impedance sensing. Gold is highly conductive, inert, biocompatible and commonly used for cell impedance sensing. However the combined gold-chromium layer is not transparent and does not allow visual inspection of the cells in the microfluidic channel. On the other hand ITO is transparent. However, its lower electrical conductivity compared to gold reduces the accuracy of measurements. ITO is stable in physiological medium and poses no threat to the cultured cells [181]. ITO electrodes are optically transparent, polarizable and highly sensitive due to the absence of insulating oxide layers [182].

The gold electrodes are fabricated by E-beam evaporation of chromium and gold on glass slides with a thickness of 10nm and 100nm, respectively. Following evaporation, the metal layer is patterned with photolithography and etched using gold and chromium etchants (Transene, Danvers, MA). To form the transparent electrodes, ITO film (100 nm) is deposited on clean glass slides at 10^{-5} torr by E-beam evaporation targeting ITO alloy pieces of 90% In_2O_3 and 10% SnO_2 composition (Kurt Lesker, Jefferson Hills, PA) in a graphite crucible (International Advanced

Materials, NY). Evaporation is performed with no substrate heating in an oxygen-rich environment with 20 sccm O₂ flow rate to make for oxygen loss during evaporation and improve the integrity of the deposited oxide layer. A 100 nm thick ITO layer was deposited with a rate of 0.5 nm/sec. After deposition the glass slides were photolithographically patterned and chemically etched with ITO etchant (Transene, Danvers, MA). The as-deposited ITO film does not have the optimal electrical and optical properties. Hence a following annealing step is needed to increase the electrical conductivity and transparency of the film [183]. Annealing is performed in furnace at 400 deg C in air for 30 minutes. It is important to pattern the ITO film before annealing since the etch rate will decrease significantly afterwards. Regardless of the electrode material, the fabricated glass and PDMS parts are cleaned with Isopropyl alcohol and DI water and irreversibly bonded together using air plasma. After bonding the parts were pressed together and heated to 70 deg C for 10 minutes. Two stainless steel needles with 0.13 mm diameter (Kingli, China) were inserted into the two ports of the culture channel for applying the pulses. The assembled device is kept in vacuum before the loading step. The fabrication steps are summarized in Figure 3-4.

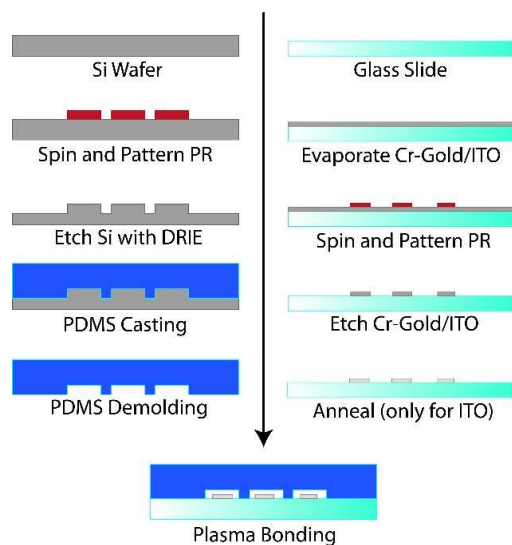


Figure 3-4. Fabrication steps of the sensor embedded microfluidic chip. The left side shows the steps to make the microfluidic part and the right side shows fabrication of the impedance sensor using either gold or ITO.

3.3. Cell culture on chip

In this study two different cell lines, bEnd3 (ATCC, Manassas, VA) and hCMEC (EMD Millipore, Billerica, MA) -from mouse and human origins, respectively - were used to construct the endothelial cell monolayer inside the chip, and their responses were compared upon application of the PEFs. Standard cell culture protocols were followed to culture both cell lines. Cells were cultured in culture flasks and maintained in complete media at 37 deg C in a water jacket incubator and were routinely passaged at 90% confluence. The complete culture medium for bEnd3 cells consisted of DMEM (ATCC) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% (v/v) penicillin-streptomycin (Life Technologies). The complete culture medium for hCMEC cells consisted of EndoGRO basal medium supplemented with EndoGRO-LS supplement (0.2%), rh EGF (5ng/ml), L-Glutamine (10mM), Hydrocortisone Hemisuccinate (1µg/ml), Heparin sulfate (0.75 U/ml), Ascorbic acid (50 µg/ml) and FBS (5%), all from EMD Millipore. The cell culture protocol on chip is similar to an earlier study [174]. Briefly, the microfluidic channel was sterilized with 70% ethanol, washed with PBS and treated with 50 µg/ml human plasma fibronectin (Cultrex) in PBS for one hour. The fibronectin solution was then removed and replaced with complete cell culture medium for another hour in the incubator. The cell suspension was then introduced into the culture channel at a concentration of 20 million cells/ml. The device was incubated for 1-2 hours to let the cells adhere to the bottom of the channel. After 2 hours the outlet of the culture channel was blocked, forcing the media to flow through the resistive channel to the outlet, maintaining a slow, gravity induced flow over the cells for the duration of cell proliferation. That provided a continuous supply of fresh media for about 12 hours without the need for a pump. Once the cells were confluent the resistive channel was blocked and continuous media flow was started through the culture channel using a peristaltic

pump (Watson Marlow, UK). The pump flow rate was adjusted to provide the physiological shear stress according to [184] (see Appendix B):

$$\tau_w = \frac{6\mu Q}{wh^2} \quad (3-2)$$

where τ_w is the wall shear stress, Q is the volumetric flow rate, w is the culture channel width and h is the culture channel height. Figure 3-5 shows the three steps for providing media in the chip during cell seeding, growth and perfusion.

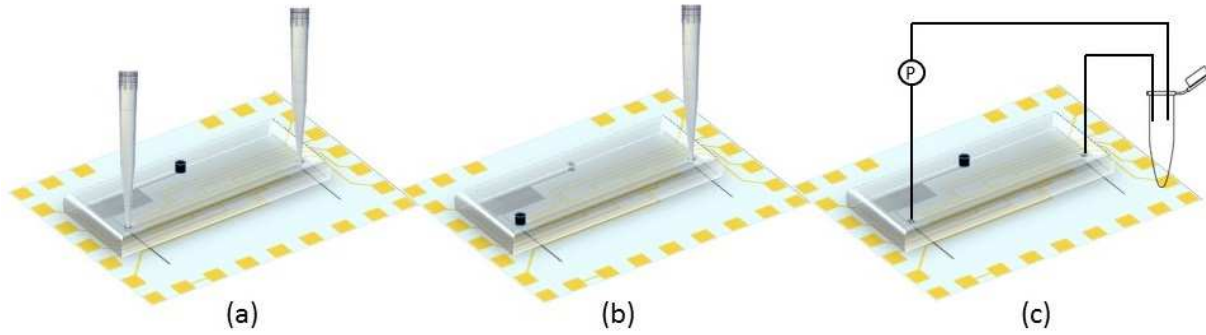


Figure 3-5. Three steps of providing media to the chip (a) static media during cell adhesion (b) low flow rate gravity perfusion during cell growth (c) media circulation with high flow rate using the peristaltic pump. The black spot represents the blocked port.

3.4. Electrical impedance spectroscopy

A bipolar impedance measurement technique is used which means current injection and voltage pickup are performed through the same pair of electrodes, which constitute the interdigitated electrodes. A printed circuit board (PCB) is designed as an interface to make the electrical connections with the microfluidic chip as shown in Figure 3-6. A rectangular portion is cut from the center of the interface board which houses the microfluidic device. Conductive silicon paste (Silicone solutions, Cat# SS-27S) is used to facilitate electrical connections between the gold/ITO pads on the chip and the terminals on the back of the PCB board.

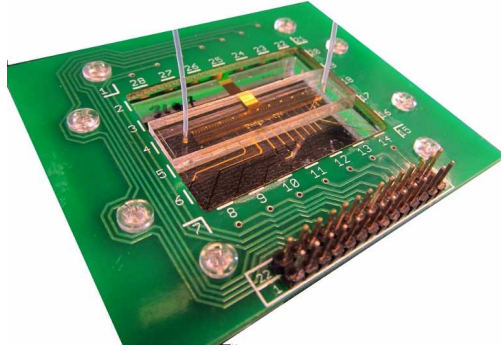


Figure 3-6. Microfluidic interface board is used to make the electrical connections with the contact pads on the microfluidic device.

A multiplexer is designed and fabricated to facilitate impedance measurements between different pairs of impedance sensors on the same chip (Appendix C). The multiplexer is controlled through the impedance analyzer. At each point along the channel the impedance spectrum (containing both phase and magnitude) is measured in a wide frequency range (0.1Hz-1MHz). The impedance spectrum contains useful information about the cell monolayer including, cell membrane capacitance, intracellular resistance, and monolayer integrity. These information could be extracted from the spectrum by fitting the data to an equivalent circuit model of the system including the electrodes, the cells and the medium inside the channel. Depending on the system under experiment, electrode material and geometry, and medium composition, different circuit models could be used for this purpose (Figure 3-7).

Gold and ITO are naturally polarizable, meaning that they do not allow any electron transfer to happen at the electrode/electrolyte interface. That causes the electrode/electrolyte interface to act as a capacitor (double layer capacitance [185]). The media inside the channel acts as a resistor which is in series with the double layer capacitor. The electrodes in combination with the medium also form a capacitor which is in parallel with the medium. That results in the electric circuit model of Figure 3-7a. the double layer capacitance C_{dl} dominates the impedance spectrum at low

frequency ($< \sim 1\text{kHz}$) and does not allow other elements in the system to show up in those frequencies. There are some methods that enable impedance measurements at low frequency, which are listed below:

Tetra-polar measurement. Having the excitation current in a separate pair of electrodes than the voltage pickup, eliminates polarization at the pickup electrodes, removing the double layer capacitance and allows measurement at low frequency.

Non-polarizable electrodes such as Ag/AgCl allow electron transfer with the electrolyte, bypassing the double layer capacitance.

Reduction/Oxidation agent. A redox agent dissolved in the electrolyte can facilitate electrode transfer with the polarizable electrode such as gold, again bypassing the double layer capacitance and reducing the impedance of the electrode/electrolyte interface. A common example of the redox probe is potassium hexacyanoferrate (II) and (III) (Sigma Aldrich). Figure 3-7b shows the circuit model for the case of electron transfer with the electrolyte. Electron transfer happens in another pathway in parallel with the double layer capacitance at the interface of the electrode. That pathway is comprised of a charge transfer resistance R_{CT} in series with a diffusion impedance Z_D . The charge transfer resistance is related to the rate of transfer of electrons between the electrode and the ions in the electrolyte. As we will see later this resistance is inversely proportional to the concentration of redox probe in the electrolyte. In most of the measurement systems, the geometric capacitance is very small, leading to its high impedance and is removed from the circuit. That simplified model is called the Randles model [186]. Several empirical models exist for the diffusion impedance including the Warburg models and the Gerischer model. Refer to Appendix D for more details about these impedance models. In all the above circuit models the capacitance is replaced with the

constant phase element (CPE), which accounts for the nonlinearities and frequency dependence of the interface [187].

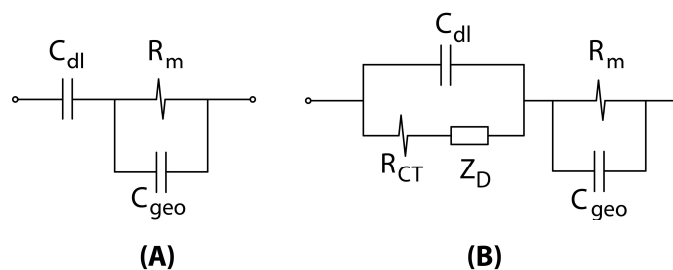


Figure 3-7. Electric circuit models (A) bare electrode in regular media (B) bare electrode in media containing redox probe. The circuit elements are: double layer capacitance (C_{dl}), medium resistance (R_m), geometric capacitance (C_{geo}), Charge transfer resistance (R_{CT}), Diffusion impedance (Z_D)

3.5. Impedance sensor characterization

In order to be able to use the fabricated impedance sensors for characterizing the cell monolayer, it is essential to first characterize the sensors in the absence of the cells. In this section we presented the readouts of the impedance sensors and investigated the effects of different conditions such as electrode material (gold/ITO) media conductivity, redox probe concentration, flow rate and elapsed time on the impedance spectra. Figure 3-8 shows the schematic of an impedance spectrum measured in a conductive medium and identifies the factors which have the most influence over specific parts of it.

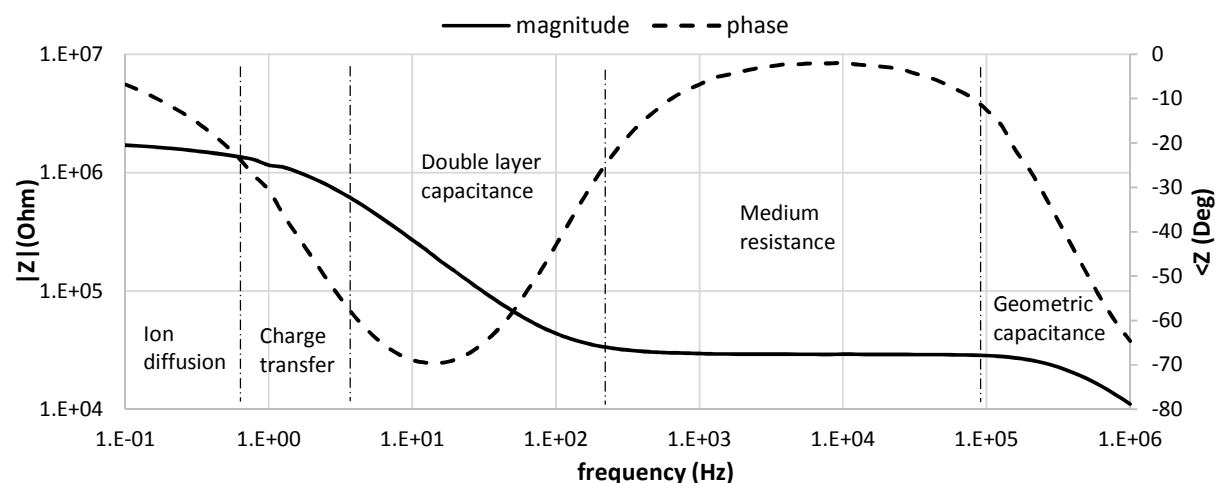


Figure 3-8. Different sections of the impedance spectrum for readings in a conductive medium containing redox agent that facilitates charge transfer across the electrode-electrolyte interface.

3.5.1. Electrode material

The electrode material makes a significant contribution to the impedance spectrum due to its electrical conductivity and charge transfer capability. Figure 3-9 and Figure 3-10 compare the acquired impedance magnitude and phase, respectively for gold and ITO electrodes in cell culture media. The electrodes have the same geometry for both materials. Therefore the observed difference is only due to the material.

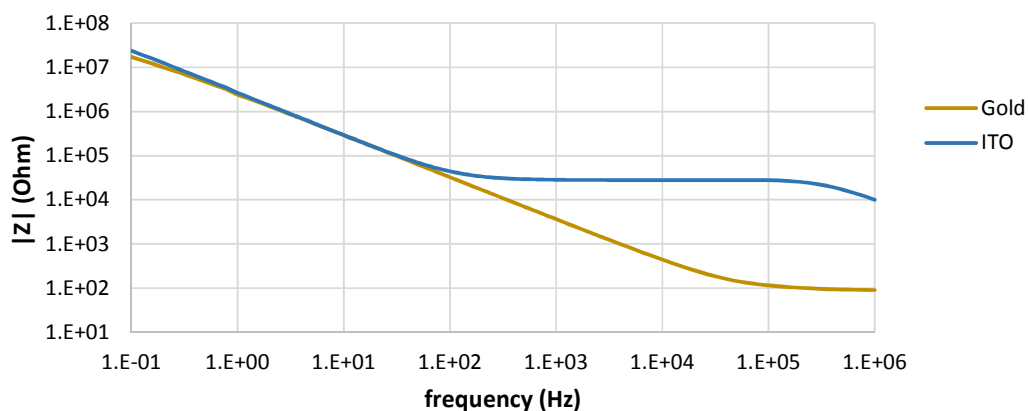


Figure 3-9. Comparison of impedance magnitude for gold and ITO in cell culture media

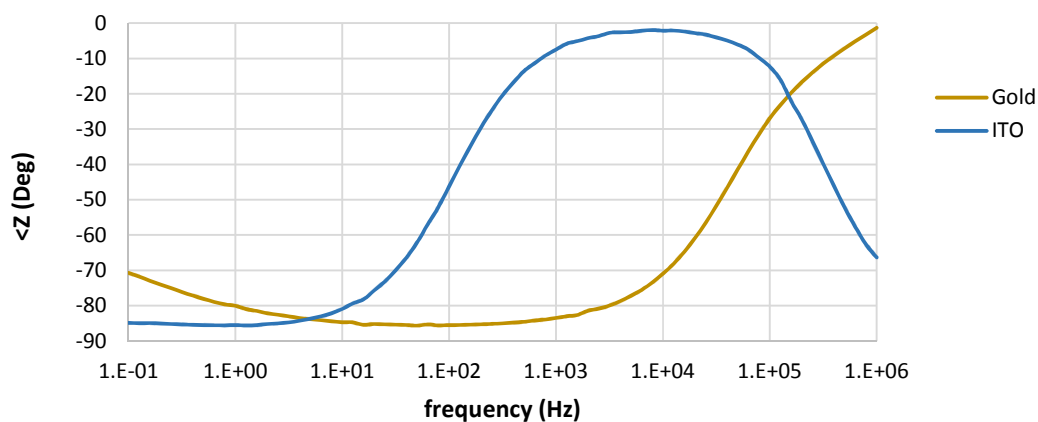


Figure 3-10. Comparison of impedance phase for gold and ITO in cell culture media

Three main differences could be observed in the impedance spectra for gold and ITO: *First*, at very low frequency the phase diagram of the gold electrode has a higher value than that of the ITO (-70° compared to -90° at 0.1Hz). That is probably due to some charge transfer that happens for gold even without a redox probe, but not for ITO. *Second*: the flat part of the impedance magnitude curve which corresponds to the resistive behavior of the sensor and electrolyte, has a higher value for ITO compared to gold. Since the same medium is used in both cases, the higher impedance is attributed to the lower electrical conductivity of ITO ($16e4$ S/m) compared to gold ($44e6$ S/m). *Third*: at very high frequency (>10 kHz) the ITO sensor tends toward capacitive behavior while

the gold sensor remains completely resistive. This behavior is discerned by looking at the decreasing trend in the spectra of the ITO electrode at high frequency and is due to the geometric capacitance of the sensor/medium combination, which is considered in circuit model of Figure 3-7a. The fact that this capacitance is absent in the impedance spectrum of gold electrode is due to its small R_{media} which does not allow for the high impedance of C_{geo} to show up.

3.5.2. Effect of electrolyte conductivity

The electrolyte conductivity has the same effect on impedance spectrum as the electrode conductivity as they are both considered in the same element of the circuit model.

3.5.3. Effect of redox probe concentrations

As mentioned before the redox probe enables charge transfer across the electrode/electrolyte interface. To find the proper amount, different concentrations of the redox pair $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (1:1) in cell culture media were introduced into the microfluidic channel and the impedance spectra were obtained. It should be noted that previous studies have used this substance in cell culture with concentrations as high as 5mM [164]. In the presence of redox probe, the circuit model of the system should be modified to account for the charge transfer capability of the electrode (Figure 3-7b). This model contains a second pathway across the electrode/electrolyte interface, in parallel with the double layer capacitance, for this purpose. This pathway contains a resistor for charge transfer, R_{CT} , and a frequency dependent impedance for diffusion of ions between the medium and the electrode surface, Z_{D} .

Increasing the redox concentration results in more resistive characteristics at the low frequency part of the spectra as shown in Figure 3-11 and Figure 3-12 for gold electrodes.

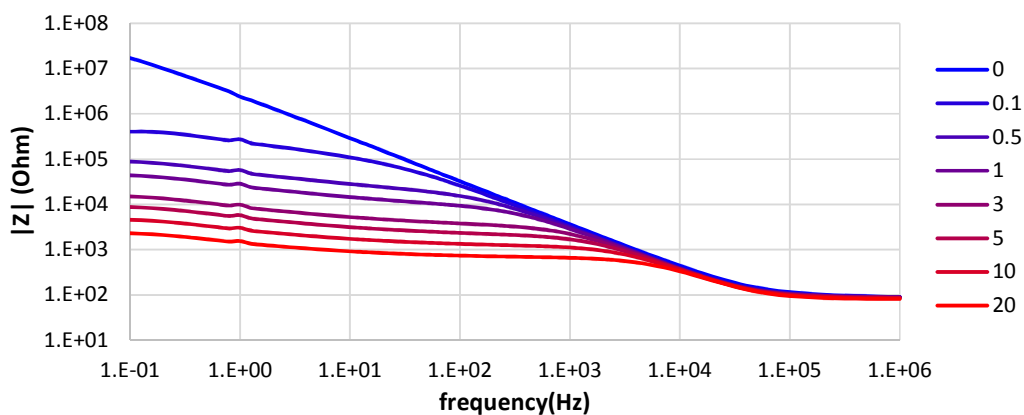


Figure 3-11. Effect of redox probe concentration (molar) on shifting the impedance magnitude for gold electrodes

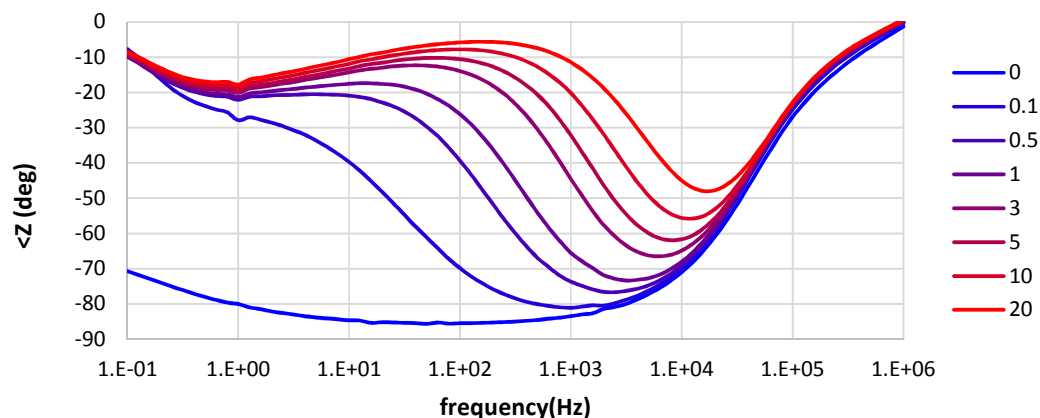


Figure 3-12. Effect of redox probe concentration (molar) on shifting the impedance phase for gold electrodes

As shown by the impedance phase spectrum (Figure 3-12) even a small amount of the redox probe has a significant effect on enhancing the charge transfer capability of the sensor. For the majority of our experiments we used 1mM redox probe, which is well tolerated by the cells.

To gain a more quantitative understanding of the sensor behavior in presence of the redox probe, the impedance spectra for gold and ITO electrodes in the presence of different concentrations of redox probe are fitted to the equivalent circuit model of Figure 3-7b. The Gerischer model is

considered for the diffusion impedance Z_D as it gives better fitting compared to the Warburg impedance.

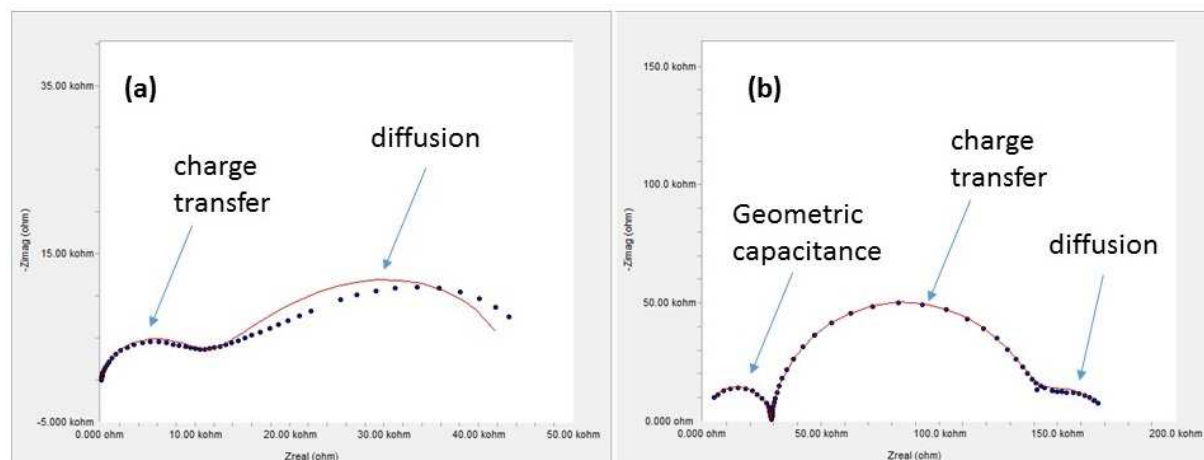


Figure 3-13. Nyquist plots of fitting of the circuit model to the impedance data acquired in the presence of 1mM redox probe for (a) gold electrodes (b) ITO electrodes. The arrows indicate what phenomena each of the half circles are corresponding to.

Figure 3-13 shows the Nyquist plots of impedance spectra for gold and ITO electrodes in the presence of 1mM redox probe in cell culture media along with the best fit of the impedance model. Both data from gold and ITO electrodes fit very well to the model. The slight deviation of the gold electrode data from the model at low frequency (diffusion section) may be attenuated by using a generalized Gerischer model which was not available in the software (Gamry EChem Analyst). The charge transfer resistances for gold and ITO are extracted from the impedance fitting and compared in Figure 3-14. Higher order regression analysis shows that for both of the electrode materials, the charge transfer resistance is inversely proportional to the redox probe concentration according to the following equations:

$$\text{Gold: } R_{CT} = \frac{12457}{[S]} \quad R^2=0.9895$$

$$\text{ITO: } R_{CT} = \frac{136847}{[S]} \quad R^2=0.9904$$

where $[S]$ is the concentration of the redox probe in mM.

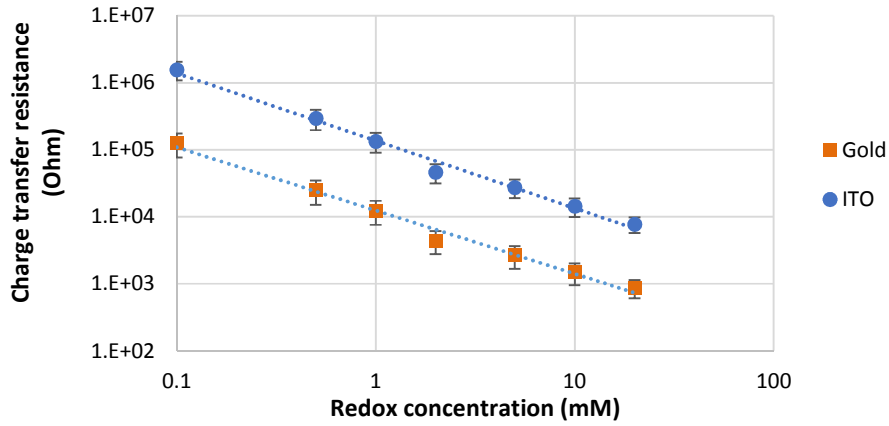


Figure 3-14. Comparison of R_{CT} for gold and ITO as a function of redox probe concentration

The obtained relationships for the charge transfer resistance is consistent with the available equation which describes this element analytically [188]:

$$R_{CT} = \frac{RT}{n^2 F^2 A k_{CT} [S]} \quad (3-3)$$

where R is the ideal gas constant, T is the absolute temperature, n is the number of transferred electrons per one molecule of the redox probe, F is the Faraday constant, A is geometric surface area of the electrode (cm^2), k_{CT} is the potential dependent charge transfer rate constant, and $[S]$ is concentration of the redox probe (mol/cm^3).

3.5.4. Effect of fluid flow over the electrodes

Due to the presence of fluid flow over the cell monolayer, it is critical to see how the measured impedance could be affected by the flow of the media. Usually mechanical motion does not affect electrical behavior of a system, mainly due to the fact that electron transfer happens at a much higher rate than such motions. However, that is not the case in fluids. Ions play the role of charge carriers in a fluid, instead of electrons. Ion movement in fluids happens orders of magnitude slower

than electron transfer in solid conductive materials. Therefore, fluid flow can significantly affect ion movements. According to equivalent circuit model of the electrode/electrolyte system in the presence of the redox probe (Figure 3-7b) and as shown in the schematic diagram of Figure 3-8, at low frequencies the impedance is dominated by the diffusion impedance Z_D .

Here we used the porous bounded Warburg model (or the “O” circuit element) for the diffusion impedance, Z_D as it gives a more physical interpretation of the diffusion impedance. Fitting of the impedance data shows that increasing the flow rate consistently reduces the Warburg time constant, B (Figure 3-16). This parameter is related to the Nernst diffusion layer thickness, δ (cm), and the diffusion coefficient of the ions, D (cm²/s), according to:

$$B = \frac{\delta}{\sqrt{D}} \quad (3-4)$$

The decreasing trend of the B parameter by increasing the flow rate is consistent with the decreasing thickness of the diffusion layer.

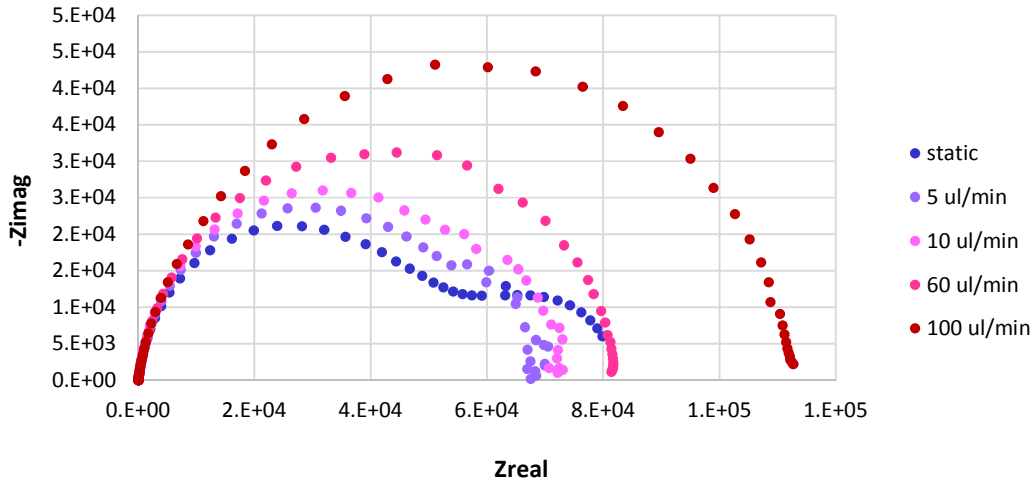


Figure 3-15. Nyquist plot of the impedance spectra of the electrode-electrolyte system for different flow rates inside the micro-channel. Cell culture medium with 1mM redox probe was used.

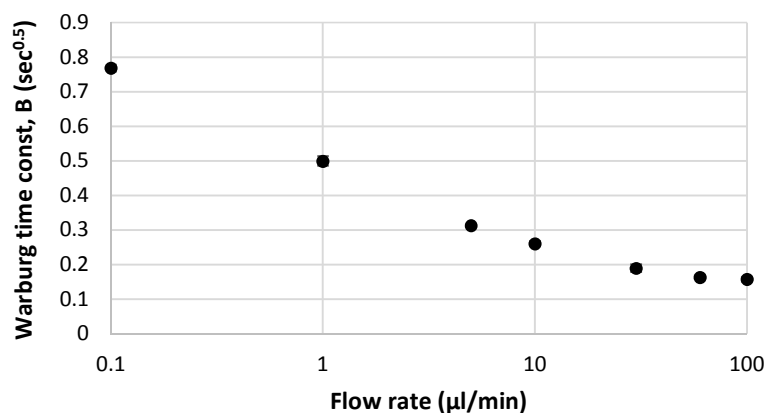


Figure 3-16. Variation of Warburg time constant, B , as a function of flow rate

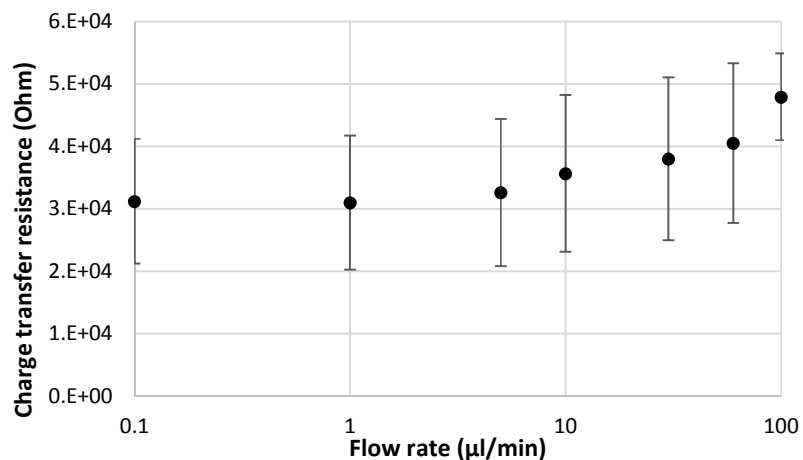


Figure 3-17. Variation of charge transfer resistance, R_{CT} as a function of flow rate

As shown about the diffusion impedance was significantly affected by the flow rate, reducing the Warburg time constant (B) from $0.77 \text{ sec}^{0.5}$ to $0.16 \text{ sec}^{0.5}$ by increasing the flow rate from zero to 100 μl/min . However the charge transfer resistance R_{CT} did not change statistically by the flow rate. That is in agreement with the fact that charge transfer resistance is affected by electrode-electrolyte interactions which are not a function of flow rate. This behavior should be considered when performing the impedance measurement in the presence of flow.

3.6. Impedance spectroscopy of endothelial cell monolayer on gold electrodes

After seeding into the microchannel, the cells were provided with complete cell culture media, slowly perfused over the cells as provided by the gravity perfusion mechanism on chip, and allowed to grow for few days until confluence. Depending upon the seeding concentration (about 20 million cells per ml) and the cell type, that may take from 2 to 5 days (Figure 3-18). The adhesion and growth of the cells on top of the electrodes are reflected in the acquired impedance from the electrodes. During the growth phase, the electrodes are covered by an increasing number of cells which causes the impedance spectrum to undergo three major changes (Figure 3-19):

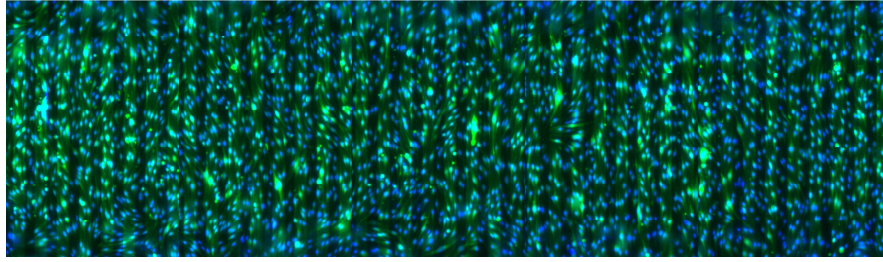


Figure 3-18. Fluorescent image of confluent bEnd3 cell monolayer cultured in the microdevice over the gold electrode array. The cells are stained using calcein AM for the cytoplasm and NucBlue for the nuclei. In order to take the fluorescent image, the device was positioned upside down on the microscope stage, avoiding the excitation light to be blocked by the opaque gold electrodes.

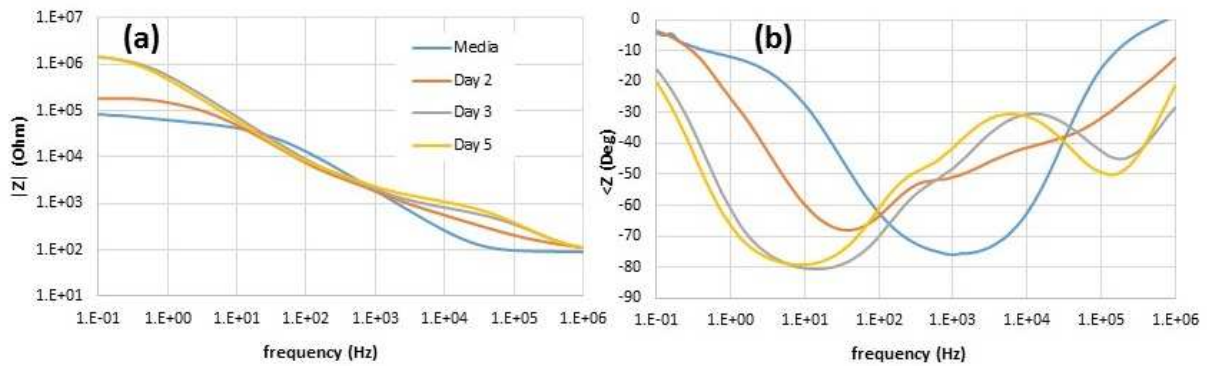


Figure 3-19. Impedance spectra of growing bEnd3 cells over gold electrodes in the presence of 1mM redox probe. (a) Magnitude (b) Phase

First: the low frequency part of the spectrum shifts toward higher magnitudes. This could be attributed to the attachment of cells to the electrode surface and increasing the charge transfer resistance (R_{CT}). It should be mentioned that this change is only visible in the presence of the redox probe.

Second: Another dispersion evolves at the frequency range of 1kHz-1MHz as visible in the impedance magnitude diagram. This dispersion is due to the impedance of the attached cell layer which is commonly modeled by a parallel combination of paracellular and transcellular impedances (Figure 3-20). The paracellular pathway is through the tight junctions and has resistive behavior (R_{TJ}). The transcellular pathway is through the cell membrane and the cytoplasm. Therefore, the transcellular impedance is a series combination of membrane capacitance (C_{mem}) and cytoplasmic resistance (R_{cyt}). The specific capacitance of the cell membrane is approximately $1 \mu F.cm^{-2}$ [189].

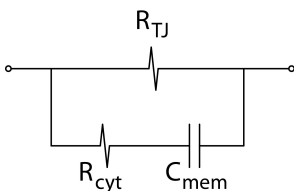


Figure 3-20. Impedance model of cell monolayer

Third: Another small dispersion also starts to form in the frequency range 100Hz-1kHz, which is more discernible in the phase diagram. It should be mentioned that this dispersion has not been detected in all endothelial cultures and seems to appear under specific growth conditions which will be discussed in what follows. It is also only detectable if the impedance measurement is performed in the presence of the redox probe. To the best of our knowledge this feature of the impedance spectrum has not been discussed in the literature, probably because in most of the studies the impedance measurements are performed in regular culture medium with no redox probe

supplement, which does not allow charge transfer with the electrode and results in a large interfacial impedance that masks this small dispersion.

Using a polarizable electrode (no redox probe and no charge transfer) the cell monolayer is commonly modeled as Figure 3-21, which is the series combination of the electrode-electrolyte impedance (Figure 3-7a) (when ignoring the geometric capacitance) and the cell monolayer impedance (Figure 3-20). A typical impedance spectrum corresponding to this model is also shown in Figure 3-22.

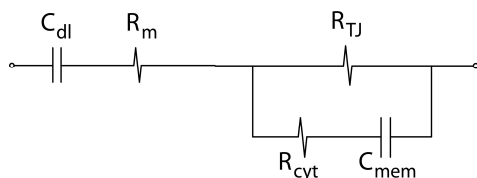


Figure 3-21. Electric circuit model of electrodes with adhered cell monolayer in a conductive medium with no redox probe.

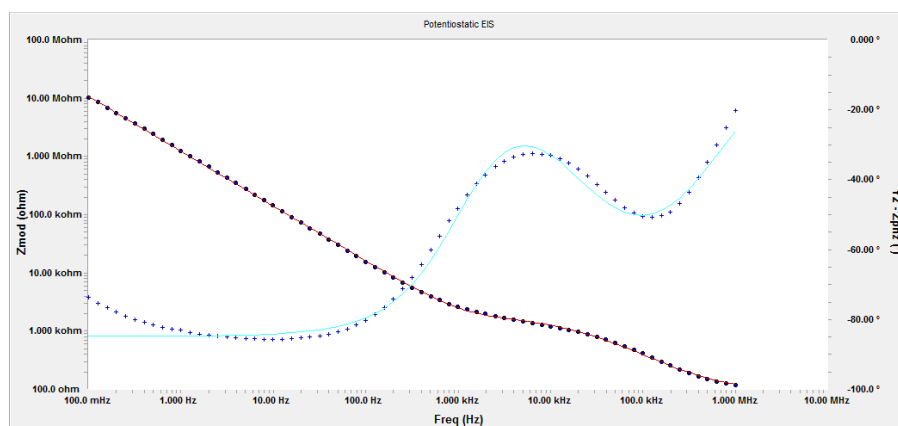


Figure 3-22. Typical impedance spectrum of confluent endothelial layer in the absence of charge transfer across the electrode and the overlaying fit to the impedance model of Figure 3-27. Fitting was performed using the Gamry Echem Analyst software. The small discrepancy in the fitting of the phase diagram at very low frequency is attributed to the small charge transfer that happens at the electrode surface even in the absence of charge transfer probes.

The addition of the redox probe to the media unveils more details about the cell impedance at low frequency which could be attributed to the morphology of the cell monolayer. By doing that, two

different types of spectra were observed for the cultured bEnd3 cells based on the appearance of the third dispersion at frequency range 100Hz-1kHz. This difference in spectra was found to be correlated with the morphology of the growing monolayer which is highly affected by the initial cell seeding density. In the following, this issue is discussed in more details:

- High density seeding

Introducing a large number of cells into the channel during the seeding step, results in the coverage of the entire surface of the channel with the cells at the beginning, leaving no more space for the cells to grow, divide and proliferate. Therefore, a tight monolayer of cells is produced within few hours. Our observations using light microscopy has shown that in such a monolayer the cells are relatively small, and interestingly aligned with the electrodes. This behavior happens regardless of the electrode material.

- Low density seeding

By introducing a low density of cells into the channel during the seeding step, the cells get a chance to spread out on the surface upon adherence and still leaving enough space for them to divide and proliferate. This way the cells grow naturally, are relatively larger in surface area and do not seem to follow the direction of the electrodes (Figure 3-23).

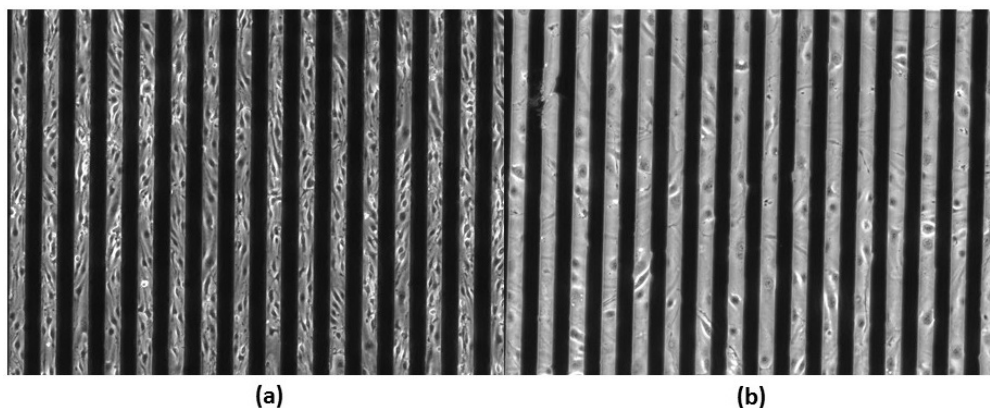


Figure 3-23. Confluent monolayer of bEnd3 cells over the interdigitated gold electrodes (a) high seeding density (b) low seeding density

Apart from the visual appearance, our experiments showed that the cells which grow from a low density population are more resilient to shear stress and last longer in the chip (data not shown here). Here we also explain the important difference in the impedance spectra of the cell monolayers under the two conditions as pointed out before. The impedance measurements for both cases are taken in the presence of 10mM redox probe to widen the range of useful frequency. It is found that for the high density seeding, only one dispersion happens after vanishing the interfacial electrode effects. However, for the low density seeding two dispersions exist in the spectrum after vanishing the interfacial electrode effects. The spectra for two cases are similar at very low and very high frequencies (Figure 3-28).

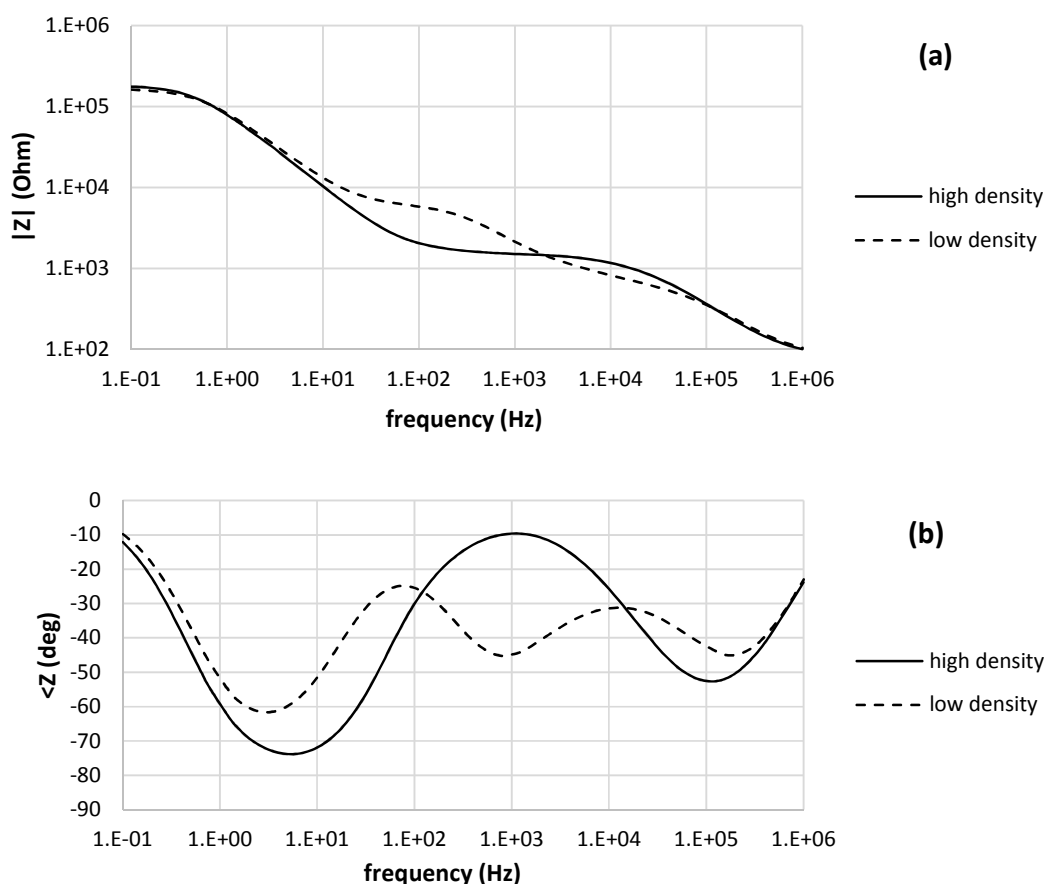


Figure 3-24. Comparison of the impedance spectra of confluent bEnad3 cells under two different seeding conditions (a) impedance magnitude (b) impedance phase, in the presence of 10mM redox probe.

3.6.1. High density seeding

For the case of high-density seeded cells, the equivalent circuit model is basically the series combination of the electrode interface, medium, and the cell layer as shown in Figure 3-25. This model gives an accurate fit to the impedance spectrum as shown in Figure 3-26.

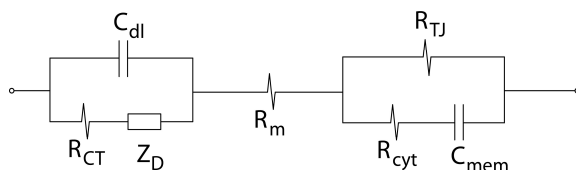


Figure 3-25. Equivalent circuit model for confluent monolayer of cells seeded with high density in the presence of redox probe

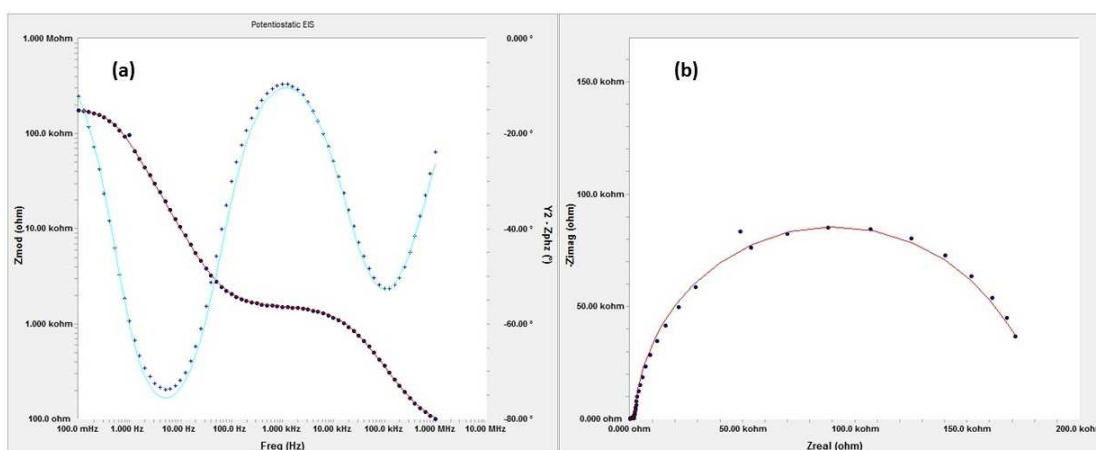


Figure 3-26. Fitting of the equivalent circuit model of Figure 3-25 to the impedance spectrum of confluent monolayer of bEnad3 cells over the interdigitated gold electrodes obtained from a high density seeding. (a) Bode plot (b) Nyquist plot of impedance spectrum

There are several important notes about the fitting of circuit models to impedance data that should always be considered. *First*, it should be noted that for an existing set of impedance data there are always infinite irrelevant circuit models that can provide an accurate fit. Therefore, the goodness of fit is not a sufficient criteria for validity of a model and proper combination of the elements should first be considered. This topic is discussed in more details in reference [190]. *Second*, the equivalent circuit model should be simplified as much as possible to result in a unique set of values

for the elements. For example in the case of circuit of Figure 3-25 the current combination of three resistors R_m , R_{TJ} , R_{cyt} , does not provide a unique solution to the fitting. Therefore, it is either suggested to remove the R_m from the circuit due to its constant value over the experiments, or to run another experiment with the bare electrodes and the same medium to find the exact value for R_m by fitting to the model of Figure 3-7b, and implement that as a constant in the model with the cells. Doing so, resulted in the media resistance of 80 Ohm.

3.6.2. Low density seeding

In order to get a better picture of the mid-frequency dispersion for low-density seeded cells, we performed impedance measurements of the confluent endothelial monolayer in the presence of a range of redox probe concentrations. (Figure 3-27)

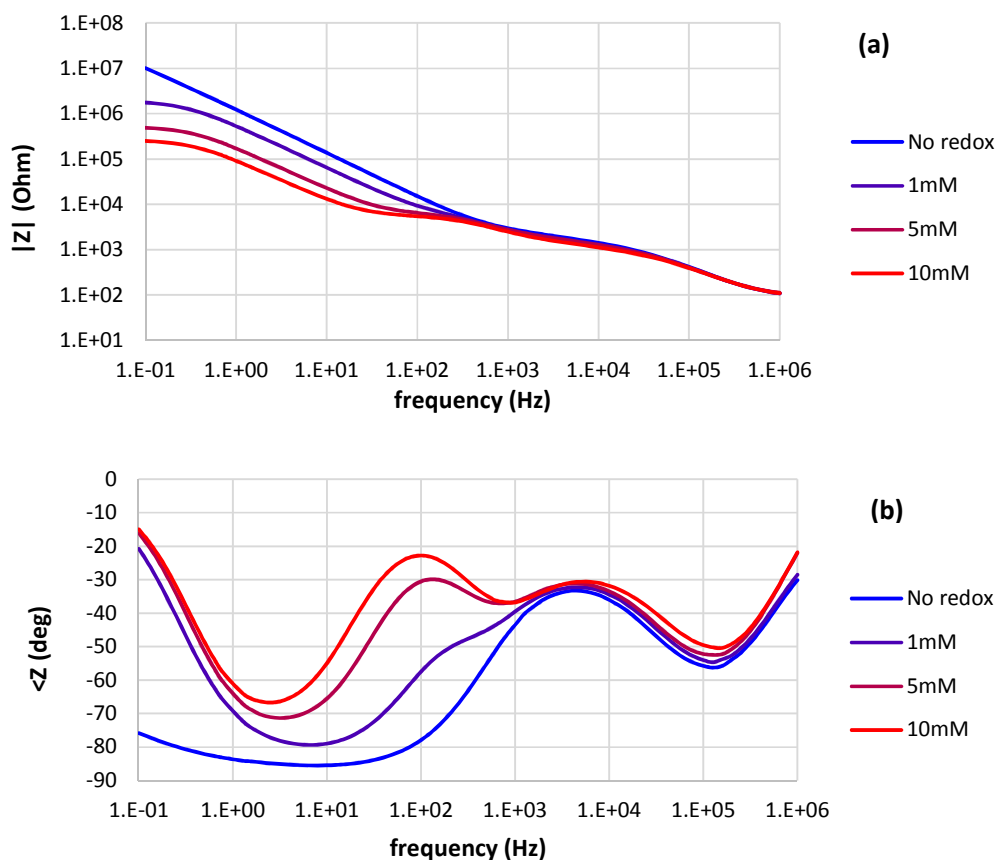


Figure 3-27. (a) Magnitude (b) Phase of impedance spectra for confluent monolayer of bEnd3 cells at day 3 in the presence of different concentrations of redox probe.

It is shown that by increasing the redox probe concentration and consequent decrease in the charge transfer resistance, more details from the impedance of the cell monolayer is revealed and the mid frequency dispersion becomes more pronounced.

Usually, the number of capacitors in the model corresponds to the number of dispersions in the spectrum. The two existing capacitors in the model, C_{dl} and C_{mem} , cause the two dispersions at low and high frequency, respectively. In order to find the proper model for the low-density seeded cells, one should note that the additional dispersion at mid frequency requires implementation of a third capacitor somewhere in the circuit model. To start, we know the complete model should contain blocks of elements which represent the electrode-electrolyte system and the cell monolayer, i.e. the Randles model and the cell model of Figure 3-20, respectively. Now the question becomes how to connect these blocks with the addition of any further elements. Applying all the requirements, considering the morphology of the cell monolayer and trying to optimize the fitting, the following circuit model was obtained.

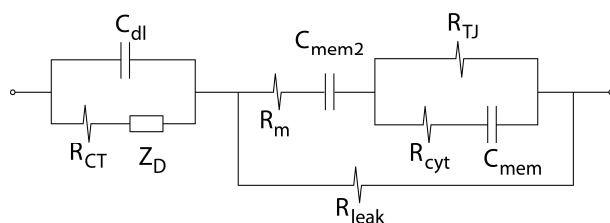


Figure 3-28. Equivalent circuit model for confluent monolayer of cells seeded with low density in the presence of redox probe.

Compared to the model of Figure 3-25, the proposed model has the following modifications:

- An additional capacitance C_{mem2} is considered to account for the cell-electrode interaction.

- After passing through the electrode-electrolyte interface another resistive pathway is considered in parallel with the cells that accounts for the small gap between the cells and the electrodes. This gap is caused by the extracellular matrix beneath the cells.

The fact that this resistive pathway does not exist in the cell monolayer after high density seeding needs to be further studied and is out of the scope of this work. However, one possibility is to correlate that to the difference between the thickness of the adsorbed fibronectin layer in the chip before cell seeding and the thickness of the secreted extracellular matrix from the cells during the growth and proliferation step. In the case of high density seeding, all the cells attach to the very thin layer of adsorbed fibronectin that was introduced during the device preparation step. However, in the case of low density seeding, the initially adhered cells attach to the existing adsorbed fibronectin layer. During proliferation the cells secrete their own extracellular matrix that is thicker than the existing fibronectin. Therefore, an additional pathway is created below the cells which corresponds to R_{leak} .

This model provides a perfect fit to the impedance spectrum of confluent cell layer over the interdigitated gold electrodes obtained from a low density seeding. (Figure 3-29)

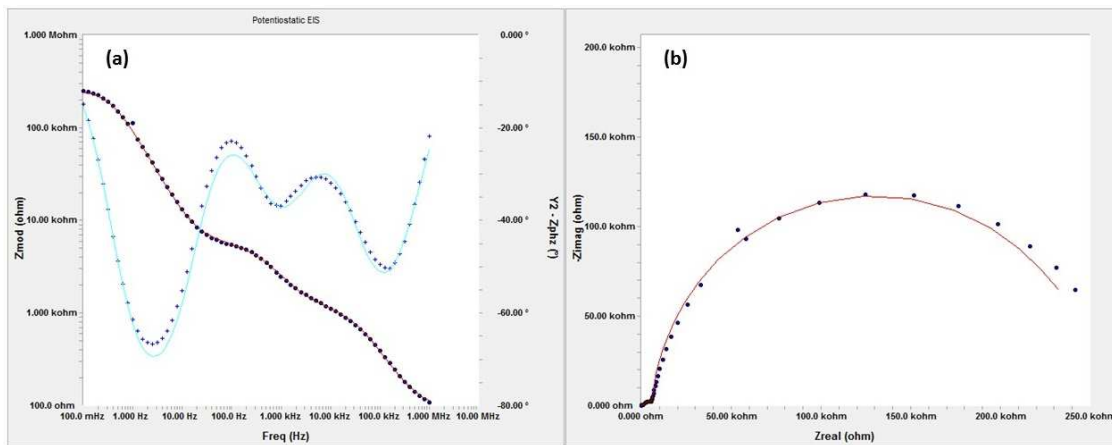


Figure 3-29. Fitting of the equivalent circuit model of Figure 3-28 to the impedance spectrum of confluent monolayer of bEnad3 cells over the interdigitated gold electrodes obtained from a low density seeding. (a) Bode plot (b) Nyquist plot of impedance spectrum

3.1. Impedance spectroscopy of endothelial cell monolayer on ITO electrodes

Due to its high resistivity, the impedance spectra obtained from ITO electrodes are higher than the ones obtained from gold electrodes. An example of a comparison between the two materials under same conditions was provided in Figure 3-9. The significantly higher value of the base of the impedance curve for ITO compared to gold (i.e. 30 kOhm vs. 90 Ohm) causes the impedance of the cell layer to be masked by the high impedance of the electrodes. Therefore, an accurate measurement of the cells is not possible. One way to resolve this issue is to decrease the resistance of the ITO electrodes by increasing their size. Therefore another impedance sensor was fabricated with wide electrodes and a common counter electrode as shown in Figure 3-30. The impedance sensor is used in conjunction with the tapered channel.

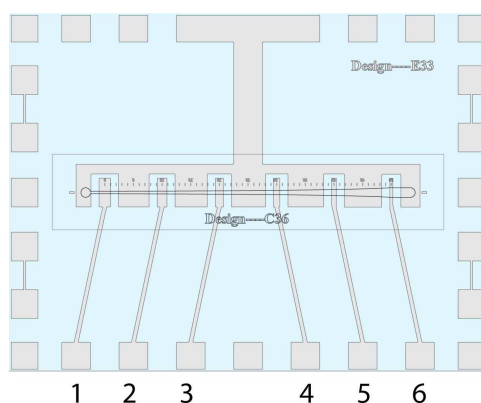


Figure 3-30. Schematic of the ITO impedance sensor with wide working electrodes and a common counter electrode.

bEnd3 cells were grown inside the device with an initial low density to give a partial coverage, and the impedance was monitored during 2 days (Figure 3-31). It is shown that the impedance spectrum is mostly affected by the cell culture at the low frequency range.

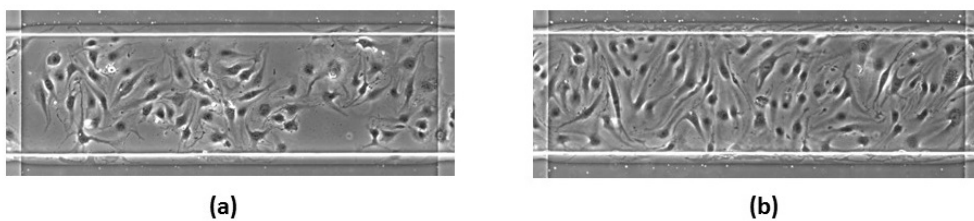


Figure 3-31. Phase contrast images of bEnd3 cells growing on ITO electrodes on chip (a) first day after seeding (b) second day. The impedance spectra for the same cells and the cell-free chip are shown in Figure 3-32

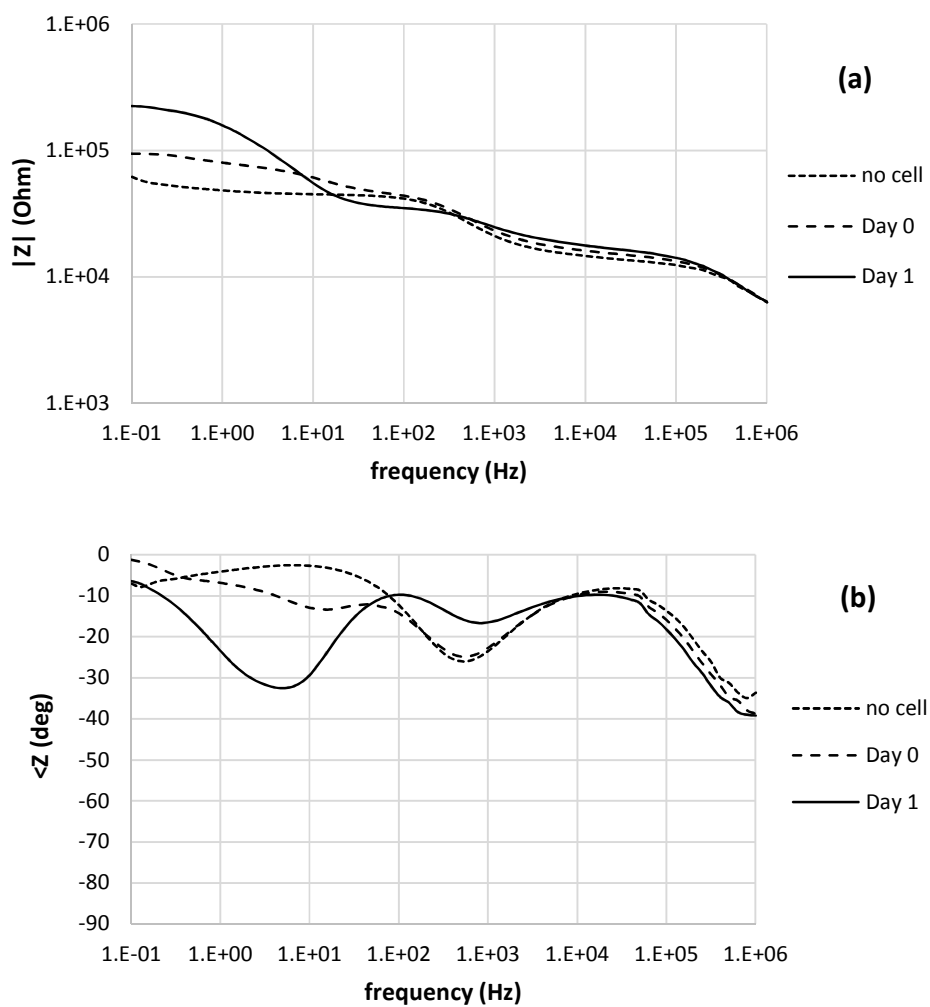


Figure 3-32. Impedance spectra of bEnd3 cell culture on ITO electrodes at different time steps in the presence of 10mM redox probe (a) impedance magnitude (b) Impedance phase

3.2. Osmotic disruption of the endothelial barrier measured visually and electrically

In order to have an idea of how the disruption of the cell monolayer would affect the impedance spectrum, the cell monolayer was osmotically disrupted and the effect of disruption on the impedance spectrum was observed. Mannitol was used for this purpose. Mannitol results in osmotic imbalance between the cells and the medium, causing an outflow of water from the cells and cell shrinkage, which results in opening of the paracellular space. Mannitol is being clinically used at concentration of 1.6M to open the BBB for drug delivery [62]. For *in vitro* studies mannitol concentrations of 0.1M [191] or 0.3 M [94] have been used. In order to have an estimate of the effect of mannitol on the disruption of the cell layer, different concentrations of mannitol were injected into separate microchannels with confluent bEnd3 cells and their behavior were monitored optically over 4 hours after injection (Figure 3-33). It was found that 0.1M mannitol did not cause any detectable change in the cell shape over the course of 4 hours. However, 1M mannitol caused the cells to round up within few minutes and detach from the surface. 0.3M resulted in deformations and round up in a fraction of the cells over time but leaving the majority of the cells intact.

According to the results obtained from different concentrations of mannitol, 0.3M was chosen to try on the cell monolayer during impedance spectroscopy. 10mM redox probe was used during impedance spectroscopy to enable capturing data in a wider frequency range. After injection of 0.3M mannitol solution into the channel, the impedance was recorded for a duration of 4 hours. As shown in Figure 3-34 it was found that the impedance changed rapidly during the first 90 minutes and reached the final shape in approximately 150 minutes.

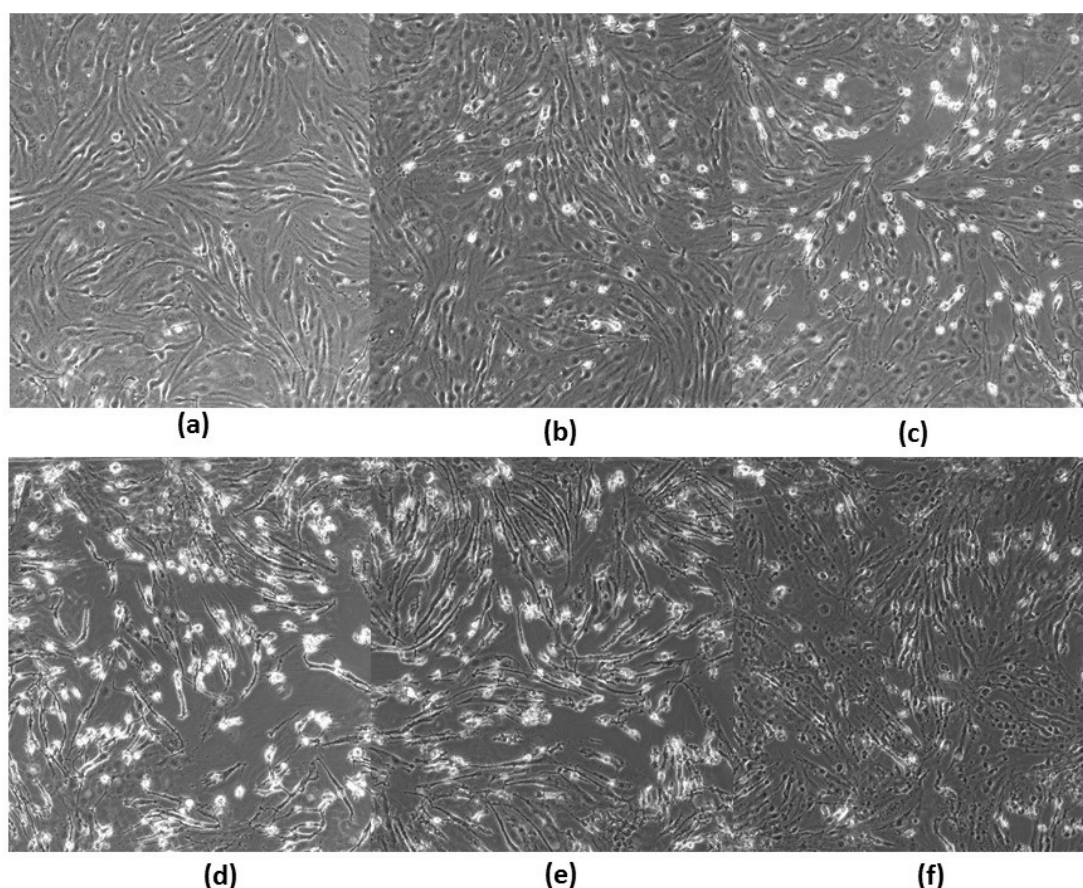


Figure 3-33. bEnd3 monolayer after exposure to different concentrations of mannitol for 4 hours (a) 0.1M, (b) 0.2M, (c) 0.3M, (d) 0.5 M, (e) 0.7 M, (f) 1 M

In a separate setup, the cell monolayer was exposed to the same concentration of mannitol and the cell shape was monitored over time using optical microscopy (Figure 3-35). Shrinkage of some cells is detectable over time. However, compared to visual inspection, the impedance measurement seems to be more accurate. For example after only 30 min, a significant shift in the impedance curve is detectable in both low and high frequency regions but almost no change in cell morphology could be seen after 1 hour. Also, still after 5 hours the general appearance of the cells seems to be fine and except some minor defects nothing is detectable. However, at that time the impedance spectrum becomes completely flat at both low and high frequencies, which corresponds to a leaky monolayer.

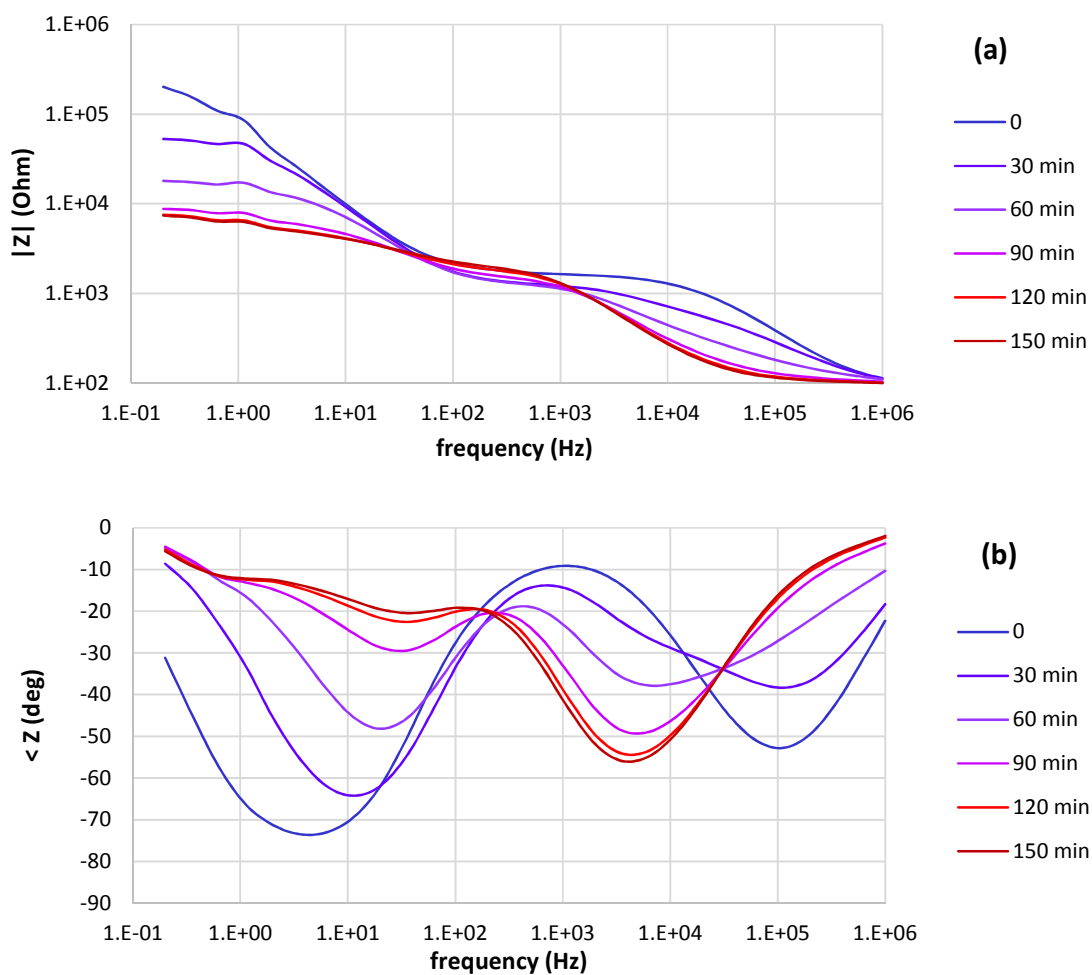


Figure 3-34. Impedance spectra of confluent monolayer of bEnd3 cells after exposure to 0.3M solution of mannitol in the presence of 10mM redox probe

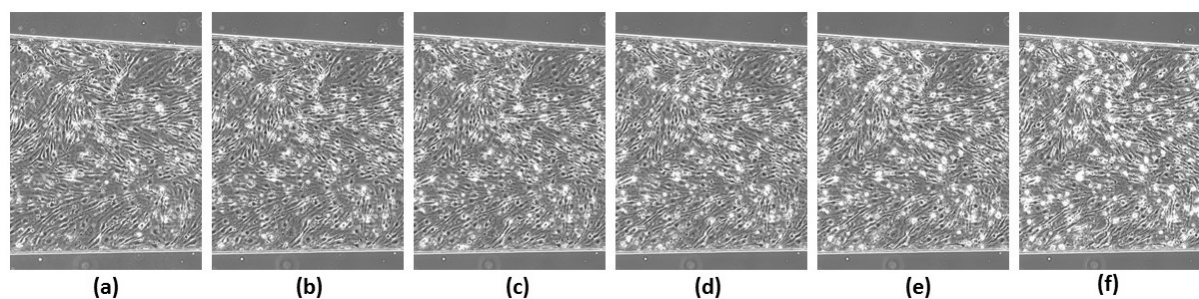


Figure 3-35. Time-lapse optical imaging of bEnd3 cell monolayer after exposure to 0.3M mannitol after (a) 0, (b) 1 hour, (c) 2 hours, (d) 3 hours, (e) 4 hours, (f) 5 hours

In order to obtain more quantitative information about the monolayer disruption process during the treatment with mannitol, the proper equivalent circuit model of Figure 3-25 (high density seeding) is fitted to the acquired impedance data and the most important parameters such as extracellular resistance and charge transfer resistance are extracted and plotted as a function of time (Figure 3-36 and Figure 3-37). It is seen that during the first 90 minutes after treatment, the extracellular resistance drops sharply, which corresponds to the disruption of TJs between the cells. However, the charge transfer resistance seems to be almost unaffected by the disruption.

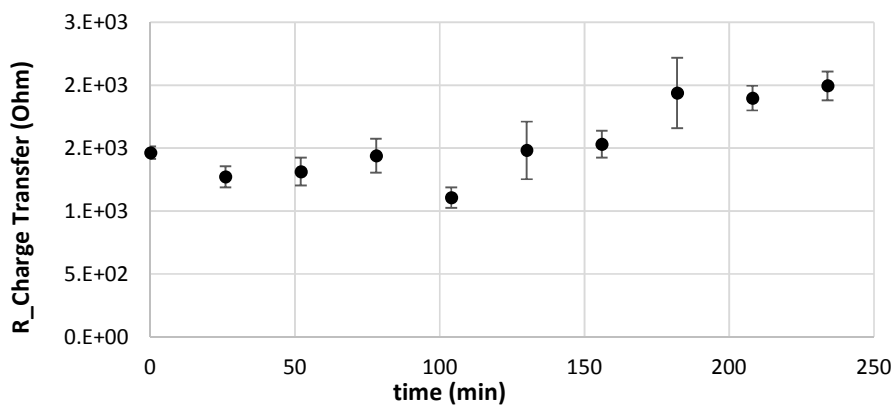


Figure 3-36. Charge transfer resistance (R_{CT}) as a function of time after treatment of bEnd3 cell monolayer with 0.3M mannitol.

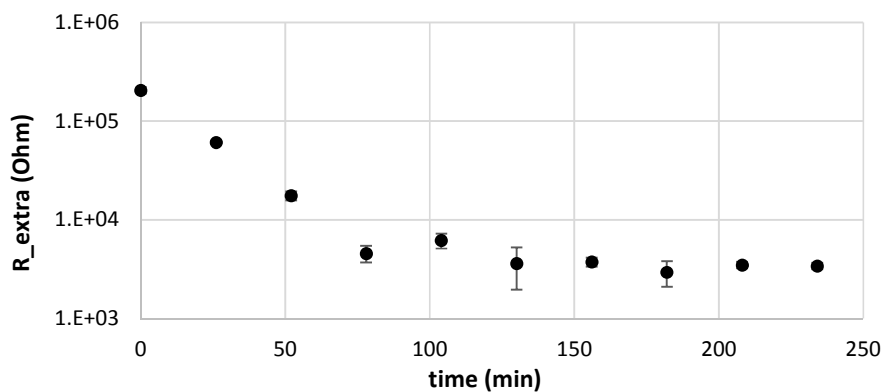


Figure 3-37. Extracellular (or tight junction) resistance (R_{TJ}) as a function of time after treatment of bEnd3 cell monolayer with 0.3M mannitol.

3.3. PEFs-induced disruption of the endothelial barrier measured electrically

PEFs are used as the second method to disrupt the endothelial cell monolayer in the chip. In Chapter 2 we showed how the disruption of the transcellular pathway could be monitored by fluorescent microscopy. The goal of this section is to see how electrical impedance spectroscopy could be used to monitor the permeabilization process. We are specifically interested in low level electric field pulses which do not cause electroporation and therefore the method of Chapter 2 could not be used for monitoring their disruptive effect.

Here we exposed the cells to bipolar pulses of electric fields with 3 V/cm magnitude and 200 Hz frequency (similar to DBS fields) and monitored their impedance over 3 hours.

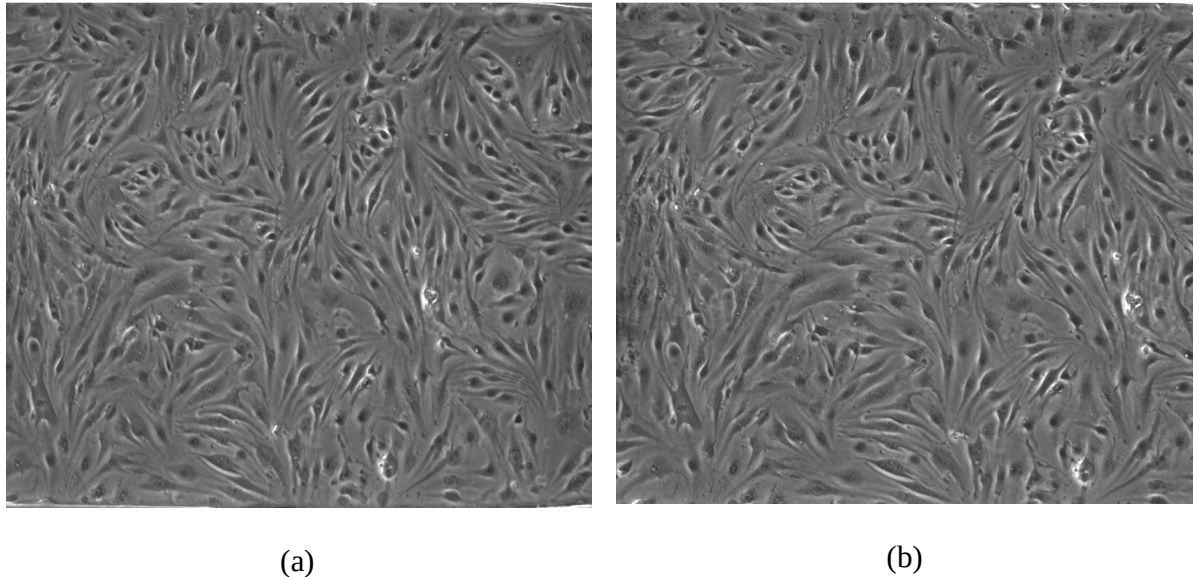


Figure 3-38. bEnd3 cell monolayer in micro-channel (a) before (b) after 3 hours exposure to PEFs

As shown in Figure 3-38 no major change is visually detectable in the configuration of the cells. However, a significant shift in both magnitude and phase diagrams of the impedance spectra is observable (Figure 3-39).

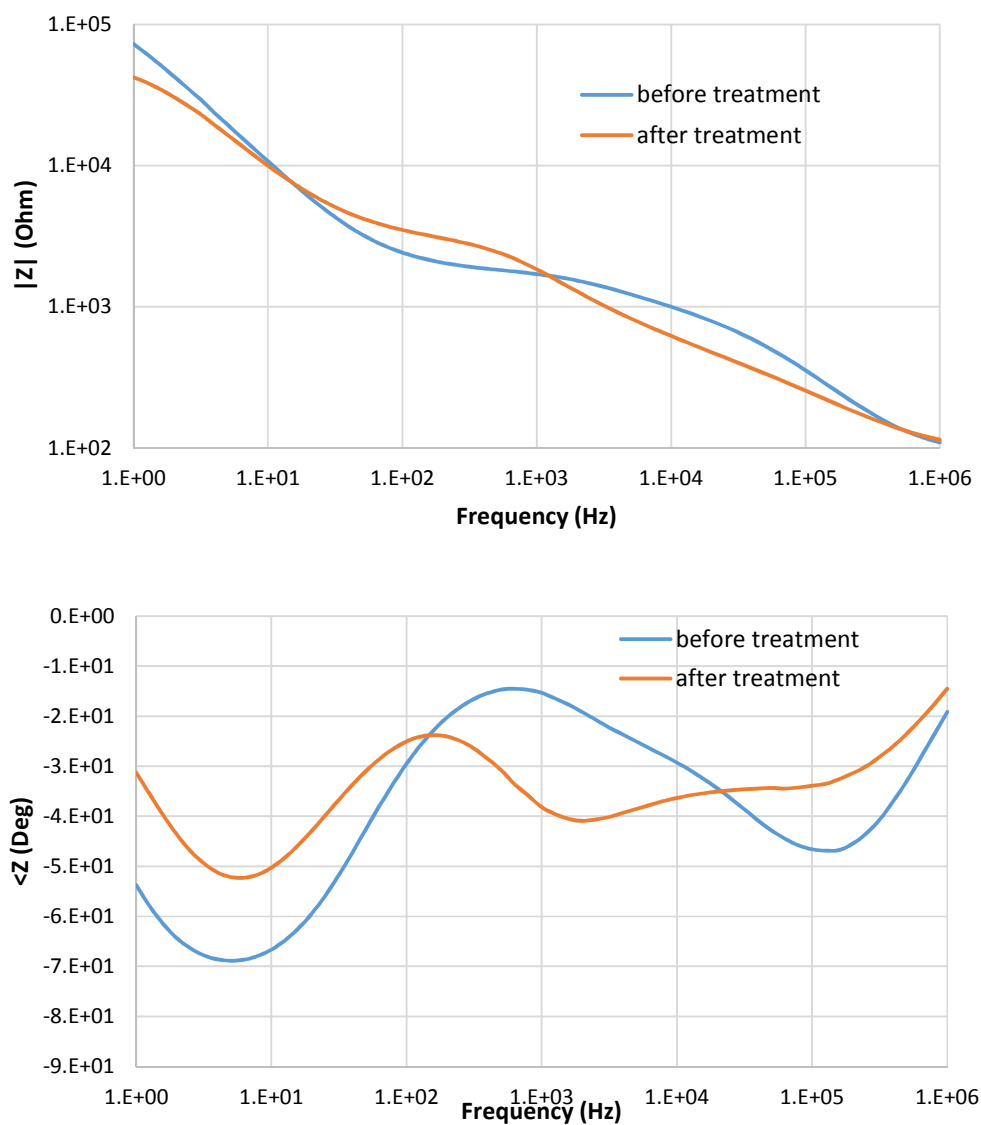


Figure 3-39. Impedance spectra of bEnd3 cell monolayer before and after exposure to low-level electric field pulses for 3 hours.

Chapter 4: Laminated microfluidic platform for studying transport across the BBB

4.1. Abstract

A laminated microfluidic platform featuring a permeable membrane is developed and used to culture brain endothelial cells as a dynamic model of the BBB. This platform provides access to both sides of the endothelial cell monolayer and facilitates direct measurement of transport across the cell monolayer. This platform could be used to investigate the effect of pulsed electric fields (PEFs) on the controlled and temporary permeabilization of the BBB to molecules of different sizes.

4.2. Introduction

This projects aims at finding the effects of PEFs on the permeability of BBB for the purpose of drug delivery. A double layer microfluidic platform was chosen to make the *in vitro* model of the BBB and perform the permeability measurements. A monolayer of brain microvascular endothelial cells was cultured in the top channel of the microdevice over a permeable membrane and exposed to physiologically relevant shear stress for the up-regulation of tight junction proteins. The luminal (top) channel was exposed to solution of a naturally impermeable substance with large molecules such as 70 kDa Dextran which is conjugated with fluorescent FITC molecule for visualization and quantification purposes. The permeability of the BBB could be measured by sampling the media in the abluminal (bottom) channel and subsequent fluorescent spectroscopy. Figure 4-1 shows the schematic of this experiment.

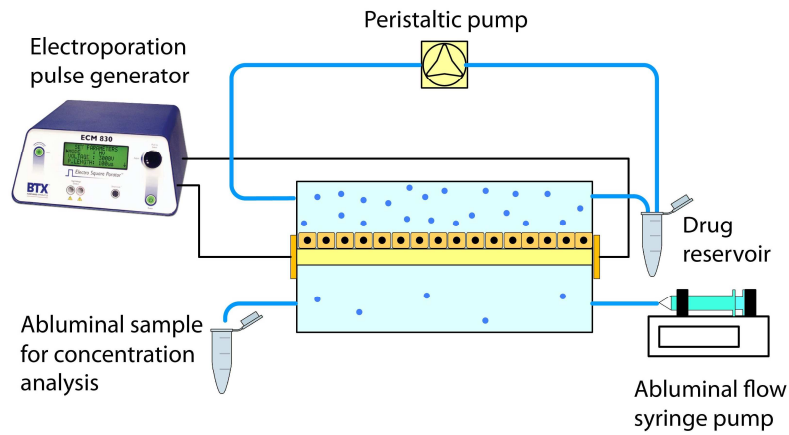


Figure 4-1. Schematic of fluid flow circuit for measuring the permeabilization of BBB on chip

4.3. Device fabrication

The microfluidic platform is comprised of two layers which are separated by a permeable membrane. The membrane is cut from the commercially available transwell inserts (Costar 3450, Fisher Scientific) and its properties are summarized in Table 1

Table 1. Properties of the permeable membrane

Material	PET
Pore size (nm)	400
Pore density (1/cm ²)	4e6
Thickness (um)	10
Appearance	translucent

Each layer of the device is fabricated separately using photolithography and replication molding as described in section 2.2.2. The device is then assembled by sandwiching the membrane in between and bonding the layer together using a PDMS mortar as described in [192] and summarized in Appendix A. The schematic of the device and the final product are shown in Figure 4-2 and Figure 4-3, respectively.

For the ease of fabrication, the top and bottom layers of the device are identical which are positioned with 90 deg angle with respect to each other. Each layer has 4 parallel channels which

after overlapping with the other layer creates 16 intersections. The 1x1 mm intersection of the two channels is where the transport phenomenon occurs.

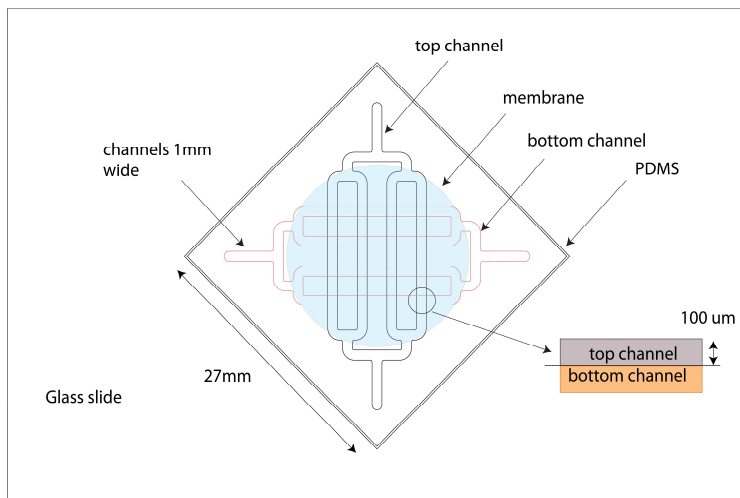


Figure 4-2. Schematic of the double layer microfluidic device

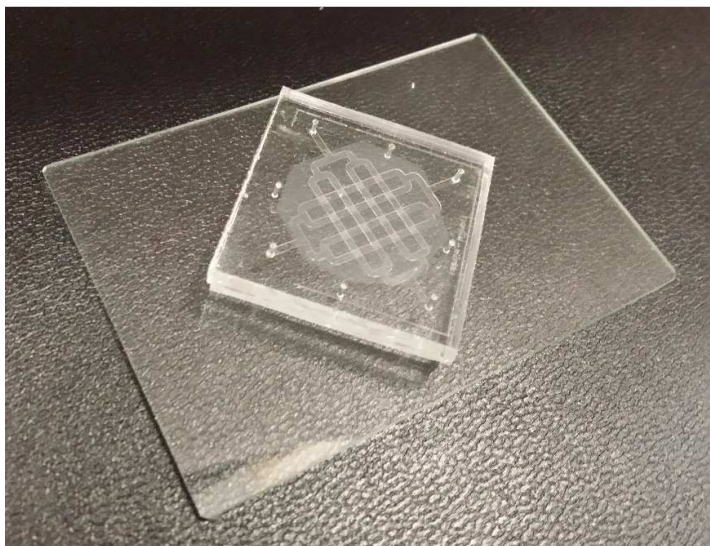


Figure 4-3. Fabricated double layer microfluidic device

4.4. Cell culture

The immortalized mouse brain capillary endothelial cell line, bEnd3, has been widely used for the *in vitro* modeling of the BBB [177, 193-196]. The bEnd.3 cells were cultured in T-75 flasks at 37

°C and 5% CO₂ and maintained in complete growth media consisting of DMEM (ATCC) supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1% (v/v) penicillin-streptomycin (Life Technologies). The cells were routinely passaged at 70-90% confluence. In order to prepare the microfluidic device for cell seeding, the PDMS channels were first sterilized with ethanol. To promote cell adhesion and proliferation, the channel was treated with 50 µg/ml human fibronectin (Trevigen, Gaithersburg, MD) for one hour in an incubator. Complete growth media was then introduced into the device and incubated for two hours. Endothelial cells were collected by washing and detaching with trypsin. The trypsin was then neutralized by the addition of media and centrifuged for five minutes at 120 × g. The trypsin solution was removed and fresh media was added to obtain a concentration of approximately 40 million cells/ml. The cells were then introduced into the top channel of the device through manual injection using a syringe and tubing until an even distribution was achieved in the channel. The device was incubated at 37 °C for two hours allowing the cells to fully attach. Then, complete media was provided to the channel using media-filled pipet tips at the inlet and outlet. The devices were incubated for two days at 37 °C and 5% CO₂ until the cells were confluent in the channel. Figure 4-4 shows the confluent monolayer of bEnd3 cells in the top channel at day 2 post seeding.

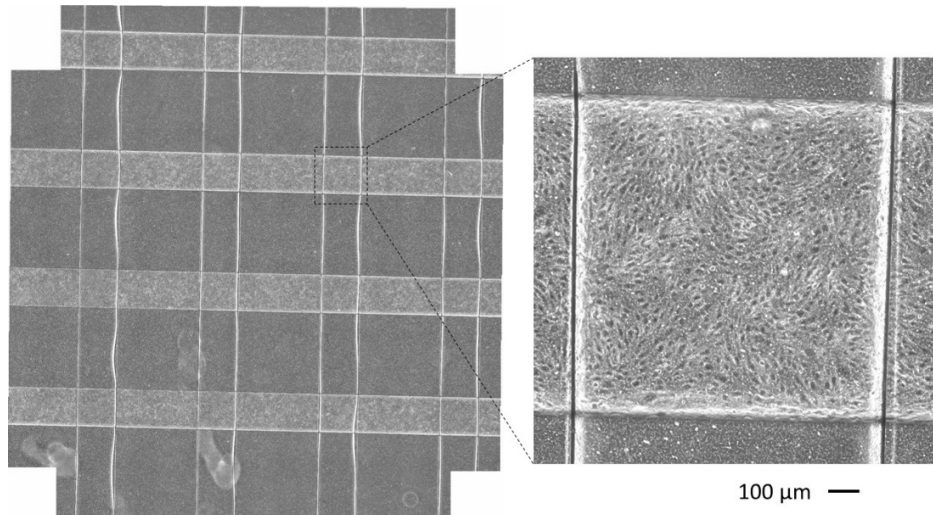


Figure 4-4. bEnd3 cells cultured in the top layer of the double layer chip at day 2 post seeding

4.5. 70kDa FITC-dextran as a tracer for permeability of BBB

70 kDa FITC dextran is commonly used as a tracer for measuring the permeability of BBB in the *in vitro* models. Due to its large size and hydrophilic nature, this molecules is not able to pass through the transcellular pathway (through the cell membrane and the intracellular space). Therefore its pathway is restricted to the paracellular space. In the case of BBB, due to the formation of intercellular tight junction, the paracellular pathway is restricted to small molecules of less than 500 Da molecular weight. Therefore 70 kDa dextran molecules see a small permeability value of $1.5\text{E-}7$ cm/s across the BBB *in vivo* [197]. Sucrose has also been used as another tracer for this purpose [194, 198].

The permeability coefficient P is calculated based on the following equation

$$P = \frac{1}{AC_0} \frac{\partial Q}{\partial t} \quad (5)$$

where A is the surface area across which transport is happening, C_0 is the concentration of the solute in the source chamber, and $\frac{\partial Q}{\partial t}$ is the solute flux. This equation is commonly being used for transport studies in transwell models in which diffusion occurs from a static source solution to a static sink solution. In this case Eq. (5) would be transformed into

$$P = \frac{1}{AC_0} \frac{CV}{\Delta t} \quad (6)$$

in which C is the final concentration of the solute in the abluminal chamber with volume V after time interval Δt .

Over the course of static experiments the concentration of the source solution decreases while that of the sink solution increases. This changes the dynamic of the transport phenomenon across the barrier and requires some assumptions for Eq. (6) to be valid. For example it is assumed that the experiments are conducted under the sink condition in which the concentration of the sink solution does not increase more than 10%.

As opposed to the transwell system, the microfluidic platform is a dynamic system with fluid flows in both luminal and abluminal channels. That dynamic nature causes the diffusion phenomenon to be different than static system with a modified permeability coefficient. In the dynamic system the solute with a fixed concentration is always being circulated in the luminal channel compensating for the amount of diffused solute. Due to the continuous fluid flow in the abluminal channel, a steady state would be achieved with a constant concentration as opposed to the static system in which the concentration of the abluminal channel increases with time. With the fresh medium with zero concentration of the solute always flowing into the abluminal channel there would be no back diffusion and the sink condition assumption is always valid.

In the dynamic case the solute flux, $\frac{\partial Q}{\partial t}$, term in Eq. (5), which means the amount of diffused solute in unit time, transforms into

$$\frac{\partial Q}{\partial t} = CU \quad (7)$$

in which C is the steady state concentration of the solute in the abluminal channel and U is the abluminal volume flow rate. The modified permeability coefficient is then

$$P = \frac{CU}{AC_0} \quad (8)$$

The concentration of FITC-dextran could be determined based on the amount of emitted fluorescent light using fluorescent spectroscopy. To do that a calibration curve is needed to relate the amount of fluorescent intensity to the known concentrations of the solute. 100 ul samples of different concentrations are placed in a 96 well-plate and the intensities are measured at excitation and emission wavelengths of 490 and 520 nm, respectively (for FITC). The calibration curve for 70 kDa FITC-dextran is shown in Figure 4-5.

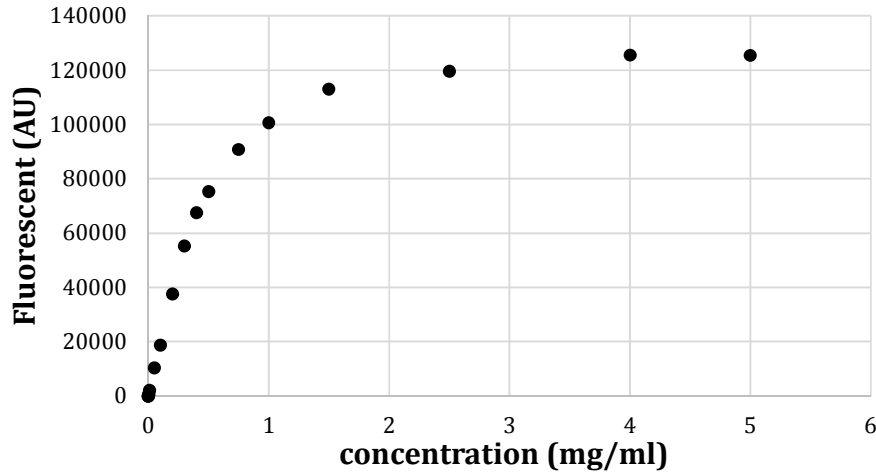


Figure 4-5. Calibration curve for 70kDa FITC-dextran

It is shown that the fluorescence follow a linear trend up to 0.5 mg/ml and after that deviates to lower values.

4.6. Permeability of the BBB-on-Chip

In the developed microfluidic model of the BBB, the apparent permeability from luminal to abluminal channel is attributed to diffusion of the solute across both the cell layer and the permeable membrane on which the cells are cultured. Considering the permeability analogous to conductivity, the three permeability coefficients are related as follows [199]

$$\frac{1}{P_a} = \frac{1}{P_c} + \frac{1}{P_m} \quad (9)$$

in which P_a is the apparent permeability, P_c is the permeability of the cell monolayer and P_m is the permeability of the membrane. We can only directly measure P_a , but since we are only interested in the permeability of the cell monolayer, the effect of the membrane should be subtracted.

A control experiment was performed with a device with no cells to find P_m . 70 kDa FITC-dextran solution in PBS with a concentration of 5 mg/ml was circulated in the luminal channel of the device with a peristaltic pump (Watson Marlow, UK) with an approximate flow rate of 5 μ l/min. PBS was injected into the abluminal channel using a syringe pump (Harvard Apparatus, Holliston, MA) with different flow rates. The PBS was collected at the outlet of the abluminal channel and collected in the wells of a 96 well plate for the consequent fluorescent spectroscopy. 8 samples of 100 μ l was collected for each flow rate. Theoretically, by running the experiment with only one flow rate, the permeability coefficient could be determined, as P is the only unknown of Eq. (8). However, by running the experiment with different flow rates we had two purposes: First to minimize the error and second to see if the permeability changes with the flow rate.

The same experiment is repeated with the device having the confluent monolayer of brain endothelial cells (bEnd3). The permeability coefficients P are calculated for each experiment and are plotted in Figure 4-6 as a function of abluminal flow rate. It is shown that when there is no cells in the device, the permeability coefficient decreases significantly by increasing the abluminal flow rate. However, in the case of the device with cultured endothelial cells, the permeability coefficient stays almost the same.

More experiments are needed to determine the exact relation between the permeability coefficient and the flow rate. However, it is hypothesized that the increased pressure in the abluminal channel due to the increase in the flow rate reduces the amount of diffused solute from top to the bottom channel.

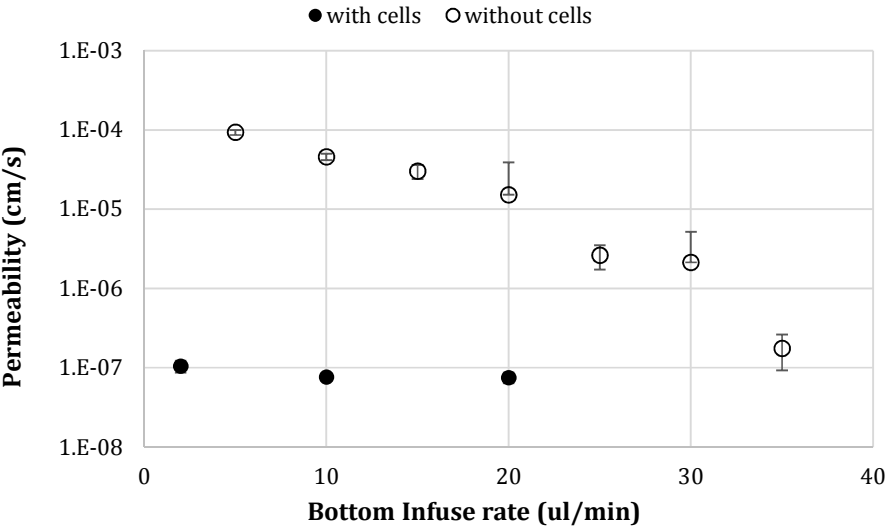


Figure 4-6. Permeability coefficient P with and without cells

4.7. Summary and Future work

A double layer microfluidic platform featuring a permeable membrane was developed. bEnd3 cells were successfully cultured in the device. Basic permeability measurements were performed by tracing large impermeable molecules (70 kDa FITC-dextran) across the cell monolayer. Monitoring the permeability of the endothelial barrier in response to different PEFs is left for future studies. This platform could also be used to monitor the permeabilization due to other stimuli such as thermal shock and ultrasound.

Chapter 5: Electrical impedance micro-sensor and its application in ablation monitoring during irreversible electroporation therapy

This Chapter has been co-authored by Eduardo Latouche and is published in the IEEE Transactions in Biomedical Engineering as ref [127] (© 2015. IEEE. Reprinted with permission, from M. Bonakdar *et al*, The Feasibility of a Smart Surgical Probe for Verification of IRE Treatments Using Electrical Impedance Spectroscopy, Transactions on Biomedical Engineering, Nov 2105)

5.1. Abstract

Irreversible electroporation (IRE) is a new focal ablation therapy that works based on delivering microsecond-long pulsed electric fields (PEFs) to the tissue. Applying the PEFs in the tissue initiates a cascade of different biophysical events at the cellular level that yields cell membrane disruption and eventually cell death. Currently, most IRE procedures are relied on pretreatment planning based on calculating electric field distribution to predict the ablation zone prior to treatment. One clinical limitation of IRE is the absence of methods for real-time treatment evaluation to actively monitor the dimensions of the induced lesion. This information is critical to ensure a complete treatment and minimize collateral damage to the surrounding healthy tissue. In this study we are taking advantage of the biophysical properties of living tissues to address this critical demand. Recent studies have shown that electrical properties of living tissues, as given by the electrical impedance spectrum, are affected during the ablation process. Using microfabrication techniques, we have developed an electrical impedance micro-sensor to collect impedance data along the length of an IRE probe for treatment verification. The probe was first tested on potato tuber, and then used to monitor the lesions in a perfused porcine liver. We used the impedance

spectra, along with an electrical model of the tissue, to obtain the conductivity of the tissue before, during, and after treatment. It is shown that electrical impedance spectroscopy could be used as a technique for treatment verification, and when combined with appropriate FEM modeling can determine the lesion dimensions. This technique has the potential to be readily translated for use with other ablation modalities already being used in clinical settings for the treatment of malignancies.

5.2. Introduction

Minimally invasive energy-based focal ablation techniques have received much attention throughout the last few decades, particularly for the treatment of aberrant masses. Radiofrequency ablation (RFA), microwave ablation (MWA), and high-intensity focused ultrasound (HIFU) ablation are among the most commonly used therapies for the management of cancerous tissue [200]. More recently, the phenomenon of electroporation (EP) has opened new windows in the field of focal ablation. Electroporation is a method used to increase the permeability of cell membranes to ions and macromolecules by exposing them to short yet intense pulsed electric fields (PEFs) [201]. Electroporation has been around for decades for *in vitro* applications but it is gaining strength for drug delivery and tumor treatment at the tissue level [202, 203]. During EP a series of short PEFs (70-100 μ s) are delivered to the cell population. These pulses affect the transmembrane potential of the cell that can create nano-scale pores on the cell membrane [204]. Depending on the pulse parameters (electric field magnitude, pulse length, number of pulses, etc.), the phenomenon can be either reversible, which is useful for cell transfection, or irreversible, which results in cell death [123]. Therefore, EP gave birth to three treatment modalities: electrochemotherapy (ECT) [205, 206], electro-gene therapy (EGT) [207, 208], and irreversible electroporation (IRE) [209, 210].

IRE is a new minimally invasive procedure for the ablation of undesirable tissue [204]. The procedure uses two or more surgical probes (electrodes) to deliver low quantities of energy into the tissue with minimal Joule heating effects and little thermally-induced cell death in the region of interest [70]. IRE lesions typically present sub-millimeter margins between dead and live tissues due to the rapidly decaying electric field surrounding the electrodes [211]. Unlike high-energy thermal-based therapies, IRE spares critical blood vessels and nerves in the periphery as well as the overall stroma surrounding the electrodes [212]. In clinical settings, surgeons have the option to use several imaging techniques such as computer tomography and ultrasound to actively monitor the position of the electrodes in the patient as well as to visualize post-treatment results [213-215]. During any focal ablation therapy, the ability to verify the lesion size is critical [216]. A small lesion may not enclose the whole tumor volume and a large one can damage the surrounding healthy tissue. For most thermal-based tissue ablation mechanisms the created lesions are commonly monitored through ultrasound, magnetic resonance, or computer tomography imaging systems, which do not give clinicians a satisfactory description of the area of treatment neither they provide information in an active manner [216]. Although IRE has enjoyed great success in the treatment of inoperable tumors, IRE lesions are moderately hard to predict due to electric field distortion caused by the heterogeneity of most tissues [217]. Currently, treatment planning is performed by finite element models of tissue and electric field distribution with *a priori* information about the tissue of interest to adjust the pulse magnitude for a proper lesion size [123]. Several groups have developed numerical models of electric field distribution during IRE treatment, taking into account the variation of tissue conductivity due to EP and temperature [218-220]. Despite all the advancements in modeling, this method could be challenging when the volume to be treated contains complex heterogeneous structures. In result, some complex tissues

such as the pancreas introduce additional challenges, which can lead to high patient to patient variability and result in under-treatment or skip ablations. This highlights the importance of a real-time feedback system for monitoring the progress of the treatment.

Electrical impedance spectroscopy (EIS) has been traditionally used in electrochemical systems for applications such as corrosion monitoring [153], coating evaluation [154] and semiconductor characterization. However in recent years, this method is gaining applications in the characterization of biological systems as a minimally invasive method. These include applications in tissue engineering, cell culture monitoring and disease diagnosis. At the cell level EIS has been used to monitor the electrical properties of single cells [159] and cell cultures in 2D and 3D [160]. Electric cell-substrate impedance spectroscopy (ECIS) is a popular technique for monitoring the growth, motion, integrity and permeability of 2D cell cultures [161, 162].

Recent studies have shown that healthy and cancerous tissues have different dielectric properties [221-223], leading to the application of EIS for cancer diagnosis. EIS could also be applied to the EP-based treatments. EP affects the dielectric properties of cells and tissues due to the alteration in transmembrane pathways and the conductance of the interstitial fluid. Several studies have shown the effect of electroporation on the passive electrical properties of single cells [137-139], cell suspension [140-142], adherent cell cultures [143, 144] and living tissues [145]. These studies have unveiled the potential application of EIS for active monitoring of the lesion during IRE treatments. Some previous studies exist using electrical impedance tomography (EIT) and magnetic resonance electrical impedance tomography (MREIT) to produce an image of the electroporated area *ex vivo* [145, 224, 225]. EIT requires the implementation of several electrodes around the treatment zone, which makes it difficult for clinical applications. Ivorra *et al* [226, 227] used plate electrodes to electroporate rat liver *in vivo* and measure the impedance spectrum of the

tissue in between the pulses. They found that the conductivity of the liver tissue increases by 9% and 43% after reversible and irreversible electroporation, respectively. Although this method is effective for determining the changes in tissue properties after uniform IRE treatment, it does not provide information about the geometry of the ablated tissue. In general, EIT is difficult to implement because it requires utilizing a large number of external electrodes which must surround the area of interest. MREIT requires no additional electrodes and is not limited to the boundaries of the object of interest, which provides an image with better spatial resolution compared to EIT. However, MREIT adds at least 15 seconds between EP pulses to reconstruct the images making it difficult to use during clinical IRE treatments that commonly utilize 5 times more pulses than EP treatments [225, 228]. Monitoring the conductivity of the tissue during electroporation is also possible by measuring the electric current passing through the tissue during the treatment [229]. This method is easy to implement and less invasive since no additional electrodes are needed for impedance measurement. However, using this method only gives the conductivity of the tissue in the vicinity of the pulsing leads and no information is obtained regarding other areas of the tissue. Although this measurement could provide some insight about the treatment at the point of pulsing, it does not reveal detailed information regarding the geometry of the lesion.

In the last decade, with the advancement of microfabrication techniques, microelectrodes are becoming more available for biomedical applications. These include microelectrode arrays for neural recording and interference [230-234], and electrical impedance micro-sensors for monitoring tissue properties in different situations [235]. Electrical conductivity and thermal conductivity micro-sensors have also been fabricated for the purpose of monitoring tissue properties during RF ablation [236, 237].

In this chapter we address the feasibility of using a custom-made micro-electrode array along a bipolar IRE probe to actively monitor changes in the impedance of tissue during IRE, and use that information to verify the extent of ablation. The flexible sensor array is made out of biocompatible materials and can be installed on probes of small diameter ($\sim 1\text{mm}$) without causing any constriction during the operation. We have developed a computational model, taking into account the change in the conductivity of the tissue during treatment. This model is used to predict the electric field distribution for different treatment procedures. Measurements taken through our microelectrode-array match the expected results from our computational model, which suggests that the proposed electrode array may be used to verify the expected outcome from treatment planning protocols. We believe that this method can impact the outcome of ablation therapies in the field of surgical oncology by providing surgeons with real-time information about the status of the therapy.

5.3. Fabrication of the impedance micro-sensor array

Advanced microfabrication techniques are used to fabricate the impedance micro-sensor array. The fabrication of the micro-sensor array was carried out at the Micro & Nano Fabrication Laboratory at Virginia Tech. A 4" Si wafer was used as the temporary substrate for the fabrication. The wafer was cleaned with acetone, Isopropyl alcohol (IPA) and DI water. Polyimide (PI) solution, PI2525 (HD Microsystems, Parlin NJ) was spin-coated on the Si wafer at 2000 rpm to yield a $13\text{ }\mu\text{m}$ thick PI base layer. To evaporate the solvent, the wafer was soft-baked on a flat hotplate at $120\text{ }^{\circ}\text{C}$ for 30 seconds followed by $150\text{ }^{\circ}\text{C}$ for another 30 seconds. For complete curing of the PI, the wafer was transferred to a furnace. The temperature was ramped up to $200\text{ }^{\circ}\text{C}$ in air environment at a rate of $4\text{ }^{\circ}\text{C}/\text{min}$. After 30 minutes a flow of nitrogen was started at 10 lit/min and the temperature was ramped up to $300\text{ }^{\circ}\text{C}$. After 1 hour the furnace was turned off and let to

cool down to room temperature. Slow heating and cooling rates were necessary to avoid cracking the PI layer. The adhesion between the PI layer and Si wafer is weak, which facilitates detachment of the structure from the wafer after fabrication but could also cause premature detachment in the middle of the fabrication steps. To avoid the later, an adhesion promoter, VM651 (HD Microsystems, Parlin NJ) was used at the edges of the wafer prior to spinning the PI solution to keep the PI layer attached without interfering with the probe structures. A 30 nm thick Cr layer was deposited as an adhesion layer followed by a 100 nm thick gold layer using E-beam evaporation (Kurt Lesker, Clairton PA). The metal layers were photolithographically patterned using AZ9260 positive photoresist (AZ Electronics, Germany) and chemically etched with Cr and gold etchants (Transene, Danvers MA) to form the conductive structures containing bonding pads, wires and sensors. As an insulation, a second layer of PI was spin-coated to 7 μm thickness and cured using the aforementioned technique. The top PI layer on the bonding pads and sensors should be etched down to the gold to expose them. All other PI layers except the designated areas for the probes were etched away to release the individual probes from the wafer. PI dry etching was performed through a titanium mask. 100 nm thick Ti film was deposited and patterned using E-beam evaporation and buffered oxide etch (BOE), respectively. PI etching was performed in oxygen plasma with 30 ml/min oxygen flow rate and 50 W of forward power. Finally, to remove the Ti layer and release the structures, the wafer was dipped into BOE for few seconds. The fabricated sensor was installed on the IRE probe and wires were soldered to the bonding pads. The fabrication steps are summarized in Appendix A.

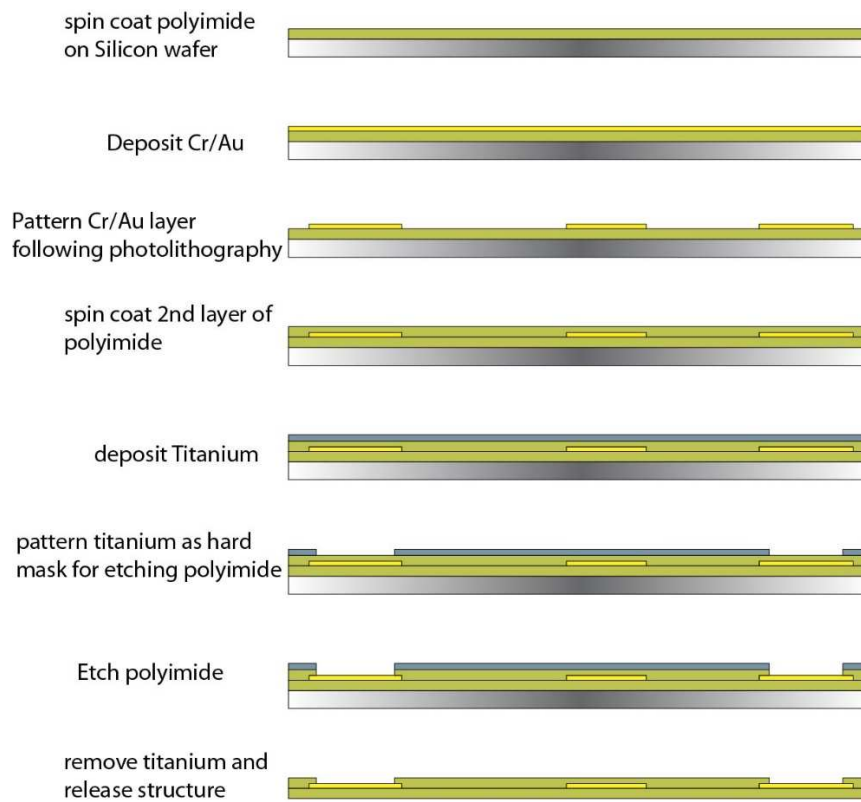


Figure 5-1 Fabrication steps for the flexible micro-sensor array

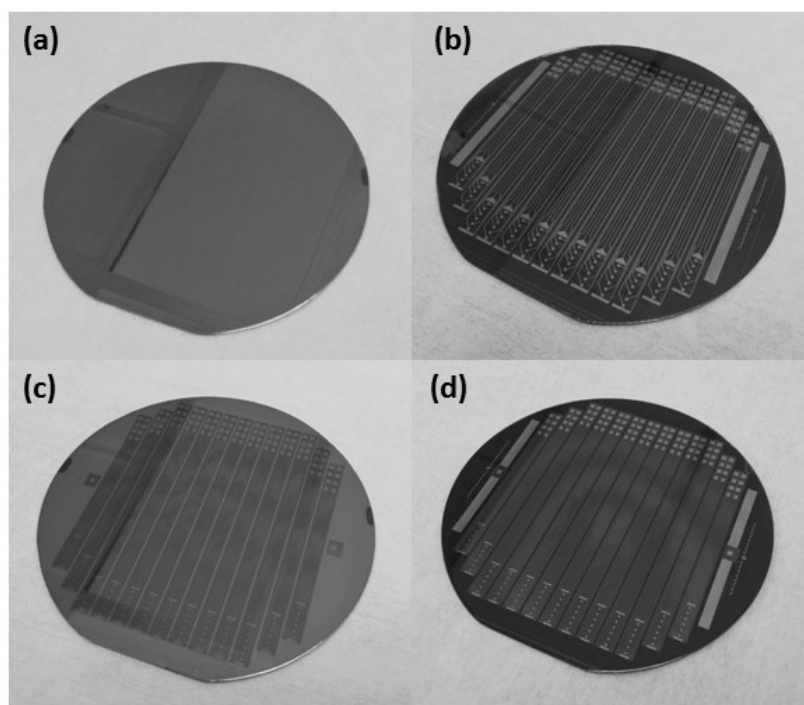


Figure 5-2. Silicon wafer's journey through consecutive steps of fabrication (a) Gold film evaporation (b) patterning electrodes in gold (c) evaporating Ti film, Photoresist patterning for the Ti mask (d) Etching Ti mask.

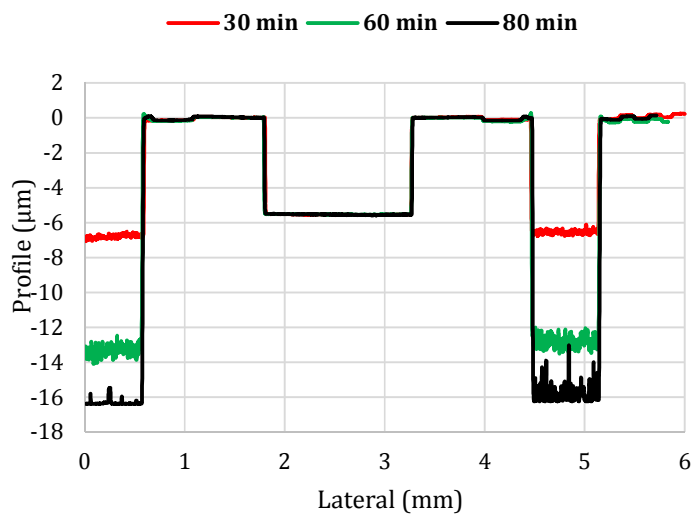
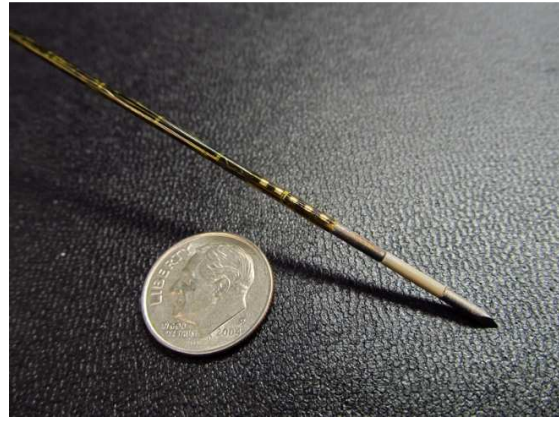


Figure 5-3. Polyimide etching progress at different times (left) along the indicated direction (right)



(a)



(b)

Figure 5-4. Fabricated impedance microsensors (a) before installation (b) after installation on the IRE bipolar probe

5.4. Different configurations of the impedance sensor

Three different configurations of the impedance sensor were designed and fabricated, each of which could be used for a specific application:

- Impedance sensors with a counter electrode
- No counter electrode
- Interdigitated sensors

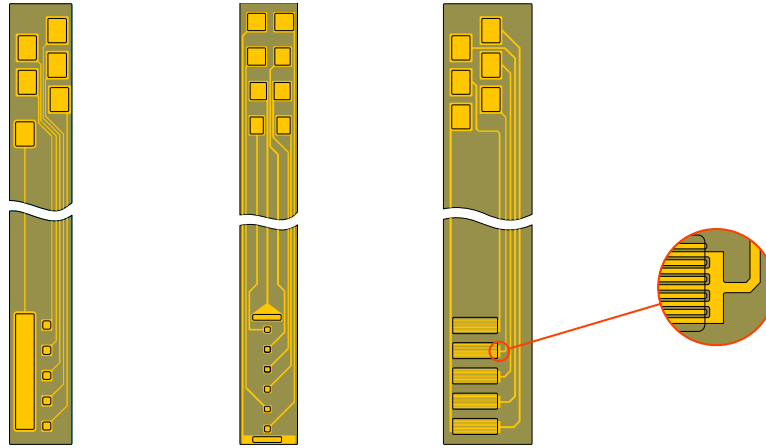


Figure 5-5. Schematics of different sensor arrays

Figure 5-5 shows schematics of these sensors. When a counter electrode is available, the impedance will be measured between any of the working electrodes and the counter electrode. Since the counter electrode is made with a very large surface area compared to the working electrodes, the contribution of the counter electrode to the measured impedance could be neglected and the measured impedance is only affected by the medium in the vicinity of the working electrode. This technique is beneficial in a sense that it allows for impedance to be measured at a very specific location as defined by the working electrode without being much affected by the counter electrode. The counter-electrode can cause some complications during the IRE therapy which are discussed in the results section of this chapter.

In the second configuration, the impedance is measured between two similar working electrodes along the probe. In this case the impedance is affected similarly by both electrodes. This

configuration has the advantage of allowing the tetra-polar impedance measurement in which the current injection and voltage pickup are performed through different pairs of electrodes. Usually the current is injected at the outer electrodes and the voltage is picked at the electrodes in the middle.

In the last configuration the interdigitated electrodes allow for impedance to be measured at a close proximity of the sensor. In this case the alterations deep into the tissue will not affect the readings from the sensors and only a thickness the same as the distance between the fingers would contribute to the readings. More details about the interdigitated electrodes will be provided in the Blood-brain barrier section.

Due to the small size of the fingers in the interdigitated configuration, the previous fabrication technic as illustrated in Figure 5-1 could not be implemented. The problem arises in the final etching step where the top PI layer is removed from the sensor areas. To accomplish that step, it is required to have a Ti mask covering the gaps between the fingers to avoid cutting through the finger gaps. However, due to the small size of the fingers and the accuracy of the instruments, it would be hard to align the gold electrodes with the Ti mask. Also undercutting during the wet etching process makes this more complicated. Therefore, we modified the procedure to accomplish this final step. An SiO_2 layer is deposited over the interdigitated electrode array and under the top PI layer to act as an etch stop during the final etch of the top PI layer. Figure 5-6 show the complete fabrication procedure for the interdigitated sensors.

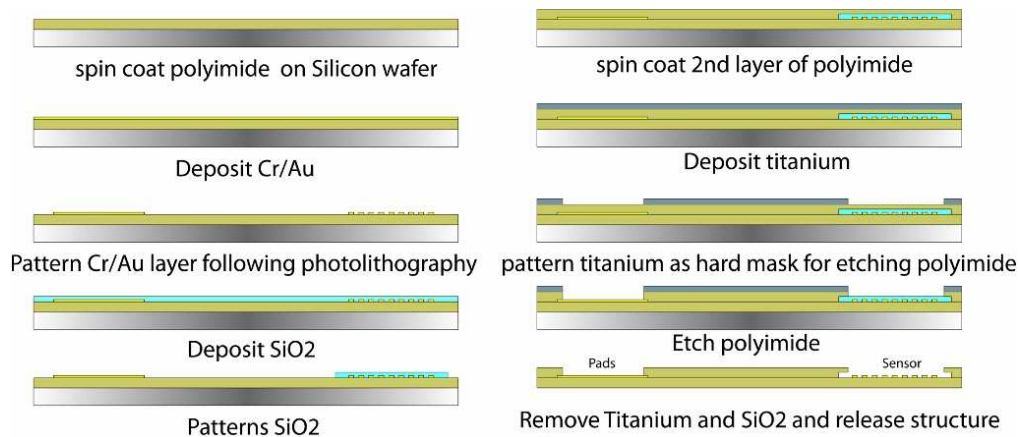


Figure 5-6. Fabrication steps for microsensor arrays with interdigitated electrodes

The impedance analyzer measures the impedance between the two fixed input wires. A multiplexer is needed in order to facilitate measurements at different sensor locations. In the case of the impedance probe with the counter electrode, one port is always connected to the counter electrode and the other port should switch between several sensors distributed along the length of the impedance probe. A second multiplexer was also designed which allows for the impedance to be measured between any combination of two sensors along the length of the probe. This multiplexer could be used in the case no counter electrode exists and also for the interdigitated electrodes. Please refer to Appendix B for more details about design and fabrication of the multiplexer.

5.5. Electrical impedance spectroscopy

The microsensor array is comprised of 5 individual working electrodes (WE) (Figure 5-5b) which are located 2.2 mm apart along the probe. The impedance is measured between the consecutive WEs. The chosen electrode placement made it possible to monitor the growth of the lesion up to 1 cm beyond the pulsing leads, which corresponds to clinically relevant lesions when using a bipolar EP probe. The impedance spectrum was measured using a Gamry Reference 600 potentiostat/galvanostat (Gamry, Warminster, PA) in the frequency range of 1Hz-1MHz at ten points per decade. The impedance probe was connected to the impedance analyzer through the custom-made multiplexer which enables switching between different sensors along the probe. The impedance analyzer and the multiplexer were controlled using Gamry Framework 6.12 data acquisition software (Gamry, Warminster, PA). To extract quantitative information about the desired electrical properties of the tissue from the impedance spectrum, it is necessary to fit the spectrum to an equivalent circuit model of the electrode/tissue system. For the best results, the model should contain elements taking into account the effect of electrode/tissue interface, intracellular and extracellular pathways. The Cole-Cole model [238, 239] had been previously used for impedance spectroscopy of tissues during electroporation [226, 240]. In this study the parallel Cole model is adopted with addition of a constant phase element (CPE) to account for electrode-tissue interface. Figure 5-7 shows the equivalent circuit model of the potato tissue.

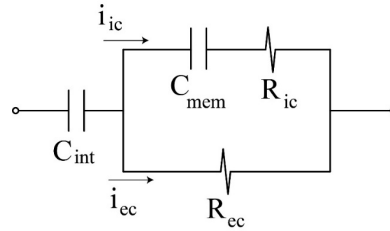


Figure 5-7. Equivalent circuit model of potato

The Cole-Cole model takes into account the intracellular and extracellular current pathways. Before EP, the cell membrane capacitance prevents the low frequency current from passing through the intracellular space. After EP, the membrane capacitance is shunted by the induced membrane pores, resulting in a lower cell resistance. At tissue level, not all cells undergo EP at the same time, hence instead of a sudden shift in impedance, a transition in impedance spectrum is observed. In this case the parameters R_{ic} and R_{ec} are no longer representative of pure intracellular and extracellular resistivities, but they include the effect of membrane permeabilization as well. However, as previous studies have shown, the Cole-Cole model is still valid for impedance analysis after EP.

5.6. Electroporation setup

To show the principles of sensor operation and be able to use relevant FEM modeling, we had to use an ideal domain for the experiments to factor out parasitic effects which exist in real tissue such as tissue heterogeneity, anisotropy, blood flow and many other factors. For these reasons potato tuber has been chosen as the platform for treatment. Potatoes are a relevant platform and well accepted model for running EP experiments [241-243]. Despite biological differences the main similarity between potato and organs, which makes these experiments relevant, is that potato cells respond to EP in a similar manner as mammalian cells. That similarity also facilitates the analogy in electrical impedance spectroscopy as potato and tissue share the common equivalent circuit model. The PEFs were generated using an ECM 830 pulse generator (Harvard apparatus, Holliston, MA) and delivered to the tissue through a commercially available IRE bipolar probe (AngioDynamics, Latham, NY). The general geometry of the probe presented a diameter of 1.63 mm with two conductive leads at the tip of 7mm in length each, separated by an 8mm long spacer. Based on clinically established protocols, all the treatments were performed delivering squared

pulses of 100 μ s in length at a repetition rate of 1 pulse per second [211, 244]. Treatments were delivered by applying 10, 30 and 100 pulses of 600, 1000 and 1500 V magnitude to the pulsing leads (total of 9 parameters).

5.7. Dynamic conductivity function and IRE threshold

Pretreatment planning of IRE therapies are usually conducted by finite element modeling of the electric field distribution within the target tissue. The FEM models give the electric field distribution generated by the applied potential at the electrode leads. It is known that an electric field threshold exists for the occurrence of IRE. In other words, the treatment zone is a region of the tissue in which the magnitude of the induced electric field is higher than a certain amount. Knowing the electric field threshold for the specific tissue is required to determine the size of the treatment zone or the ablation size. Different techniques could be used to find the IRE threshold, such as discoloration of the tissue, live/dead staining after treatment or measuring the electrical conductivity of the tissue.

It is known that the electroporation phenomenon changes the electrical conductivity of the tissue. The cell membrane disruption allows the electric current to flow through the cell and reduces the resistivity of the tissue at low frequency range.

To characterize the electrical properties of potato in response to EP, and to find the IRE threshold for different number of pulses, a set of 1-dimensional experiments were performed similar to the procedure implemented in [242], in which cylindrical samples of potato are exposed to uniform PEFs between two parallel plate electrodes and the impedance is measured across the same electrodes before and after treatment (Figure 5-8). In addition to that all samples were quantitatively assessed based on the darkening of the sample with respect to its initial color.

Potato tuber tissue was removed using a surgical biopsy needle resulting in cylindrical samples of 6mm in diameter and 5mm in height. Potato samples were exposed to uniform PEFs using parallel aluminum plate electrodes. Connected to the impedance analyzer, the same electrodes were also used for impedance measurements pre- and post-electroporation. A variety of pulse-numbers and magnitude permutations were applied to the samples with $n=4$ for each combination. Resistance values from the impedance measurements were solved for using an equivalent circuit model previously described and used to solve for conductivity of tissue by using Eq. (5-1).

$$R = \frac{L}{A\sigma} \quad (5-1)$$

where R is the resistance value obtained from impedance measurements, L is the length of the cylindrical sample, A is the cross sectional area of the sample, and σ is the conductivity of the potato. In order to assess electroporation, treated samples were stored in petri dishes for 12 hours at which point the samples were placed on a white background and a picture was taken using a digital CCD camera (Canon PowerShot SX110is). All images were converted to grayscale and assessed based on the darkening of the sample with respect to its initial color (control sample) using the ImageJ software (NIH, Bethesda, MD).

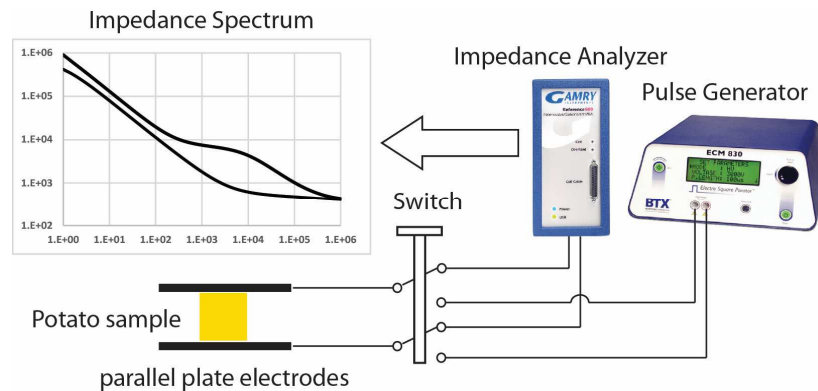


Figure 5-8. Schematic of the experimental setup for determination of conductivity function and IRE threshold

The results of the 1-D experiments were used to find a model for the variation of conductivity as a function of electric field magnitude and the number of pulses, *i.e.*, $\sigma_d(E)$. For this purpose a sigmoidal equation was developed for each particular number of pulses by fitting to the experimentally obtained conductivity values. Sigmoid functions have been used in the past to describe the change in tissue conductivity due to electroporation [245]. We found that the following equation fits our impedance data very well.

$$\sigma_d = \sigma_0 + a \exp(-e^{b(c-E)}) \quad (5-2)$$

In this equation σ_0 is the conductivity of the non-electroporated tissue and E is the applied electric field. The values for parameters a , b , and c were obtained following the best fit of the equation to the experimentally obtained data points for conductivity. Parameter a is the amplitude of the change in conductivity for irreversibly electroporated tissue, b is a constant regulating the growth rate and c is the location of the transition zone.

Figure 5-9 shows the results from the visual inspection of samples after 1-D electroporation experiments. Potato samples exposed to a uniform electric field presented the expected change in coloration when a sufficiently high electric field or number of pulses was delivered. The parallel plate electrodes ensure a uniform electric field across the sample, which results in a uniform treatment. As shown in Figure 5-9.A the color of the treated samples serves as an indication for the occurrence of IRE. The colors of the potato samples were quantitatively analyzed in ImageJ and the normalized contrast with respect to the fully electroporated sample is plotted in Figure 5-9.B. As shown in Figure 5-9, an acute contrast can be observed between samples exposed to 100V/cm and the rest of the samples. This is in agreement with the data in Figure 5-10.B, as 100V/cm is located below the inflection points of all curves for different number of pulses. The

sharp contrast between electroporated and non-electroporated samples is in agreement with the lesions observed inside the treated potatoes as shown later in Figure 5-13.

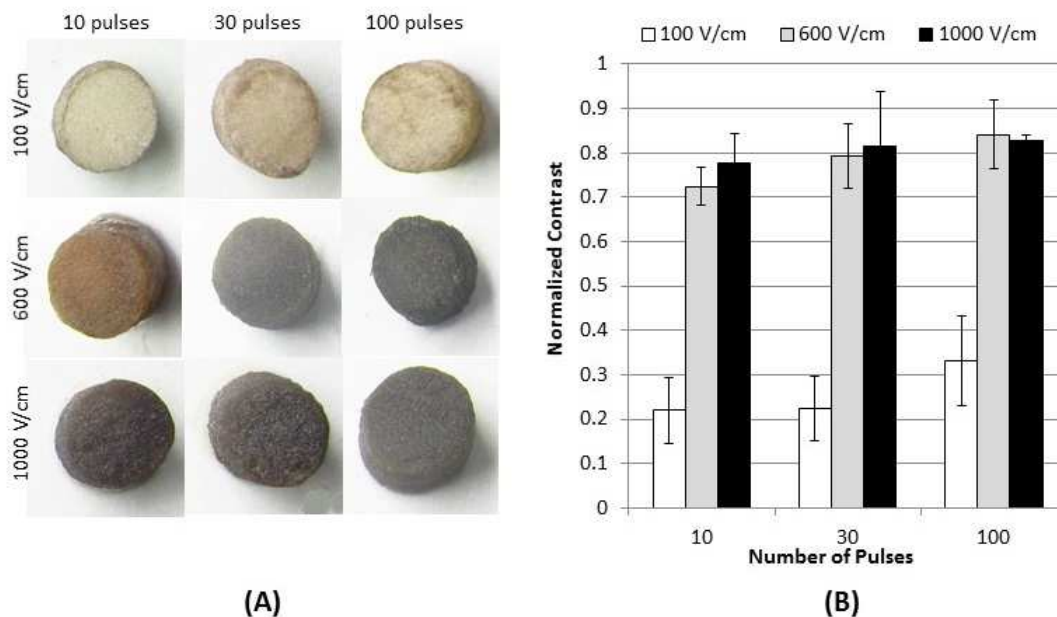


Figure 5-9. (A) Potato tuber samples (B) Normalized contrast after 12 hours post-treatment. Data is shown as average $\pm$ SD.

The rapid change in the color of potato samples after even a small number of pulses makes it difficult to use the color criterion to find the IRE threshold. It is believed that the electrical conductivity of the tissue increases significantly after IRE treatment. Hence, the increase in the electrical conductivity value is used as the criterion for determination of the IRE threshold.

The shifting in the electrical impedance spectra of potato samples exposed to PEFs of 400 V/cm magnitude and increasing pulse numbers is shown in Figure 5-10.A as an example. For all samples the impedance spectra are analyzed using the equivalent circuit model of Figure 5-7. After fitting, the R_{ec} resistance is extracted as a measure of the sample resistance at low frequency [235]. The obtained resistance is then converted to electrical conductivity using Eq. (5-1). The fact that

electroporation pulses are short DC pulses justifies using the low frequency electrical conductivity for modeling purposes. Figure 5-10.B shows the data points and the fitted $\sigma_d(E)$ functions as in Eq.(5-2) for 10, 30 and 100 pulses. The inflection point of each curve corresponds to the electric field threshold for the occurrence of electroporation [241, 242], which are found to be 581 V/cm, 298 V/cm, and 227 V/cm for 10 pulses, 30 pulses, and 100 pulses, respectively. The inflection points are shown with hollow circles on the graph.

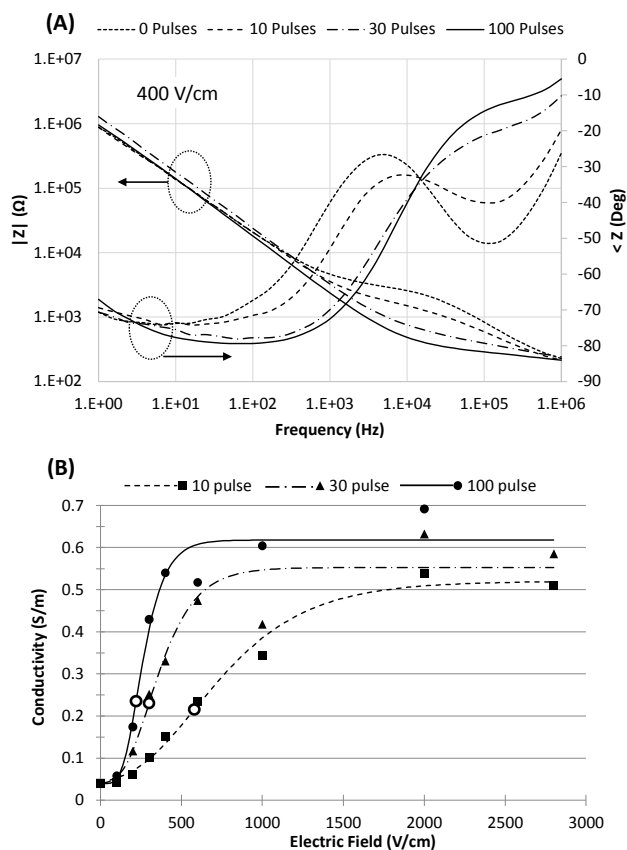


Figure 5-10. (A) Comparison of impedance spectra of potato tuber samples after exposure PEFs of 400V/cm magnitude and increasing number of pulses (B). Electrical conductivity of potato as a function of electric field for different pulse numbers. The inflection points are shown with hollow circles. The inflection points are considered as the IRE thresholds and are equal to 581 V/cm, 298 V/cm and 227 V/cm for 10 pulses, 30 pulses and 100 pulses, respectively.

Figure 5-10.B shows how the electrical conductivity increases after exposure to different pulse numbers of increasing electric field magnitude. It is shown that the transition zone between initial and final conductivity values narrows down as the number of pulses increase, resulting in a sharp increase in electrical conductivity for 100 pulses. But the transition for 10 pulses happens gradually. It should be noted that the inflection points of all three curves correspond to the same conductivity value (~ 0.22 S/m).

5.8. Finite element analysis

A finite element model (FEM) was developed in COMSOL Multiphysics® V4.3 (COMSOL, Stockholm, Sweden) to find the electric field distribution inside the tissue for each treatment. A 2-D axisymmetric platform was used to develop a model that mimics our experimental setup. The geometry used to obtain numerical results was comprised of an ellipsoid with the average dimensions of all treated potatoes along with a simplified geometry of the experimental bipolar probe with both geometries sharing center of mass at the origin. In order to keep the problem general, it was further assumed that the tissue is isotropic and macroscopically homogeneous. The physics involved in solving for the electric field distribution is governed by the following differential equation:

$$\nabla \cdot (\sigma_d \nabla \varphi) = 0 \quad (5-3)$$

where σ_d represents the electrical conductivity of the tissue as described by Figure 5-10.B and φ is the electric potential [123]. Boundary conditions for the electrode-tissue interface were set to $\varphi = V_0$ on one electrode while setting the other electrode boundary to $\varphi = 0$. All boundaries with no direct contact with an electrode were assumed thermally and electrically insulated. Heat conduction and generation during the treatment is governed by the following equation.

$$\rho_p \frac{\partial T}{\partial t} = k_p \nabla^2 T + \sigma_d |\nabla \varphi|^2 \frac{d}{\tau} = \quad (5-4)$$

where k_p is the thermal conductivity, T is the temperature, c_p is the specific heat capacity, and ρ is the density of the potato (tissue). The Joule heating term, $\sigma_d |\nabla \varphi|^2$, is simplified by using a scale factor directly related to the ratio of pulse duration d , and pulse interval τ . This simplification exemplifies a duty cycle approach in which the total energy delivered to the tissue is averaged throughout the duration of the treatment [246]. IRE is expected to take place in potato tissue

exposed to a marginal electric field threshold, which was found by matching experimental lesion dimensions to electric field values from our finite element analysis [242]. The material properties of potato are given in Table 5-1.

Table 5-1- Tissue properties and constants used in computational

Property	Symbol	Value	Ref
Heat capacity, J/(kg·K)	c_p	3033	[247]
Density, kg/m ³	ρ_p	1040	[247]
Thermal conductivity, W/(m·K)	k_p	0.552	[247]

The computational model is solved for 10, 30 and 100 consecutive pulses of 100V, 600V and 1500V to the pulsing leads (total of 9 analyses). Figure 5-11 shows the induced electric field magnitude along the length of the IRE probe for each treatment. Generally, the electric field magnitude decreases slightly by increasing the number of applied pulses which is attributed to the increased tissue conductivity during EP treatments as shown in Figure 5-10.B. Each graph also shows the IRE threshold for each number of pulses, as obtained from the dynamic conductivity function. For each number of pulses, the areas of the potato with a higher electric field magnitude than the indicated threshold become irreversibly electroporated. For instance, for 10 pulses of 1500V and 1000V the lesion grows 1.5mm and 0.6mm beyond probe 1, respectively. 10 pulses of 600V will not create any ablation beyond probe 1. By increasing the number of pulses a lower threshold is needed for IRE, as proved by 1-D experiments and shown in Figure 5-10.B. 30 pulses of 1500V, 1000V and 600V will create lesions 3mm, 1.8mm and 0.6mm beyond probe 1. In this case for 1500V the lesion boundary is located halfway between probe 2 and 3 and for 1000V the lesion is very close to probe 2. For 100 pulses of 1500V, 1000V and 600V, the lesion grows 4mm, 2.5mm and 1mm, respectively, beyond probe 1. In this case for 1500V and 1000V pulses the lesion grow close to probe 3 and probe 2, respectively.

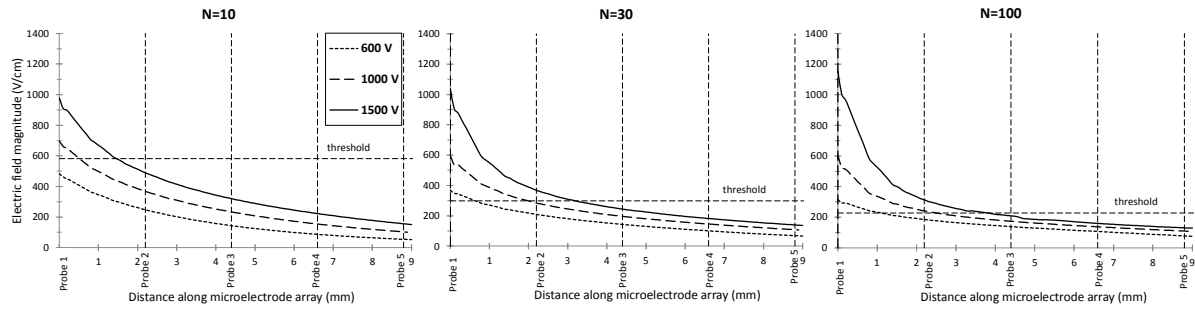


Figure 5-11. FEM results for electric field distribution along the length of the probe for different pulse parameters

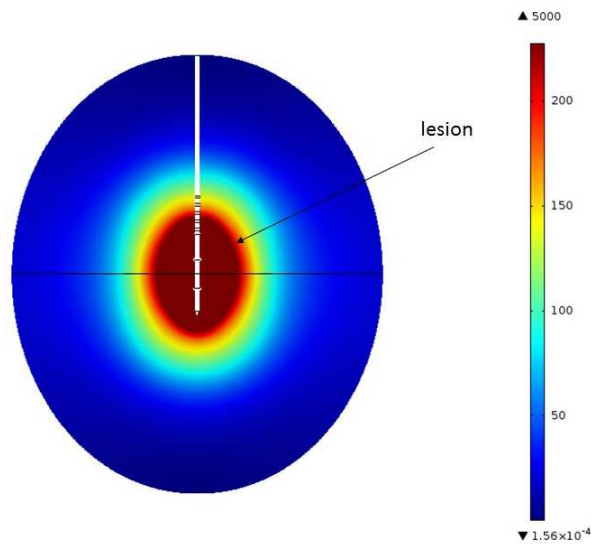


Figure 5-12. FEM modeling of electric field distribution for 1500V and 100 pulses. The dark red region at the center represents the lesion which is identified by $EF > 227$ V/cm

5.9. Lesion monitoring during IRE treatment in potato

The impedance spectrum is measured between consecutive sensors of the impedance probe before and after applying a total number of 10, 30 and 100 pulses for each potato. The impedance spectra before and after pulsing at each sensor are fitted to the equivalent circuit model of Figure 5-7 to find the circuit parameters. The tissue resistance, R_{ec} , is then converted into the conductivity using the calibration equation. Geometrical factors as well as electrical heterogeneity across different potatoes induce differences in the measured impedance before running any treatment. For this reason the tissue resistances were normalized to their initial values for better comparison.

Figure 5-13.A shows the percent change in tissue conductivity between sensor pairs for different treatments. Each diagram shows the change in conductivity after applying a certain number of pulses with different magnitudes. It should be noted that potatoes are heterogeneous and as revealed by our measurements, the conductivity of potato varies up to 24% at different points in different potatoes. Hence, instead of the absolute value of the conductivity, the relative change in tissue conductivity is plotted, which is obtained by normalizing the conductivity after each treatment to the conductivity of bare potato at the same point. As shown in Figure 5-13.A, depending on the applied pulse number and magnitude, certain sensors along the probe undergo a significant increase in the measured electrical conductivity. The results from the 1-D experiments, as shown in Figure 5-10.B, show how a certain increase in electrical conductivity could be related to the occurrence of IRE for the case of homogeneous treatment. However, during the treatment with the bipolar probe, due to the non-uniform electric field, both the ablated and non-ablated areas of the tissue contribute to the impedance measurement, hence a lower increase in electrical conductivity is expected at sensors within the same electric field compared to the 1-D experiment. As shown in Figure 5-13.A, for each number of pulses, the normalized conductivity decreases by

moving away from the pulsing leads. This value is equal to 100% (i.e., no increase) for points sufficiently away from the lesion.

Figure 5-13.B shows the induced lesion in potato after 10, 30 and 100 pulses of 1500V. It is shown that for 10 and 30 pulses the lesion edge is located between probes 1&2 and 2&3, respectively. For 100 pulses the lesion has grown up to probe 3. These results are consistent with the FEM results presented in Figure 5-11.

Based on the equivalent circuit model of Figure 5-7 the impedance spectrum could be divided into three frequency intervals. At low frequencies ($f < 1$ KHz) the impedance spectrum is dominated by the capacitive behavior of electric double layer at the electrode-tissue interface which is shown by the linearly decreasing impedance magnitude in Figure 5-10.A. At very high frequencies ($f > 100$ KHz) the impedance of all capacitive elements vanish, simplifying the equivalent circuit model to the parallel combination of R_{ic} and R_{ec} as verified by the resistive characteristics of the impedance spectrum at this range. As depicted in Figure 5-10.A, EP does not change the high and low frequency regions of the spectrum. The electric double layer at the electrode interface is not affected by electroporation, resulting in the same capacitance and the same impedance at low frequency range. At high frequency, the cell membrane capacitance is bypassed and the effect of EP on the membrane resistance is not reflected in this part of the spectrum. On the other hand the combination of cytoplasmic and the extracellular resistances which contribute to the high frequency impedance, do not change after EP, resulting in the same impedance at high frequency. At mid frequency range ($1 \text{ KHz} < f < 100 \text{ KHz}$) the electrode effects are vanished, however the existing cell membrane capacitance results in part of the intracellular current to pass through the induced membrane pores. Hence, this part of the spectrum is mostly affected by the membrane

resistance and undergoes the most significant shift in magnitude after EP due to the decreasing resistance of the cell membrane.

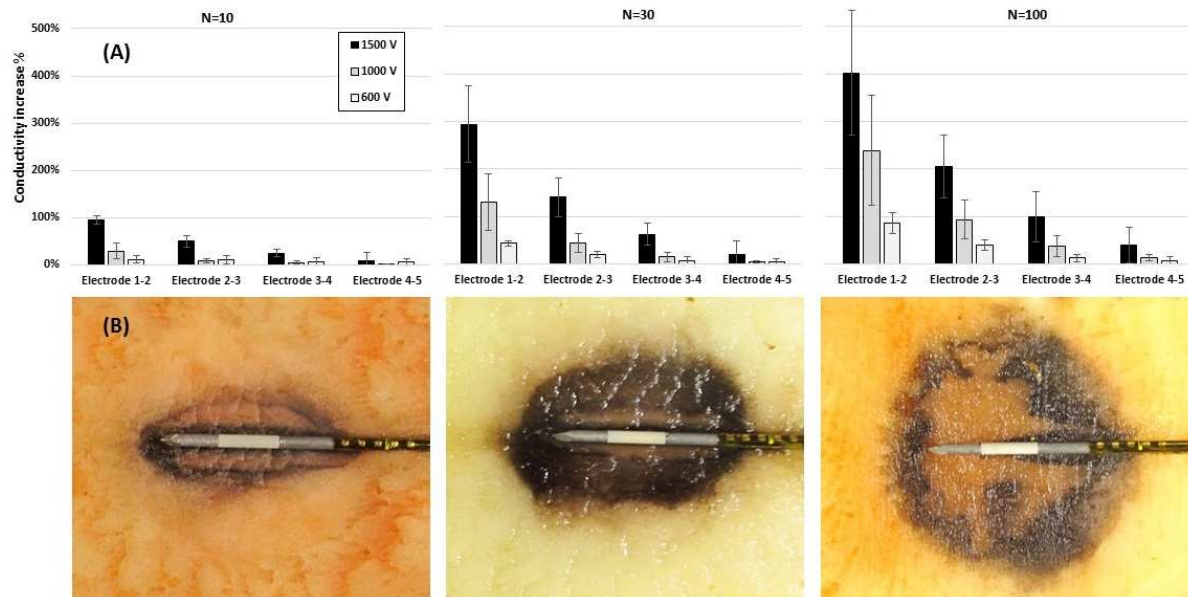


Figure 5-13. FE (A) Change in tissue resistivity at different points along the probe during treatment with different number of pulses (B) The ablated areas inside the potatoes after treating with 10, 30 and 100 pulses of 1500V.

As shown in Figure 5-13.B, the lesion starts in an elliptical shape around the pulsing leads of the bipolar probe which further grows into a circular shape after applying sufficient number of pulses (i.e., 100 in this case). This behavior which is confirmed by our FEM modeling, is a result of the change in conductivity after EP.

The impedance data gives feedback about the size of the lesion in both axial and perpendicular directions of the probe. This feedback is quantitative and accurate for determining lesion size in the axial direction yet it allows for qualitative interpretation of lesion growth in the perpendicular direction. In general sensors which are embedded more into the lesion, show a higher increase in the measured conductivity. According to the impedance data and depicted lesions in Figure 5-13, sensors which read a doubling in the measured conductivity are located at the lesion boundary

along the probe. For example in case of 10 pulses of 1500V, the E12 and E23 measurements show 100% and 50% increase in conductivity, respectively. This means that the lesion has reached E1 but not E2. In case of 30 pulses of 1500V, E23 and E34, measure 150% and 50% increase in conductivity, respectively, which corresponds to the lesion growing up to E2. In case of 100 pulses of 1500V, E34 and E45 measure 100% and 30% increase in conductivity, respectively, which corresponds to the lesion growing close to E4. These measurements are in agreement with the observed lesions in Figure 5-13.B and the predicted lesion size by FEM (Figure 5-11).

Lesion growth in the perpendicular direction of the probe is also reflected in the impedance measurement by the probe. For example it is predicted by FEM model (Figure 5-11, solid line) and observed in Figure 5-13.B that for 30 and 100 pulse treatments, E1 and E2 would fall within the lesion. However, the corresponding impedance measurement shows 400% and 500% increase in conductivity for 30 and 100 pulses, respectively. This difference is attributed to the depth of the lesion in the perpendicular direction. For the case of 10 pulses of 1500V, the small depth of the lesion in perpendicular direction and the marginal location of E2 compared with the lesion, results in 100% increase in conductivity for E12 measurement. For all treatments, the measurements showing 0% increase in conductivity correspond to electrodes completely outside of the lesion. The results show that the impedance probe is not only capable of monitoring the lesion length along the probe, but also gives relevant information regarding its other dimensions. In this study the correlation of lesion depth and impedance data is shown more qualitatively, however this information when combined with FEM modeling can give accurate shape and size of the lesion in all dimensions.

The FEM model was developed under the assumption of isotropic domains, which is relevant in case of a potato tuber. However some tissues like skeletal muscles are anisotropic, showing

different electrical conductivities and IRE thresholds in different directions. This behavior affects the distribution of the electric field within the tissue and consequently the ablated area, as shown in previous studies [248]. However, we believe that, when combined with the proper FEM model, the same electrode setup could be used for anisotropic tissue as well. This issue is out of the scope of this paper and will be addressed in our future studies.

In our experiments, acquisition of the complete impedance spectrum took about 20 seconds. However, the 10 points per decade resolution of the obtained spectra is far beyond the requirement for fitting the data to the model. Our results indicate that only a few impedance measurements distributed along the frequency range (depending on the shape of the spectrum) could give us an accurate fitting to the model, reducing the measurement time to a fraction of a second. Therefore, the entire impedance measurement sequence can fit in the inter-pulse time gap of 1 second, facilitating real-time monitoring during the treatment.

5.10. Validation of a perfused organ model for verification of irreversible electroporation treatments

Once the impedance probe was proven to be functional in a potato tuber, the next step was to try the functionality of the probe in an animal tissue model. For that purpose we first investigated the validity of a perfused porcine liver to reproduce the outcomes of IRE treatments performed *in vivo*.

5.10.1. Organ preparations

Freshly harvested porcine livers were acquired from a local slaughterhouse. Connections were made to the portal vein, hepatic artery, and major hepatic vein, using Luer lock connectors. The organs were then perfused with modified PBS containing NaCl (137mM), KCl (2.7mM), Na₂HPO₄ (10mM), KH₂PO₄ (1.8mM), CaCl₂ (1mM) and MgCl₂ (0.5mM). Upon arrival to the lab, the organs were connected to the VasoWave™ Perfusion System (Smart Perfusion, Denver, NC) for active

perfusion. The VasoWave system has been used before to enhance organ preservation [249]. Using this system the perfusate was delivered to the liver through the portal vein and hepatic artery and was recycled back via the hepatic vein (Figure 5-14).



Figure 5-14. Porcine liver connected to the VasoWave system for active perfusion with modified PBS.

5.10.2. Comparison of the ablation sizes in the perfused organ and in vivo

IRE pulses were applied to the perfused liver through a pair of monopolar electrodes using the BTX pulse generator. A variety of different treatments comprised of different pulse parameters were applied to the perfused liver. After the treatment, the liver was sliced through the mid plane of the ablation and stained using triphenyl tetrazolium chloride (TTC) (MP Biomedicals, Santa Ana, CA) in PBS (10 gr/l) for 5-10 minutes which allows visualization of the dead tissue. The ablation sizes were compared to previously published data in *in vivo* porcine livers [250] and another set of data that was previously obtained in *in vivo* canine livers and kindly provided by Dr. Robert Neal (AngioDynamics Inc.). A linear regression analysis was performed to check the correlation between the ablation sizes for perfused and *in vivo* organs (Figure 5-15).

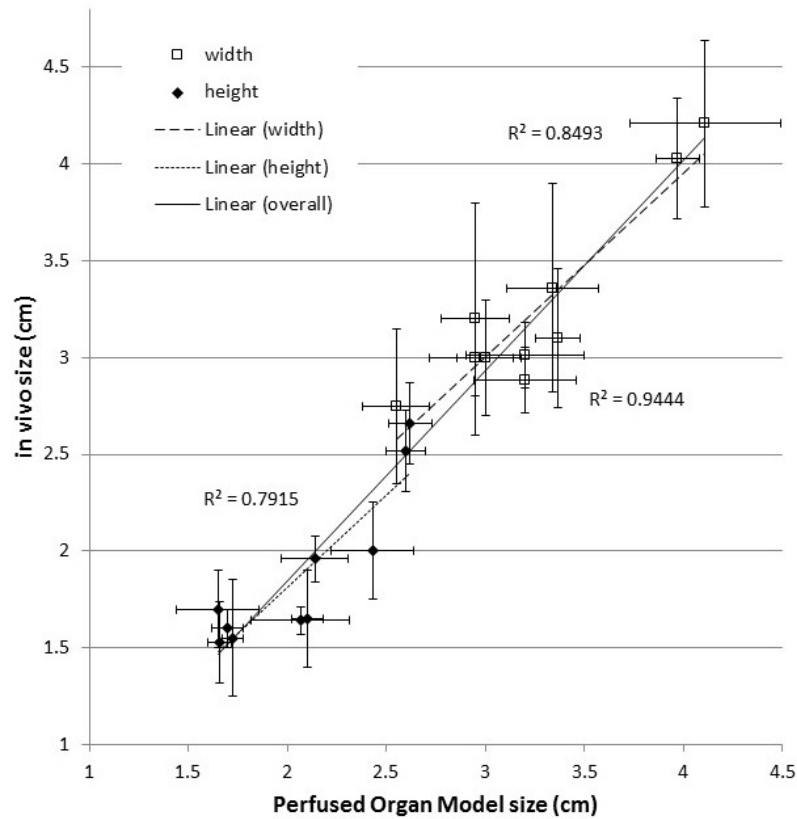
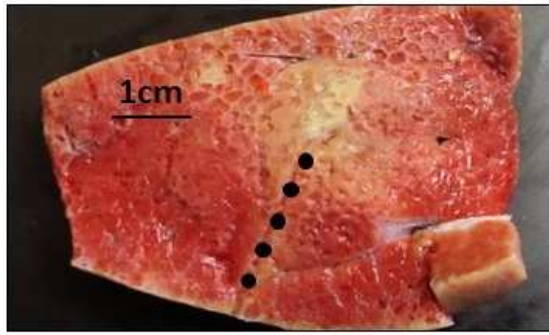


Figure 5-15. Comparison of in vivo and ex vivo lesion sizes for all experiments.

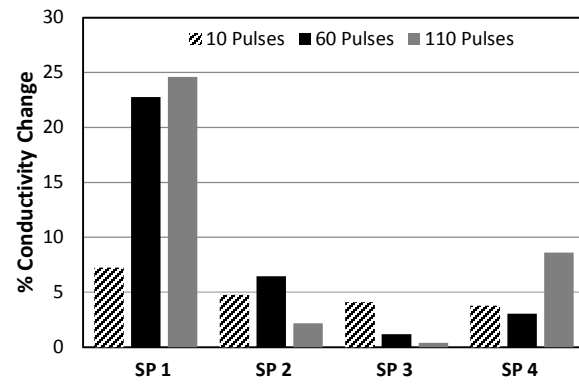
As shown above, a strong correlation exists between the ablations in the perfused model and the corresponding ones in vivo. ($R^2=0.94$), which proves the validity of the perfused organ model for investigation of IRE treatments.

5.11. Lesion monitoring in liver

A preliminary experiment was performed to show the feasibility of using the impedance technique to monitor the lesion growth in the liver tissue. IRE treatments were performed in the perfused liver with the same setup as the treatments in the potato tuber and the impedance data was collected after delivery of 10, 60 and 110 pulses. After completion of the treatment the ablation zone was sliced and stained with TTC to visualize the lesion.



(a)



(b)

Figure 5-16. (a) The induced lesion in the perfused porcine liver after delivery of 110 pulses and (b) The corresponding changes in the tissue resistance after delivery of 10, 60 and 110 pulses.

The induced lesion after delivery of 110 pulses is shown in Figure 5-16a. The impedance spectrum was measured between adjacent sensor pairs (SP1-SP4) and the tissue conductivity was calculated and plotted for each of the sensor pairs (Figure 5-16b). It is shown that sensors 1 and 2 are located inside the lesion and therefore, the corresponding conductivity reading (SP1) shows a significant increase during and after delivery of treatment.

It should be noted that the data presented here are considered to be preliminary and for the feasibility study only. Further experiments on perfused organs with variable treatment parameters are required to fully characterize the impedance readings in tissue and correlate those to the size of the ablation.

5.12. Conclusions

Taking advantage of the characteristic changes in conductivity during IRE, we have created a sensor that can be used to collect impedance data during treatments in potato tuber. We performed experiments applying a uniform electric field across cylindrical tissue samples to characterize the IRE thresholds and bulk conductivity changes during treatments. The results from our

computational model, which was constructed based on uniform electric field data, correlated well with the lesion dimensions. Using advanced microfabrication techniques, we fabricated a 20 μ m thick, flexible impedance micro-sensor array. We used impedance data collected through the sensor array to estimate the extent of insult to the tissue for a variety of treatments. Finally, agreement between the FEM and impedance data collected through the sensor array was verified by physically measuring the induced lesion which showed excellent agreement for different protocols.

The system presented herein can be adapted for intra-pulse measurements, which could potentially eliminate overtreatment of tissue and reduce unnecessary thermal effects. Furthermore, the device presented in this study could be readily implemented in the clinic as the proposed micro-sensor is fabricated from biocompatible materials and is relatively easy to manufacture. Finally, this method of active ablation monitoring could potentially be translated for use with other therapies involving probes as energy foci.

Chapter 6: Summary and future work

Several microfluidic platforms were developed for mimicking the permeability and transport across the BBB, each of which were intended to investigate the permeabilization process of the BBB model using different tools and from different perspectives. In Chapter 2 a microfluidic device was designed to expose the brain endothelial cell monolayer to a gradient of pulsed electric fields. Using this platform the threshold for reversible and irreversible cell membrane permeabilization due to electroporation was found. The threshold for uptake of naturally impermeable molecules due to PEFs was also determined and the approximate concentration of uptaken molecules was found. This information is useful for estimating the extent of transcellular transport across the cell monolayer. In Chapter 3 a microfluidic device embedded with impedance micro-sensors was fabricated. The response of impedance sensors in different chemical conditions was characterized using equivalent circuit models of the bare sensor. Using this platform the integrity of the endothelial barrier was monitored in normal conditions as well as after disruption with mannitol and PEFs. Equivalent circuit models of cell monolayer were used to translate the experimental impedance data into useful information. In order to make a more physiologically relevant model of the BBB and find the permeability in a direct way, a double layer microfluidic device was fabricated and presented in Chapter 4. In this device brain endothelial cells were cultured on a permeable membrane between two micro-channels and the permeability was measured by exposing the top (luminal) side of the cell monolayer to solution of a tracer molecule and measuring the diffused concentration in the bottom (abluminal) channel. The micro-devices presented in Chapters 2, 3 and 4 were intended to investigate the effects of PEFs at the cellular level. Chapter 5 shows how micro-device and specifically micro-sensors could be used to monitor the effects of PEFs at the tissue level. The impedance micro-sensors presented in this chapter were

successfully used to monitor the electrical impedance spectrum and give information about the size of the ablated tissue during IRE therapy.

The aforementioned achievements in this study and the developed microengineered platforms pave the way to many future experiments to extent our knowledge about PEFs-based therapies. Here we have presented a list of potential studies that could be done in continuation of this study to complement that from different aspects:

Biological aspects of the BBB

The main focus of our study on the BBB was to develop the required platforms for stimulating the cells and monitoring their response. These platforms were tested on cell lines such as bEnd3 and hCMEC. However, more physiologically relevant studies could be performed by using primary cells which give a better representation of the BBB. Having a co-culture of endothelial cells with other important cells of the BBB such as astrocytes, pericytes or neurons is also considered a significant improvement.

TJ disruption

In continuation of the work performed in Chapter 2 and by using the same platform, it would be possible to find the thresholds required to disrupt the TJs by staining the TJ-related proteins such as Occludin or Claudins after application of the PEFs. The results presented in Chapter 2 were corresponding to the transcellular transport across the BBB. On the other hand, disruption of the TJ could be correlated with the paracellular transport across the BBB.

Brain tumor-on-Chip

As a long term vision, the BBB model could be integrated into a more general model for studying brain tumor treatments (Figure 6-1). This model may have the BBB on top of a hydrogel layer which embeds the brain tumor cells. Neurons could be cultured as an underlying layer over

transparent electrodes. This model could be used to study the simultaneous effects of different therapies on the tumor cells and neurons, by visual observation of tumor cells and electrical stimulation/recording of neurons, respectively.

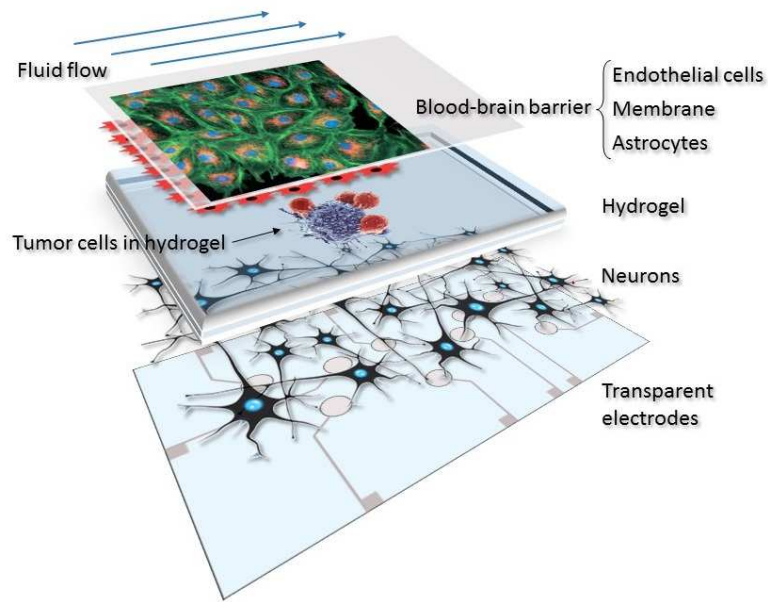


Figure 6-1. Comprehensive model of brain tumor-on-chip including BBB model on top, tumor cells embedded in hydrogel in the middle and neurons over electrodes at the bottom.

Ablation monitoring in animal tissue

The impedance sensor of Chapter 5 could be tested in animal tissue either *ex vivo* or *in vivo* to monitor the lesion during IRE therapy. From a technical standpoint, a tetra-polar measurement could increase the useful frequency range of acquired impedance spectra, which along with the proper circuit model of the tissue could be used to give detailed information about the physiological changes that undergo during the treatment. This information could eventually be used to determine the regions of reversible and irreversible ablations during the treatment.

References

- [1] J. W. Ivey, M. Bonakdar, A. Kanitkar, R. V. Davalos, and S. S. Verbridge, "Improving cancer therapies by targeting the physical and chemical hallmarks of the tumor microenvironment," *Cancer letters*, 2015.
- [2] C. Bettinger, J. Borenstein, and S. Tao, *Microfluidic cell culture systems*, 1st ed. Oxford ; Waltham, MA: William Andrew, 2013.
- [3] P. Neuzi, S. Giselsbrecht, K. Lange, T. J. Huang, and A. Manz, "Revisiting lab-on-a-chip technology for drug discovery," *Nat Rev Drug Discov*, vol. 11, pp. 620-32, Aug 2012.
- [4] E. W. Young and D. J. Beebe, "Fundamentals of microfluidic cell culture in controlled microenvironments," *Chemical Society Reviews*, vol. 39, pp. 1036-1048, 2010.
- [5] L. De Bartolo, J. V. Schweder, A. Haverich, and A. Bader, "A Novel Full - Scale Flat Membrane Bioreactor Utilizing Porcine Hepatocytes: Cell Viability and Tissue - Specific Functions," *Biotechnology progress*, vol. 16, pp. 102-108, 2000.
- [6] R. Booth and H. Kim, "Characterization of a microfluidic in vitro model of the blood-brain barrier (mu BBB)," *Lab on a Chip*, vol. 12, pp. 1784-1792, 2012.
- [7] N. J. Douville, Y.-C. Tung, R. Li, J. D. Wang, M. E. El-Sayed, and S. Takayama, "Fabrication of two-layered channel system with embedded electrodes to measure resistance across epithelial and endothelial barriers," *Analytical chemistry*, vol. 82, pp. 2505-2511, 2010.
- [8] B. Zhang, M.-C. Kim, T. Thorsen, and Z. Wang, "A self-contained microfluidic cell culture system," *Biomedical microdevices*, vol. 11, pp. 1233-1237, 2009.
- [9] Y.-C. Toh, T. C. Lim, D. Tai, G. Xiao, D. van Noort, and H. Yu, "A microfluidic 3D hepatocyte chip for drug toxicity testing," *Lab on a Chip*, vol. 9, pp. 2026-2035, 2009.
- [10] S. N. Bhatia and D. E. Ingber, "Microfluidic organs-on-chips," *Nature biotechnology*, 2014.
- [11] D. Huh, G. A. Hamilton, and D. E. Ingber, "From 3D cell culture to organs-on-chips," *Trends in cell biology*, vol. 21, pp. 745-754, 2011.
- [12] S. A. Lee, Y. No da, E. Kang, J. Ju, D. S. Kim, and S. H. Lee, "Spheroid-based three-dimensional liver-on-a-chip to investigate hepatocyte-hepatic stellate cell interactions and flow effects," *Lab Chip*, vol. 13, pp. 3529-37, Sep 21 2013.
- [13] V. N. Goral and P. K. Yuen, "Microfluidic platforms for hepatocyte cell culture: new technologies and applications," *Ann Biomed Eng*, vol. 40, pp. 1244-54, Jun 2012.
- [14] D. Huh, B. D. Matthews, A. Mammoto, M. Montoya-Zavala, H. Y. Hsin, and D. E. Ingber, "Reconstituting organ-level lung functions on a chip," *Science*, vol. 328, pp. 1662-8, Jun 25 2010.
- [15] D. Huh, B. D. Matthews, A. Mammoto, M. Montoya-Zavala, and D. E. Ingber, "A human breathing lung-on-a-chip for drug screening and nanotoxicology applications," *Drug Metabolism Reviews*, vol. 43, pp. 6-6, Nov 2011.
- [16] X. Zhang, Q. Wang, B. Gablaski, X. Zhang, P. Lucchesi, and Y. Zhao, "A microdevice for studying intercellular electromechanical transduction in adult cardiac myocytes," *Lab on a Chip*, vol. 13, pp. 3090-3097, 2013.
- [17] H. Kimura, T. Yamamoto, H. Sakai, Y. Sakai, and T. Fujii, "An integrated microfluidic system for long-term perfusion culture and on-line monitoring of intestinal tissue models," *Lab Chip*, vol. 8, pp. 741-746, 2008.

- [18] G. Zeck and P. Fromherz, "Noninvasive neuroelectronic interfacing with synaptically connected snail neurons immobilized on a semiconductor chip," *Proceedings of the National Academy of Sciences*, vol. 98, pp. 10457-10462, 2001.
- [19] K.-J. Jang, H. S. Cho, W. G. Bae, T.-H. Kwon, and K.-Y. Suh, "Fluid-shear-stress-induced translocation of aquaporin-2 and reorganization of actin cytoskeleton in renal tubular epithelial cells," *Integrative Biology*, vol. 3, pp. 134-141, 2011.
- [20] S. Srigunapalan, C. Lam, A. R. Wheeler, and C. A. Simmons, "A microfluidic membrane device to mimic critical components of the vascular microenvironment," *Biomicrofluidics*, vol. 5, p. 013409, 2011.
- [21] J. W. Song, S. P. Cavnar, A. C. Walker, K. E. Luker, M. Gupta, Y.-C. Tung, *et al.*, "Microfluidic endothelium for studying the intravascular adhesion of metastatic breast cancer cells," *PloS one*, vol. 4, p. e5756, 2009.
- [22] D. Wlodkowic and J. M. Cooper, "Tumors on chips: oncology meets microfluidics," *Current Opinion in Chemical Biology*, vol. 14, pp. 556-567, Oct 2010.
- [23] W. M. Pardridge, "Why is the global CNS pharmaceutical market so under-penetrated?," *Drug Discovery Today*, vol. 7, pp. 5-7, 1/1/ 2002.
- [24] P. Ehrlich, "Das sauerstoffbedürfnis des organismus," *Eine Farbenanalytische Studie*, 1885.
- [25] N. J. Abbott, L. Rönnbäck, and E. Hansson, "Astrocyte–endothelial interactions at the blood–brain barrier," *Nature Reviews Neuroscience*, vol. 7, pp. 41-53, 2006.
- [26] C. H. Lai and K. H. Kuo, "The critical component to establish in vitro BBB model: Pericyte," *Brain Research Reviews*, vol. 50, pp. 258-265, Dec 15 2005.
- [27] B. T. Hawkins and T. P. Davis, "The blood-brain barrier/neurovascular unit in health and disease," *Pharmacol Rev*, vol. 57, pp. 173-85, Jun 2005.
- [28] Y. Chen and L. Liu, "Modern methods for delivery of drugs across the blood–brain barrier," *Advanced drug delivery reviews*, vol. 64, pp. 640-665, 2012.
- [29] G. Li, M. Simon, L. Cancel, Z.-D. Shi, X. Ji, J. Tarbell, *et al.*, "Permeability of Endothelial and Astrocyte Cocultures: In Vitro Blood–Brain Barrier Models for Drug Delivery Studies," *Annals of Biomedical Engineering*, vol. 38, pp. 2499-2511, 2010/08/01 2010.
- [30] B. M. Fu, "Experimental methods and transport models for drug delivery across the blood-brain barrier," *Curr Pharm Biotechnol*, vol. 13, pp. 1346-59, Jun 2012.
- [31] U. Kniesel and H. Wolburg, "Tight junctions of the blood–brain barrier," *Cellular and molecular neurobiology*, vol. 20, pp. 57-76, 2000.
- [32] H. Wolburg and A. Lippoldt, "Tight junctions of the blood–brain barrier: development, composition and regulation," *Vascular pharmacology*, vol. 38, pp. 323-337, 2002.
- [33] M. Furuse, T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, and S. Tsukita, "Occludin: a novel integral membrane protein localizing at tight junctions," *The Journal of cell biology*, vol. 123, pp. 1777-1788, 1993.
- [34] M. Furuse, K. Fujita, T. Hiiragi, K. Fujimoto, and S. Tsukita, "Claudin-1 and-2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin," *The Journal of cell biology*, vol. 141, pp. 1539-1550, 1998.
- [35] B. R. Stevenson, J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough, "Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia," *The Journal of cell biology*, vol. 103, pp. 755-766, 1986.

- [36] L. A. Jesaitis and D. A. Goodenough, "Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the *Drosophila* discs-large tumor suppressor protein," *The Journal of cell biology*, vol. 124, pp. 949-961, 1994.
- [37] M. S. Balda, L. Gonzalez-Mariscal, K. Matter, M. Cereijido, and J. M. Anderson, "Assembly of the tight junction: the role of diacylglycerol," *The Journal of Cell Biology*, vol. 123, pp. 293-302, 1993.
- [38] Y. Zhong, K. Enomoto, H. Isomura, N. Sawada, T. Minase, M. Oyamada, *et al.*, "Localization of the 7H6 antigen at tight junctions correlates with the paracellular barrier function of MDCK cells," *Experimental cell research*, vol. 214, pp. 614-620, 1994.
- [39] S. Citi, H. Sabanay, J. Kendrick-Jones, and B. Geiger, "Cingulin: characterization and localization," *Journal of Cell Science*, vol. 93, pp. 107-122, 1989.
- [40] I. Martìn-Padura, S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, *et al.*, "Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration," *The Journal of cell biology*, vol. 142, pp. 117-127, 1998.
- [41] M. Itoh, M. Furuse, K. Morita, K. Kubota, M. Saitou, and S. Tsukita, "Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins," *The Journal of cell biology*, vol. 147, pp. 1351-1363, 1999.
- [42] A. G. Howarth, M. R. Hughes, and B. R. Stevenson, "Detection of the tight junction-associated protein ZO-1 in astrocytes and other nonepithelial cell types," *American Journal of Physiology-Cell Physiology*, vol. 262, pp. C461-C469, 1992.
- [43] B. R. Stevenson, J. M. Anderson, D. A. Goodenough, and M. S. Mooseker, "Tight junction structure and ZO-1 content are identical in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance," *The Journal of cell biology*, vol. 107, pp. 2401-2408, 1988.
- [44] J. D. Huber, R. D. Egleton, and T. P. Davis, "Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier," *Trends in Neurosciences*, vol. 24, pp. 719-725, 12/1/ 2001.
- [45] B. M Fu, "Experimental methods and transport models for drug delivery across the blood-brain barrier," *Current pharmaceutical biotechnology*, vol. 13, pp. 1346-1359, 2012.
- [46] W. M. Pardridge, "Drug and gene delivery to the brain: the vascular route," *Neuron*, vol. 36, pp. 555-558, 2002.
- [47] W. M. Pardridge, "Drug and gene targeting to the brain with molecular trojan horses," *Nat Rev Drug Discov*, vol. 1, pp. 131-139, 02//print 2002.
- [48] W. Pardridge, "Blood-brain barrier genomics and the use of endogenous transporters to cause drug penetration into the brain," *Current opinion in drug discovery & development*, vol. 6, pp. 683-691, 2003.
- [49] R. A. Kroll and E. A. Neuwelt, "Outwitting the blood-brain barrier for therapeutic purposes: osmotic opening and other means," *Neurosurgery*, vol. 42, pp. 1083-99; discussion 1099-100, May 1998.
- [50] K. Hynynen, N. McDannold, N. A. Sheikov, F. A. Jolesz, and N. Vykhodtseva, "Local and reversible blood-brain barrier disruption by noninvasive focused ultrasound at frequencies suitable for trans-skull sonications," *Neuroimage*, vol. 24, pp. 12-20, Jan 1 2005.
- [51] L. H. Treat, N. McDannold, N. Vykhodtseva, Y. Z. Zhang, K. Tam, and K. Hynynen, "Targeted delivery of doxorubicin to the rat brain at therapeutic levels using MRI-guided focused ultrasound," *International Journal of Cancer*, vol. 121, pp. 901-907, Aug 2007.

- [52] N. I. Vykhodtseva, K. Hynynen, and C. Damianou, "Histologic effects of high intensity pulsed ultrasound exposure with subharmonic emission in rabbit brain in vivo," *Ultrasound in Medicine & Biology*, vol. 21, pp. 969-979, // 1995.
- [53] N. D. Doolittle, M. E. Miner, W. A. Hall, T. Siegal, E. J. Hanson, E. Osztie, *et al.*, "Safety and efficacy of a multicenter study using intraarterial chemotherapy in conjunction with osmotic opening of the blood-brain barrier for the treatment of patients with malignant brain tumors," *Cancer*, vol. 88, pp. 637-647, 2000.
- [54] E. C. Unger, T. Porter, W. Culp, R. Labell, T. Matsunaga, and R. Zutshi, "Therapeutic applications of lipid-coated microbubbles," *Advanced Drug Delivery Reviews*, vol. 56, pp. 1291-1314, May 2004.
- [55] S. V. Lopez-Quintero, A. Datta, R. Amaya, M. Elwassif, M. Bikson, and J. M. Tarbell, "DBS-relevant electric fields increase hydraulic conductivity of in vitro endothelial monolayers," *Journal of Neural Engineering*, vol. 7, Feb 2010.
- [56] C. B. Arena, P. A. Garcia, M. B. Sano, J. D. Olson, T. Rogers-Cotrone, J. H. Rossmeisl Jr, *et al.*, "Focal blood-brain-barrier disruption with high-frequency pulsed electric fields," *TECHNOLOGY*, vol. 2, pp. 206-213, 2014.
- [57] P. A. Garcia, J. H. Rossmeisl, J. L. Robertson, J. D. Olson, A. J. Johnson, T. L. Ellis, *et al.*, "7.0-T Magnetic Resonance Imaging Characterization of Acute Blood-Brain-Barrier Disruption Achieved with Intracranial Irreversible Electroporation," *Plos One*, vol. 7, Nov 2012.
- [58] M. Hjouj, D. Last, D. Guez, D. Daniels, S. Sharabi, J. Lavee, *et al.*, "MRI study on reversible and irreversible electroporation induced blood brain barrier disruption," *PLoS One*, vol. 7, p. e42817, 2012.
- [59] N. McDannold, N. Vykhodtseva, and K. Hynynen, "Targeted disruption of the blood-brain barrier with focused ultrasound: association with cavitation activity," *Physics in medicine and biology*, vol. 51, p. 793, 2006.
- [60] H.-L. Liu, M.-Y. Hua, P.-Y. Chen, P.-C. Chu, C.-H. Pan, H.-W. Yang, *et al.*, "Blood-Brain Barrier Disruption with Focused Ultrasound Enhances Delivery of Chemotherapeutic Drugs for Glioblastoma Treatment," *Radiology*, vol. 255, pp. 415-425, 2010.
- [61] K. Warren, R. Jakacki, B. Widemann, A. Aikin, M. Libucha, R. Packer, *et al.*, "Phase II trial of intravenous lobaradimil and carboplatin in childhood brain tumors: a report from the Children's Oncology Group," *Cancer Chemotherapy and Pharmacology*, vol. 58, pp. 343-347, 2006/09/01 2006.
- [62] S. I. Rapoport, "Osmotic opening of the blood-brain barrier: principles, mechanism, and therapeutic applications," *Cellular and molecular neurobiology*, vol. 20, pp. 217-230, 2000.
- [63] K. Prokai-Tatrai, L. Prokai, and N. Bodor, "Brain-targeted delivery of a leucine-enkephalin analogue by retrometabolic design," *J Med Chem*, vol. 39, pp. 4775-82, Nov 22 1996.
- [64] J. Huwyler, D. Wu, and W. M. Pardridge, "Brain drug delivery of small molecules using immunoliposomes," *Proceedings of the National Academy of Sciences*, vol. 93, pp. 14164-14169, November 26, 1996 1996.
- [65] F. Agnesi, M. D. Johnson, and J. L. Vitek, "Chapter 4 - Deep brain stimulation: how does it work?," in *Handbook of Clinical Neurology*. vol. Volume 116, M. L. Andres and H. Mark, Eds., ed: Elsevier, 2013, pp. 39-54.

- [66] R. Sandyk, "Treatment with electromagnetic fields reverses the long-term clinical course of a patient with chronic progressive multiple sclerosis," *International Journal of Neuroscience*, vol. 90, pp. 177-185, 1997.
- [67] L. G. Salford, B. R. R. Persson, A. Brun, C. P. Ceberg, P. C. Kongstad, and L. M. Mir, "A New Brain Tumor Therapy Combining Bleomycin with in Vivo Electroporation," *Biochemical and Biophysical Research Communications*, vol. 194, pp. 938-943, 7/30/1993.
- [68] B. Agerholm-Larsen, H. K. Iversen, P. Ibsen, J. M. Moller, F. Mahmood, K. S. Jensen, *et al.*, "Preclinical validation of electrochemotherapy as an effective treatment for brain tumors," *Cancer Res*, vol. 71, pp. 3753-62, Jun 1 2011.
- [69] R. V. Davalos, I. L. Mir, and B. Rubinsky, "Tissue ablation with irreversible electroporation," *Ann Biomed Eng*, vol. 33, pp. 223-31, Feb 2005.
- [70] P. A. Garcia, T. Pancotto, J. H. Rossmeisl, N. Henao-Guerrero, N. R. Gustafson, G. B. Daniel, *et al.*, "Non-Thermal Irreversible Electroporation (N-TIRE) and Adjuvant Fractionated Radiotherapeutic Multimodal Therapy for Intracranial Malignant Glioma in a Canine Patient," *Technology in Cancer Research & Treatment*, vol. 10, pp. 73-83, Feb 2011.
- [71] J. Chunlan, R. V. Davalos, and J. C. Bischof, "A Review of Basic to Clinical Studies of Irreversible Electroporation Therapy," *Biomedical Engineering, IEEE Transactions on*, vol. 62, pp. 4-20, 2015.
- [72] C. Kanthou, S. Kranjc, G. Sersa, G. Tozer, A. Zupanic, and M. Cemazar, "The endothelial cytoskeleton as a target of electroporation-based therapies," *Molecular cancer therapeutics*, vol. 5, pp. 3145-3152, 2006.
- [73] S. V. Lopez-Quintero, A. Datta, R. Amaya, M. Elwassif, M. Bikson, and J. M. Tarbell, "DBS-relevant electric fields increase hydraulic conductivity of in vitro endothelial monolayers," *J Neural Eng*, vol. 7, p. 16005, Feb 2010.
- [74] R. Stam, "Electromagnetic fields and the blood-brain barrier," *Brain research reviews*, vol. 65, pp. 80-97, 2010.
- [75] A. H. Frey and S. R. Feld, "Neural function and behavior: defining the relationship," *Annals of the New York Academy of Sciences*, vol. 247, pp. 433-439, 1975.
- [76] G. Ding, K. Li, X. Wang, Y. Zhou, L. Qiu, J. Tan, *et al.*, "Effects of exposure to electromagnetic pulse on the permeability of brain micro vascular in rats," *Biochem Environ Sci*, vol. 22, pp. 255-258, 2009.
- [77] J. X. Zhou, G. R. Ding, J. Zhang, Y. C. Zhou, Y. J. Zhang, and G. Z. Guo, "Detrimental effect of electromagnetic pulse exposure on permeability of in vitro blood-brain-barrier model," *Biomed Environ Sci*, vol. 26, pp. 128-37, Feb 2013.
- [78] L. G. Salford, A. Brun, K. Stureson, J. L. Eberhardt, and B. R. Persson, "Permeability of the blood - brain barrier induced by 915 MHz electromagnetic radiation, continuous wave and modulated at 8, 16, 50, and 200 Hz," *Microscopy research and technique*, vol. 27, pp. 535-542, 1994.
- [79] C. B. Arena, P. A. Garcia, M. B. Sano, J. D. Olson, T. Rogers-Cotrone, J. H. Rossmeisl, *et al.*, "Focal blood-brain-barrier disruption with high-frequency pulsed electric fields," *TECHNOLOGY*, vol. 0, pp. 1-8.
- [80] P. Perel, I. Roberts, E. Sena, P. Wheble, C. Briscoe, P. Sandercock, *et al.*, "Comparison of treatment effects between animal experiments and clinical trials: systematic review," *Bmj*, vol. 334, p. 197, 2007.

- [81] J. Bicker, G. Alves, A. Fortuna, and A. Falcão, "Blood–brain barrier models and their relevance for a successful development of CNS drug delivery systems: a review," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 87, pp. 409-432, 2014.
- [82] I. Wilhelm, C. Fazakas, and I. A. Krizbai, "In vitro models of the blood-brain barrier," *Acta Neurobiol Exp (Wars)*, vol. 71, pp. 113-28, 2011.
- [83] E. P. Eddy, B. E. Maleef, T. K. Hart, and P. L. Smith, "In vitro models to predict blood-brain barrier permeability," *Advanced Drug Delivery Reviews*, vol. 23, pp. 185-198, Jan 15 1997.
- [84] H. Franke, H.-J. Galla, and C. T. Beuckmann, "An improved low-permeability in vitro-model of the blood–brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol," *Brain Research*, vol. 818, pp. 65-71, 2/6/ 1999.
- [85] C. D. Hue, S. Q. Cao, S. F. Haider, K. V. Vo, G. B. Effgen, E. Vogel, *et al.*, "Blood-Brain Barrier Dysfunction after Primary Blast Injury in vitro," *Journal of Neurotrauma*, vol. 30, pp. 1652-1663, Oct 1 2013.
- [86] W. Fauquette, C. Amourette, M. P. Dehouck, and M. Diserbo, "Radiation-induced blood-brain barrier damages: an in vitro study," *Brain Res*, vol. 1433, pp. 114-26, Jan 18 2012.
- [87] A. Schirmacher, S. Winters, S. Fischer, J. Goeke, H. J. Galla, U. Kullnick, *et al.*, "Electromagnetic fields (1.8 GHz) increase the permeability to sucrose of the blood-brain barrier in vitro," *Bioelectromagnetics*, vol. 21, pp. 338-45, Jul 2000.
- [88] H. Franke, J. Streckert, A. Bitz, J. Goeke, V. Hansen, E. B. Ringelstein, *et al.*, "Effects of Universal Mobile Telecommunications System (UMTS) electromagnetic fields on the blood-brain barrier in vitro," *Radiat Res*, vol. 164, pp. 258-69, Sep 2005.
- [89] M. P. Chan, S. Morisawa, A. Nakayama, Y. Kawamoto, and M. Yoneda, "Development of an in vitro blood–brain barrier model to study the effects of endosulfan on the permeability of tight junctions and a comparative study of the cytotoxic effects of endosulfan on rat and human glial and neuronal cell cultures," *Environmental toxicology*, vol. 21, pp. 223-235, 2006.
- [90] S. Nakagawa, M. A. Deli, H. Kawaguchi, T. Shimizudani, T. Shimono, A. Kittel, *et al.*, "A new blood–brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes," *Neurochemistry international*, vol. 54, pp. 253-263, 2009.
- [91] J. Barar and Y. Omid, "Bioelectrical and permeability properties of brain microvasculature endothelial cells: Effects of tight junction modulators," *J Biol Sci*, vol. 8, pp. 556-562, 2008.
- [92] S. H. Ma, L. A. Lepak, R. J. Hussain, W. Shain, and M. L. Shuler, "An endothelial and astrocyte co-culture model of the blood–brain barrier utilizing an ultra-thin, nanofabricated silicon nitride membrane," *Lab on a chip*, vol. 5, pp. 74-85, 2005.
- [93] L. Cucullo, M. S. McAllister, K. Kight, L. Krizanac-Bengez, M. Marroni, M. R. Mayberg, *et al.*, "A new dynamic in vitro model for the multidimensional study of astrocyte-endothelial cell interactions at the blood-brain barrier," *Brain Research*, vol. 951, pp. 243-254, Oct 4 2002.
- [94] J. A. Kim, H. N. Kim, S.-K. Im, S. Chung, J. Y. Kang, and N. Choi, "Collagen-based brain microvasculature model in vitro using three-dimensional printed template," *Biomicrofluidics*, vol. 9, p. 024115, 2015.
- [95] F. R. Walter, S. Valkai, A. Kincses, A. Petneházi, T. Czeller, S. Veszélka, *et al.*, "A versatile lab-on-a-chip tool for modeling biological barriers," *Sensors and Actuators B: Chemical*, vol. 222, pp. 1209-1219, 2016.

- [96] L. M. Griep, F. Wolbers, B. de Wagenaar, P. M. ter Braak, B. B. Weksler, I. A. Romero, *et al.*, "BBB ON CHIP: microfluidic platform to mechanically and biochemically modulate blood-brain barrier function," *Biomedical Microdevices*, vol. 15, pp. 145-150, Feb 2013.
- [97] B. Prabhakarparandian, M. C. Shen, J. B. Nichols, I. R. Mills, M. Sidoryk-Wegrzynowicz, M. Aschner, *et al.*, "SyM-BBB: a microfluidic blood brain barrier model," *Lab on a Chip*, vol. 13, pp. 1093-1101, 2013.
- [98] S. Nag, "Blood-brain barrier permeability using tracers and immunohistochemistry," *The Blood-Brain Barrier: Biology and Research Protocols*, pp. 133-144, 2003.
- [99] D. H. Jo, R. Lee, J. H. Kim, H. O. Jun, T. G. Lee, and J. H. Kim, "Real-time estimation of paracellular permeability of cerebral endothelial cells by capacitance sensor array," *Scientific reports*, vol. 5, 2015.
- [100] Á. Könczöl, J. Müller, E. Földes, Z. Béni, K. Végh, Á. Kéry, *et al.*, "Applicability of a Blood-Brain Barrier Specific Artificial Membrane Permeability Assay at the Early Stage of Natural Product-Based CNS Drug Discovery," *Journal of Natural Products*, vol. 76, pp. 655-663, 2013/04/26 2013.
- [101] O. C. Colgan, N. T. Collins, G. Ferguson, R. P. Murphy, Y. A. Birney, P. A. Cahill, *et al.*, "Influence of basolateral condition on the regulation of brain microvascular endothelial tight junction properties and barrier function," *Brain Research*, vol. 1193, pp. 84-92, 2/8/2008.
- [102] R. H. Booth and H. Kim, "A parallel array microfluidic blood-brain barrier model for high-throughput quantitation of shear stress effects," in *Chemical and Biological Microsystems Society*, 2012.
- [103] V. Siddharthan, Y. V. Kim, S. Liu, and K. S. Kim, "Human astrocytes/astrocyte-conditioned medium and shear stress enhance the barrier properties of human brain microvascular endothelial cells," *Brain research*, vol. 1147, pp. 39-50, 2007.
- [104] L. Cucullo, M. Hossain, V. Puvenna, N. Marchi, and D. Janigro, "The role of shear stress in Blood-Brain Barrier endothelial physiology," *BMC neuroscience*, vol. 12, p. 40, 2011.
- [105] R. Booth, S. Noh, and H. Kim, "A multiple-channel, multiple-assay platform for characterization of full-range shear stress effects on vascular endothelial cells," *Lab on a Chip*, vol. 14, pp. 1880-1890, 2014.
- [106] A. K. H. Achyuta, A. J. Conway, R. B. Crouse, E. C. Bannister, R. N. Lee, C. P. Katnik, *et al.*, "A modular approach to create a neurovascular unit-on-a-chip," *Lab on a Chip*, vol. 13, pp. 542-553, 2013.
- [107] B. Rubinsky, *Irreversible electroporation*: Springer Science & Business Media, 2009.
- [108] R. V. Davalos, L. Mir, and B. Rubinsky, "Tissue ablation with irreversible electroporation," *Annals of biomedical engineering*, vol. 33, pp. 223-231, 2005.
- [109] Available: www.nanoknife.com
- [110] S. N. Goldberg, "Radiofrequency tumor ablation: principles and techniques," *European Journal of Ultrasound*, vol. 13, pp. 129-147, 2001.
- [111] G. ter Haar and C. Coussios, "High intensity focused ultrasound: physical principles and devices," *International Journal of Hyperthermia*, vol. 23, pp. 89-104, 2007.
- [112] B. G. Patel, C. L. Parsons, M. Bidair, and J. D. Schmidt, "Review: Cryoablation for carcinoma of the prostate," *Journal of surgical oncology*, vol. 63, pp. 256-264, 1996.
- [113] G. Carrafiello, D. Laganà, M. Mangini, F. Fontana, G. Dionigi, L. Boni, *et al.*, "Microwave tumors ablation: principles, clinical applications and review of preliminary experiences," *International Journal of Surgery*, vol. 6, pp. S65-S69, 2008.

- [114] M. Pech, A. Janitzky, J. J. Wendler, C. Strang, S. Blaschke, O. Dudeck, *et al.*, "Irreversible electroporation of renal cell carcinoma: a first-in-man phase I clinical study," *Cardiovascular and interventional radiology*, vol. 34, pp. 132-138, 2011.
- [115] R. Cannon, S. Ellis, D. Hayes, G. Narayanan, and R. C. Martin, "Safety and early efficacy of irreversible electroporation for hepatic tumors in proximity to vital structures," *Journal of surgical oncology*, vol. 107, pp. 544-549, 2013.
- [116] W. Cheung, H. Kavnoudias, S. Roberts, B. Szkandera, W. Kemp, and K. Thomson, "Irreversible electroporation for unresectable hepatocellular carcinoma: initial experience and review of safety and outcomes," *Technology in cancer research & treatment*, vol. 12, pp. 233-241, 2013.
- [117] V. Kasivisvanathan, A. Thapar, Y. Oskrochi, J. Picard, and E. L. Leen, "Irreversible electroporation for focal ablation at the porta hepatis," *Cardiovascular and interventional radiology*, vol. 35, pp. 1531-1534, 2012.
- [118] R. C. Martin, K. McFarland, S. Ellis, and V. Velanovich, "Irreversible electroporation therapy in the management of locally advanced pancreatic adenocarcinoma," *Journal of the American College of Surgeons*, vol. 215, pp. 361-369, 2012.
- [119] R. C. Martin II, K. McFarland, S. Ellis, and V. Velanovich, "Irreversible electroporation in locally advanced pancreatic cancer: potential improved overall survival," *Annals of surgical oncology*, vol. 20, pp. 443-449, 2013.
- [120] G. Narayanan, P. J. Hosein, G. Arora, K. J. Barbary, T. Froud, A. S. Livingstone, *et al.*, "Percutaneous irreversible electroporation for downstaging and control of unresectable pancreatic adenocarcinoma," *Journal of Vascular and Interventional Radiology*, vol. 23, pp. 1613-1621, 2012.
- [121] M. Usman, W. Moore, R. Talati, K. Watkins, and T. V. Bilfinger, "Irreversible electroporation of lung neoplasm: a case series," *Medical science monitor: international medical journal of experimental and clinical research*, vol. 18, p. CS43, 2012.
- [122] H. J. Scheffer, K. Nielsen, M. C. de Jong, A. A. van Tilborg, J. M. Vieveen, A. R. Bouwman, *et al.*, "Irreversible electroporation for nonthermal tumor ablation in the clinical setting: a systematic review of safety and efficacy," *Journal of Vascular and Interventional Radiology*, vol. 25, pp. 997-1011, 2014.
- [123] J. F. Edd and R. V. Davalos, "Mathematical Modeling of irreversible Electroporation for treatment planning," *Technology in Cancer Research & Treatment*, vol. 6, pp. 275-286, Aug 2007.
- [124] A. Zupanic, B. Kos, and D. Miklavcic, "Treatment planning of electroporation-based medical interventions: electrochemotherapy, gene electrotransfer and irreversible electroporation," *Physics in medicine and biology*, vol. 57, p. 5425, 2012.
- [125] D. Sel and D. Miklavcic, "Feasibility of employing model-based optimization of pulse amplitude and electrode distance for effective tumor electroporomeabilization," *Biomedical Engineering, IEEE Transactions on*, vol. 54, pp. 773-781, 2007.
- [126] P. Garcia, T. Pancotto, J. Rossmeisl, N. Henao-Guerrero, N. Gustafson, G. Daniel, *et al.*, "Non-thermal irreversible electroporation (N-TIRE) and adjuvant fractionated radiotherapeutic multimodal therapy for intracranial malignant glioma in a canine patient," *Technology in cancer research & treatment*, vol. 10, pp. 73-83, 2011.
- [127] M. Bonakdar, E. Latouche, R. Mahajan, and R. Davalos, "The Feasibility of a Smart Surgical Probe for Verification of IRE Treatments Using Electrical Impedance Spectroscopy," 2015.

- [128] R. Stämpfli, "Reversible electrical breakdown of the excitable membrane of a Ranvier node," *Ann. Acad. Brasil. Ciens*, vol. 30, pp. 57-63, 1958.
- [129] J. Teissie and M.-P. Rols, "An experimental evaluation of the critical potential difference inducing cell membrane electroporation," *Biophysical journal*, vol. 65, p. 409, 1993.
- [130] K. C. Melikov, V. A. Frolov, A. Shcherbakov, A. V. Samsonov, Y. A. Chizmadzhev, and L. V. Chernomordik, "Voltage-induced nonconductive pre-pores and metastable single pores in unmodified planar lipid bilayer," *Biophysical journal*, vol. 80, pp. 1829-1836, 2001.
- [131] A. Ivorra, "Tissue electroporation as a bioelectric phenomenon: Basic concepts," in *Irreversible Electroporation*, ed: Springer, 2010, pp. 23-61.
- [132] H. P. Schwan, "Electrical properties of tissue and cell suspensions," *Advances in biological and medical physics*, vol. 5, pp. 147-209, 1956.
- [133] K. Kinoshita, I. Ashikawa, N. Saita, H. Yoshimura, H. Itoh, K. Nagayama, *et al.*, "Electroporation of cell membrane visualized under a pulsed-laser fluorescence microscope," *Biophysical journal*, vol. 53, pp. 1015-1019, 1988.
- [134] E. Tekle, R. D. Astumian, and P. B. Chock, "Selective and asymmetric molecular transport across electroporated cell membranes," *Proceedings of the National Academy of Sciences*, vol. 91, pp. 11512-11516, 1994.
- [135] T. Kotnik and D. Miklavčič, "Analytical description of transmembrane voltage induced by electric fields on spheroidal cells," *Biophysical Journal*, vol. 79, pp. 670-679, 2000.
- [136] C. E. Miller and C. S. Henriquez, "Three-dimensional finite element solution for biopotentials: erythrocyte in an applied field," *Biomedical Engineering, IEEE Transactions on*, vol. 35, pp. 712-718, 1988.
- [137] Y. Huang and B. Rubinsky, "Micro-Electroporation: Improving the Efficiency and Understanding of Electrical Permeabilization of Cells," *Biomedical Microdevices*, vol. 2, pp. 145-150, 1999/03/01 1999.
- [138] A. Meir and B. Rubinsky, "Electrical impedance tomographic imaging of a single cell electroporation," *Biomedical Microdevices*, vol. 16, pp. 427-437, 2014/06/01 2014.
- [139] R. Davalos, Y. Huang, and B. Rubinsky, "Electroporation: Bio-electrochemical mass transfer at the nano scale," *Microscale Thermophysical Engineering*, vol. 4, pp. 147-159, 2000.
- [140] M. Pavlin, M. Kandušer, M. Reberšek, G. Pucihar, F. X. Hart, Magjarević, *et al.*, "Effect of Cell Electroporation on the Conductivity of a Cell Suspension," *Biophysical Journal*, vol. 88, pp. 4378-4390, 6// 2005.
- [141] J. Zhuang, A. Steuer, Y. Jing, and J. F. Kolb, *Pulsed Electric Field Induced Dielectric Evolution of Mammalian Cells*, 2012.
- [142] J. Zhuang, W. Ren, Y. Jing, and J. F. Kolb, "Dielectric Evolution of Mammalian Cell Membranes after Exposure to Pulsed Electric Fields," *Ieee Transactions on Dielectrics and Electrical Insulation*, vol. 19, pp. 609-622, Apr 2012.
- [143] J. A. Stolwijk, C. Hartmann, P. Balani, S. Albermann, C. R. Keese, I. Giaever, *et al.*, "Impedance analysis of adherent cells after in situ electroporation: Non-invasive monitoring during intracellular manipulations," *Biosensors and Bioelectronics*, vol. 26, pp. 4720-4727, 8/15/ 2011.
- [144] T. García-Sánchez, M. Guitart, J. Rosell-Ferrer, A. Gómez-Foix, and R. Bragós, "A new spiral microelectrode assembly for electroporation and impedance measurements of

- adherent cell monolayers," *Biomedical Microdevices*, vol. 16, pp. 575-590, 2014/08/01 2014.
- [145] R. V. Davalos, D. M. Otten, L. M. Mir, and B. Rubinsky, "Electrical impedance tomography for imaging tissue electroporation," *Ieee Transactions on Biomedical Engineering*, vol. 51, pp. 761-767, May 2004.
 - [146] C. Jiang, Q. Shao, and J. Bischof, "Pulse Timing During Irreversible Electroporation Achieves Enhanced Destruction in a Hindlimb Model of Cancer," *Annals of biomedical engineering*, pp. 1-9, 2014.
 - [147] C. B. Arena, R. L. Mahajan, M. N. Rylander, and R. V. Davalos, "An Experimental and Numerical Investigation of Phase Change Electrodes for Therapeutic Irreversible Electroporation," *Journal of biomechanical engineering*, vol. 135, p. 111009, 2013.
 - [148] L. Appelbaum, E. Ben-David, M. Faroja, Y. Nissenbaum, J. Sosna, and S. N. Goldberg, "Irreversible electroporation ablation: creation of large-volume ablation zones in in vivo porcine liver with four-electrode arrays," *Radiology*, vol. 270, pp. 416-424, 2014.
 - [149] S. K. Frandsen, H. Gissel, P. Hojman, T. Tramm, J. Eriksen, and J. Gehl, "Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis," *Cancer research*, vol. 72, pp. 1336-1341, 2012.
 - [150] C. B. Arena, M. B. Sano, J. H. Rossmeisl, Jr., J. L. Caldwell, P. A. Garcia, M. N. Rylander, *et al.*, "High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction," *Biomed Eng Online*, vol. 10, p. 102, 2011.
 - [151] C. B. Arena, M. B. Sano, M. N. Rylander, and R. V. Davalos, "Theoretical considerations of tissue electroporation with high-frequency bipolar pulses," *IEEE Trans Biomed Eng*, vol. 58, pp. 1474-82, May 2011.
 - [152] E. Barsoukov and J. R. Macdonald, *Impedance spectroscopy: theory, experiment, and applications*: John Wiley & Sons, 2005.
 - [153] Y. Chen and W. P. Jepson, "EIS measurement for corrosion monitoring under multiphase flow conditions," *Electrochimica Acta*, vol. 44, pp. 4453-4464, 7/31/ 1999.
 - [154] F. Mansfeld and C. H. Tsai, "Determination of Coating Deterioration with EIS: I. Basic Relationships," *Corrosion*, vol. 47, pp. 958-963, 1991/12/01 1991.
 - [155] A. Eddahech, O. Briat, N. Bertrand, J.-Y. Delétage, and J.-M. Vinassa, "Behavior and state-of-health monitoring of Li-ion batteries using impedance spectroscopy and recurrent neural networks," *International Journal of Electrical Power & Energy Systems*, vol. 42, pp. 487-494, 2012.
 - [156] M. À. Oliver, I. Gobantes, J. Arnau, J. Elvira, P. Riu, N. s. Grèbol, *et al.*, "Evaluation of the electrical impedance spectroscopy (EIS) equipment for ham meat quality selection," *Meat science*, vol. 58, pp. 305-312, 2001.
 - [157] J.-L. Damez, S. Clerjon, S. Abouelkaram, and J. Lepetit, "Beef meat electrical impedance spectroscopy and anisotropy sensing for non-invasive early assessment of meat ageing," *Journal of Food Engineering*, vol. 85, pp. 116-122, 2008.
 - [158] A. R. Varlan and W. Sansen, "Nondestructive electrical impedance analysis in fruit: normal ripening and injuries characterization," *Electromagnetic Biology and Medicine*, vol. 15, pp. 213-227, 1996.
 - [159] T. Sun, S. Gawad, C. Bernabini, N. G. Green, and H. Morgan, "Broadband single cell impedance spectroscopy using maximum length sequences: theoretical analysis and practical considerations," *Measurement Science & Technology*, vol. 18, pp. 2859-2868, Sep 2007.

- [160] A. H. Kyle, C. T. O. Chan, and A. I. Minchinton, "Characterization of Three-Dimensional Tissue Cultures Using Electrical Impedance Spectroscopy," *Biophysical Journal*, vol. 76, pp. 2640-2648, 5// 1999.
- [161] I. Giaever and C. R. Keese, "Use of Electric Fields to Monitor the Dynamical Aspect of Cell Behavior in Tissue Culture," *Biomedical Engineering, IEEE Transactions on*, vol. BME-33, pp. 242-247, 1986.
- [162] C. Tiruppathi, A. B. Malik, P. J. Del Vecchio, C. R. Keese, and I. Giaever, "Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function," *Proceedings of the National Academy of Sciences*, vol. 89, pp. 7919-7923, September 1, 1992 1992.
- [163] A. Bouafssoun, S. Helali, S. Mebarek, C. Zeiller, A.-F. Prigent, A. Othmane, *et al.*, "Electrical probing of endothelial cell behaviour on a fibronectin/polystyrene/thiol/gold electrode by Faradaic electrochemical impedance spectroscopy (EIS)," *Bioelectrochemistry*, vol. 70, pp. 401-407, 2007.
- [164] A. Bouafssoun, S. Helali, A. Othmane, A. Kerkeni, A. F. Prigent, N. Jaffrézic - Renault, *et al.*, "Evaluation of endothelial cell adhesion onto different protein/gold electrodes by EIS," *Macromolecular bioscience*, vol. 7, pp. 599-610, 2007.
- [165] J. Seebach, P. Dieterich, F. Luo, H. Schillers, D. Vestweber, H. Oberleithner, *et al.*, "Endothelial barrier function under laminar fluid shear stress," *Laboratory investigation*, vol. 80, pp. 1819-1831, 2000.
- [166] T. A. Nguyen, T.-I. Yin, D. Reyes, and G. A. Urban, "Microfluidic chip with integrated electrical cell-impedance sensing for monitoring single cancer cell migration in three-dimensional matrixes," *Analytical chemistry*, vol. 85, pp. 11068-11076, 2013.
- [167] E. Gersing, "Impedance spectroscopy on living tissue for determination of the state of organs," *Bioelectrochemistry and bioenergetics*, vol. 45, pp. 145-149, 1998.
- [168] M. Schaefer, W. Gross, J. Ackemann, and M. Gebhard, "The complex dielectric spectrum of heart tissue during ischemia," *Bioelectrochemistry*, vol. 58, pp. 171-180, 2002.
- [169] M. Schäfer, H. J. KIRLUM, C. Schlegel, and M. Gebhard, "Dielectric properties of skeletal muscle during ischemia in the frequency range from 50 Hz to 200 MHz," *Annals of the New York Academy of Sciences*, vol. 873, pp. 59-64, 1999.
- [170] Y. Zou and Z. Guo, "A review of electrical impedance techniques for breast cancer detection," *Medical Engineering & Physics*, vol. 25, pp. 79-90, 3// 2003.
- [171] R. J. Halter, A. Schned, J. Heaney, A. Hartov, S. Schutz, and K. D. Paulsen, "Electrical Impedance Spectroscopy of Benign and Malignant Prostatic Tissues," *The Journal of Urology*, vol. 179, pp. 1580-1586, 4// 2008.
- [172] A. Molckovsky and B. C. Wilson, "Monitoring of cell and tissue responses to photodynamic therapy by electrical impedance spectroscopy," *Physics in Medicine and Biology*, vol. 46, p. 983, 2001.
- [173] J. Wegener, S. Zink, P. Rösen, and H.-J. Galla, "Use of electrochemical impedance measurements to monitor β -adrenergic stimulation of bovine aortic endothelial cells," *Pflügers Archiv*, vol. 437, pp. 925-934, 1999.
- [174] M. Bonakdar, E. M. Wasson, Y. W. Lee, and R. V. Davalos, "Electroporation of Brain Endothelial Cells on Chip toward Permeabilizing the Blood-Brain Barrier," *Biophysical journal*, vol. 110, pp. 503-513, 2016.

- [175] M. Gumbleton and K. L. Audus, "Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier," *Journal of Pharmaceutical Sciences*, vol. 90, pp. 1681-1698, 2001.
- [176] G. Li, M. J. Simon, L. M. Cancel, Z.-D. Shi, X. Ji, J. M. Tarbell, *et al.*, "Permeability of Endothelial and Astrocyte Cocultures: In Vitro Blood–Brain Barrier Models for Drug Delivery Studies," *Annals of biomedical engineering*, vol. 38, pp. 2499-2511, 04/02 2010.
- [177] Y. Omid, L. Campbell, J. Barar, D. Connell, S. Akhtar, and M. Gumbleton, "Evaluation of the immortalised mouse brain capillary endothelial cell line, b. End3, as an in vitro blood–brain barrier model for drug uptake and transport studies," *Brain research*, vol. 990, pp. 95-112, 2003.
- [178] R. C. Brown, A. P. Morris, and R. G. O'Neil, "Tight junction protein expression and barrier properties of immortalized mouse brain microvessel endothelial cells," *Brain research*, vol. 1130, pp. 17-30, 2007.
- [179] N. Soga, J. O. Connolly, M. Chellaiah, J. Kawamura, and K. A. Hruska, "Rac regulates vascular endothelial growth factor stimulated motility," *Cell communication & adhesion*, 2009.
- [180] G. R. Ding, L. B. Qiu, X. W. Wang, K. C. Li, Y. C. Zhou, Y. Zhou, *et al.*, "EMP-induced alterations of tight junction protein expression and disruption of the blood-brain barrier," *Toxicol Lett*, vol. 196, pp. 154-60, Jul 15 2010.
- [181] G. W. Gross, W. Y. Wen, and J. W. Lin, "Transparent indium-tin oxide electrode patterns for extracellular, multisite recording in neuronal cultures," *Journal of Neuroscience Methods*, vol. 15, pp. 243-252, 11// 1985.
- [182] H. Hillebrandt, A. Abdelghani, C. Abdelghani-Jacquín, M. Aepfelbacher, and E. Sackmann, "Electrical and optical characterization of thrombin-induced permeability of cultured endothelial cell monolayers on semiconductor electrode arrays," *Applied Physics A*, vol. 73, pp. 539-546, 2001/11/01 2001.
- [183] H. Kobayashi, T. Ishida, K. Nakamura, Y. Nakato, and H. Tsubomura, "Properties of Indium Tin Oxide-Films Prepared by the Electron-Beam Evaporation Method in Relation to Characteristics of Indium Tin Oxide Silicon-Oxide Silicon Junction Solar-Cells," *Journal of Applied Physics*, vol. 72, pp. 5288-5293, Dec 1 1992.
- [184] R. K. Shah and A. L. London, *Laminar flow forced convection in ducts : a source book for compact heat exchanger analytical data*. New York: Academic Press, 1978.
- [185] Z. Stojek, "The electrical double layer and its structure," in *Electroanalytical methods*, ed: Springer, 2010, pp. 3-9.
- [186] R. P. Janek, W. R. Fawcett, and A. Ulman, "Impedance Spectroscopy of Self-Assembled Monolayers on Au(111): Sodium Ferrocyanide Charge Transfer at Modified Electrodes," *Langmuir*, vol. 14, pp. 3011-3018, 1998/05/01 1998.
- [187] E. T. McAdams, A. Lacknermeier, J. A. McLaughlin, D. Macken, and J. Jossinet, "The linear and non-linear electrical properties of the electrode-electrolyte interface," *Biosensors and Bioelectronics*, vol. 10, pp. 67-74, // 1995.
- [188] E. Katz and I. Willner, "Probing biomolecular interactions at conductive and semiconductive surfaces by impedance spectroscopy: routes to impedimetric immunosensors, DNA - sensors, and enzyme biosensors," *Electroanalysis*, vol. 15, pp. 913-947, 2003.
- [189] K. S. Cole, *Membranes, ions, and impulses; a chapter of classical biophysics*. Berkeley,: University of California Press, 1968.

- [190] M. E. Orazem, P. Agarwal, and L. H. Garcia-Rubio, "Critical issues associated with interpretation of impedance spectra," *Journal of Electroanalytical Chemistry*, vol. 378, pp. 51-62, 1994.
- [191] A. M. Malek, G. G. Goss, L. Jiang, S. Izumo, and S. L. Alper, "Mannitol at clinical concentrations activates multiple signaling pathways and induces apoptosis in endothelial cells," *Stroke*, vol. 29, pp. 2631-2640, 1998.
- [192] B.-h. Chueh, D. Huh, C. R. Kyrtsos, T. Houssin, N. Futai, and S. Takayama, "Leakage-free bonding of porous membranes into layered microfluidic array systems," *Analytical chemistry*, vol. 79, pp. 3504-3508, 2007.
- [193] G. Li, M. J. Simon, L. M. Cancel, Z.-D. Shi, X. Ji, J. M. Tarbell, *et al.*, "Permeability of endothelial and astrocyte cocultures: in vitro blood–brain barrier models for drug delivery studies," *Annals of biomedical engineering*, vol. 38, pp. 2499-2511, 2010.
- [194] M. Gumbleton and K. L. Audus, "Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood - brain barrier," *Journal of pharmaceutical sciences*, vol. 90, pp. 1681-1698, 2001.
- [195] S. Wagner, A. Zensi, S. L. Wien, S. E. Tschickardt, W. Maier, T. Vogel, *et al.*, "Uptake mechanism of ApoE-modified nanoparticles on brain capillary endothelial cells as a blood-brain barrier model," *PloS one*, vol. 7, p. e32568, 2012.
- [196] R. Booth and H. Kim, "Characterization of a microfluidic in vitro model of the blood-brain barrier (μ BBB)," *Lab on a chip*, vol. 12, pp. 1784-1792, 2012.
- [197] W. Yuan, Y. Lv, M. Zeng, and B. M. Fu, "Non-invasive measurement of solute permeability in cerebral microvessels of the rat," *Microvascular Research*, vol. 77, pp. 166-173, 3// 2009.
- [198] L. Cucullo, M. Hossain, E. Rapp, T. Manders, N. Marchi, and D. Janigro, "Development of a humanized in vitro blood–brain barrier model to screen for brain penetration of antiepileptic drugs," *Epilepsia*, vol. 48, pp. 505-516, 2007.
- [199] M. Boveri, V. Berezowski, A. Price, S. Slupek, A. M. Lenfant, C. Benaud, *et al.*, "Induction of blood - brain barrier properties in cultured brain capillary endothelial cells: Comparison between primary glial cells and C6 cell line," *Glia*, vol. 51, pp. 187-198, 2005.
- [200] K. F. Chu and D. E. Dupuy, "Thermal ablation of tumours: biological mechanisms and advances in therapy," *Nat Rev Cancer*, vol. 14, pp. 199-208, Mar 2014.
- [201] A. Ivorra and B. Rubinsky, "Historical Review of Irreversible Electroporation in Medicine," in *Irreversible Electroporation*, B. Rubinsky, Ed., ed: Springer Berlin Heidelberg, 2010, pp. 1-21.
- [202] L. M. Mir, S. Orlowski, J. Belehradek, Jr., and C. Paoletti, "Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses," *Eur J Cancer*, vol. 27, pp. 68-72, 1991.
- [203] L. C. Heller and R. Heller, "In vivo electroporation for gene therapy," *Hum Gene Ther*, vol. 17, pp. 890-7, Sep 2006.
- [204] R. V. Davalos, L. M. Mir, and B. Rubinsky, "Tissue ablation with irreversible electroporation," *Annals of Biomedical Engineering*, vol. 33, pp. 223-231, Feb 2005.
- [205] M. L. Yarmush, A. Golberg, G. Sersa, T. Kotnik, and D. Miklavcic, "Electroporation-Based Technologies for Medicine: Principles, Applications, and Challenges," *Annual Review of Biomedical Engineering*, Vol 16, vol. 16, pp. 295-320, 2014.

- [206] D. Miklavcic, B. Mali, B. Kos, R. Heller, and G. Sersa, "Electrochemotherapy: from the drawing board into medical practice," *Biomedical Engineering Online*, vol. 13, Mar 12 2014.
- [207] D. A. Dean, "Nonviral gene transfer to skeletal, smooth, and cardiac muscle in living animals," *Am J Physiol Cell Physiol*, vol. 289, pp. C233-45, Aug 2005.
- [208] M. Jaroszeski, R. Gilbert, C. Nicolau, and R. Heller, "Delivery of Genes In Vivo Using Pulsed Electric Fields," in *Electrochemotherapy, Electrogenetherapy, and Transdermal Drug Delivery*. vol. 37, M. Jaroszeski, R. Heller, and R. Gilbert, Eds., ed: Humana Press, 2000, pp. 173-186.
- [209] C. L. Jiang, R. V. Davalos, and J. C. Bischof, "A Review of Basic to Clinical Studies of Irreversible Electroporation Therapy," *Ieee Transactions on Biomedical Engineering*, vol. 62, pp. 4-20, Jan 2015.
- [210] H. J. Scheffer, K. Nielsen, M. C. de Jong, A. A. J. M. van Tilborg, J. M. Vieveen, A. R. A. Bouwman, *et al.*, "Irreversible Electroporation for Nonthermal Tumor Ablation in the Clinical Setting: A Systematic Review of Safety and Efficacy," *Journal of Vascular and Interventional Radiology*, vol. 25, pp. 997-1011, Jul 2014.
- [211] B. Al-Sakere, F. Andre, C. Bernat, E. Connault, P. Opolon, R. V. Davalos, *et al.*, "Tumor Ablation with Irreversible Electroporation," *Plos One*, vol. 2, Nov 7 2007.
- [212] B. Rubinsky, "Irreversible Electroporation in Medicine," *Technology in Cancer Research & Treatment*, vol. 6, pp. 255-259, August 1, 2007 2007.
- [213] E. W. Lee, C. T. Loh, and S. T. Kee, "Imaging guided percutaneous irreversible electroporation: Ultrasound and immunohistological correlation," *Technology in Cancer Research & Treatment*, vol. 6, pp. 287-293, Aug 2007.
- [214] B. Rubinsky, G. Onik, and P. Mikus, "Irreversible electroporation: A new ablation modality - Clinical implications," *Technology in Cancer Research & Treatment*, vol. 6, pp. 37-48, Feb 2007.
- [215] E. W. Lee, C. Chen, V. E. Prieto, S. M. Dry, C. T. Loh, and S. T. Kee, "Advanced Hepatic Ablation Technique for Creating Complete Cell Death : Irreversible Electroporation," *Radiology*, vol. 255, pp. 426-433, May 2010.
- [216] S. N. Goldberg, G. S. Gazelle, and P. R. Mueller, "Thermal ablation therapy for focal malignancy: a unified approach to underlying principles, techniques, and diagnostic imaging guidance," *AJR Am J Roentgenol*, vol. 174, pp. 323-31, Feb 2000.
- [217] C. Daniels and B. Rubinsky, "Electrical Field and Temperature Model of Nonthermal Irreversible Electroporation in Heterogeneous Tissues," *Journal of Biomechanical Engineering-Transactions of the Asme*, vol. 131, Jul 2009.
- [218] R. E. Neal, P. A. Garcia, J. L. Robertson, and R. V. Davalos, "Experimental Characterization and Numerical Modeling of Tissue Electrical Conductivity during Pulsed Electric Fields for Irreversible Electroporation Treatment Planning," *Ieee Transactions on Biomedical Engineering*, vol. 59, pp. 1076-1085, Apr 2012.
- [219] S. Corovic, L. M. Mir, and D. Miklavcic, "In vivo muscle electroporation threshold determination: realistic numerical models and in vivo experiments," *J Membr Biol*, vol. 245, pp. 509-20, Sep 2012.
- [220] R. V. Davalos and B. Rubinsky, "Temperature considerations during irreversible electroporation," *International Journal of Heat and Mass Transfer*, vol. 51, pp. 5617-5622, Nov 2008.

- [221] J. Estrela da Silva, J. P. Marques de Sá, and J. Jossinet, "Classification of breast tissue by electrical impedance spectroscopy," *Medical and Biological Engineering and Computing*, vol. 38, pp. 26-30, 2000/01/01 2000.
- [222] J. Jossinet, "Variability of impedivity in normal and pathological breast tissue," *Medical and Biological Engineering and Computing*, vol. 34, pp. 346-350, 1996/09/01 1996.
- [223] S. Laufer, A. Ivorra, V. E. Reuter, B. Rubinsky, and S. B. Solomon, "Electrical impedance characterization of normal and cancerous human hepatic tissue," *Physiol Meas*, vol. 31, pp. 995-1009, Jul 2010.
- [224] Y. Granot, A. Ivorra, E. Maor, and B. Rubinsky, "In vivo imaging of irreversible electroporation by means of electrical impedance tomography," *Phys Med Biol*, vol. 54, pp. 4927-4943, Aug 21 2009.
- [225] M. Kranjc, F. Bajd, I. Sersa, and D. Miklavcic, "Magnetic resonance electrical impedance tomography for measuring electrical conductivity during electroporation," *Physiological Measurement*, vol. 35, pp. 985-96, Jun 2014.
- [226] A. Ivorra and B. Rubinsky, "In vivo electrical impedance measurements during and after electroporation of rat liver," *Bioelectrochemistry*, vol. 70, pp. 287-295, May 2007.
- [227] A. Ivorra, B. Al-Sakere, B. Rubinsky, and L. M. Mir, "In vivo electrical conductivity measurements during and after tumor electroporation: conductivity changes reflect the treatment outcome," *Phys Med Biol*, vol. 54, pp. 5949-5963, Oct 7 2009.
- [228] R. C. G. Martin, "Irreversible Electroporation of Locally Advanced Pancreatic Head Adenocarcinoma," *Journal of Gastrointestinal Surgery*, vol. 17, pp. 1850-1856, Oct 2013.
- [229] E. M. Dunki-Jacobs, P. Philips, and R. C. G. Martin, "Evaluation of Resistance as a Measure of Successful Tumor Ablation During Irreversible Electroporation of the Pancreas," *Journal of the American College of Surgeons*, vol. 218, pp. 179-187, 2// 2014.
- [230] Y.-H. Kim, C. Lee, K.-M. Ahn, M. Lee, and Y.-J. Kim, "Robust and real-time monitoring of nerve regeneration using implantable flexible microelectrode array," *Biosensors and Bioelectronics*, vol. 24, pp. 1883-1887, 2009.
- [231] P. Norlin, M. Kindlundh, A. Mouroux, K. Yoshida, and U. G. Hofmann, "A 32-site neural recording probe fabricated by DRIE of SOI substrates," *Journal of Micromechanics and Microengineering*, vol. 12, p. 414, 2002.
- [232] A. L. Owens, T. J. Denison, H. Versnel, M. Rebbert, M. Peckerar, and S. A. Shamma, "Multi-electrode array for measuring evoked potentials from surface of ferret primary auditory cortex," *Journal of neuroscience methods*, vol. 58, pp. 209-220, 1995.
- [233] I. P. Clements, V. J. Mukhatyar, A. Srinivasan, J. T. Bentley, D. S. Andreasen, and R. V. Bellamkonda, "Regenerative scaffold electrodes for peripheral nerve interfacing," *Neural Systems and Rehabilitation Engineering, IEEE Transactions on*, vol. 21, pp. 554-566, 2013.
- [234] J. Ordonez, M. Schuettler, C. Boehler, T. Boretius, and T. Stieglitz, "Thin films and microelectrode arrays for neuroprosthetics," *MRS bulletin*, vol. 37, pp. 590-598, 2012.
- [235] A. Ivorra, R. Gomez, N. Noguera, R. Villa, A. Sola, L. Palacios, *et al.*, "Minimally invasive silicon probe for electrical impedance measurements in small animals," *Biosensors and bioelectronics*, vol. 19, pp. 391-399, 2003.
- [236] Y. Ming, H. V. Panchawagh, R. J. Podhajsky, and R. L. Mahajan, "Micromachined Hot-Wire Thermal Conductivity Probe for Biomedical Applications," *Biomedical Engineering, IEEE Transactions on*, vol. 56, pp. 2477-2484, 2009.

- [237] M. Yi, H. V. Panchawagh, R. L. Mahajan, Z. Liu, and S. N. Goldberg, "Micromachined electrical conductivity probe for RF ablation of tumors," in *ASME 2005 International Mechanical Engineering Congress and Exposition*, 2005, pp. 53-56.
- [238] K. S. Cole, "Permeability and impermeability of cell membranes for ions," in *Cold Spring Harbor Symposia on Quantitative Biology*, 1940, pp. 110-122.
- [239] O. G. Martinsen and S. Grimnes, *Bioimpedance and bioelectricity basics*: Academic press, 2011.
- [240] R. V. Davalos, B. Rubinsky, and D. M. Otten, "A feasibility study for electrical impedance tomography as a means to monitor tissue electroporation for molecular medicine," *Ieee Transactions on Biomedical Engineering*, vol. 49, pp. 400-403, Apr 2002.
- [241] R. E. Neal, P. A. Garcia, J. L. Robertson, and R. V. Davalos, "Experimental Characterization and Numerical Modeling of Tissue Electrical Conductivity during Pulsed Electric Fields for Irreversible Electroporation Treatment Planning," *Biomedical Engineering, IEEE Transactions on*, vol. 59, pp. 1076-1085, 2012.
- [242] A. Ivorra, L. M. Mir, and B. Rubinsky, "Electric Field Redistribution due to Conductivity Changes during Tissue Electroporation: Experiments with a Simple Vegetal Model," *World Congress on Medical Physics and Biomedical Engineering, Vol 25, Pt 13*, vol. 25, pp. 59-62, 2009.
- [243] M. Hjouj and B. Rubinsky, "Magnetic Resonance Imaging Characteristics of Nonthermal Irreversible Electroporation in Vegetable Tissue," *The Journal of Membrane Biology*, vol. 236, pp. 137-146, 2010/07/01 2010.
- [244] M. Bower, L. Sherwood, Y. Li, and R. Martin, "Irreversible Electroporation of the Pancreas: Definitive Local Therapy Without Systemic Effects," *Journal of Surgical Oncology*, vol. 104, pp. 22-28, Jul 1 2011.
- [245] P. A. Garcia, R. V. Davalos, and D. Miklavcic, "A Numerical Investigation of the Electric and Thermal Cell Kill Distributions in Electroporation-Based Therapies in Tissue," *Plos One*, vol. 9, Aug 12 2014.
- [246] P. A. Garcia, J. H. Rossmeisl, R. E. Neal, T. L. Ellis, and R. V. Davalos, "A Parametric Study Delineating Irreversible Electroporation from Thermal Damage Based on a Minimally Invasive Intracranial Procedure," *Biomedical Engineering Online*, vol. 10, Apr 30 2011.
- [247] M. Rao, J. Barnard, and J. Kenny, "Thermal conductivity and thermal diffusivity of process variety squash and white potatoes.," *Transactions of the ASAE*, 1975.
- [248] S. Čorović, A. Županič, S. Kranjc, B. Al Sakere, A. Leroy-Willig, L. M. Mir, *et al.*, "The influence of skeletal muscle anisotropy on electroporation: in vivo study and numerical modeling," *Medical & biological engineering & computing*, vol. 48, pp. 637-648, 2010.
- [249] T. N. Zu, A. I. Athamneh, E. Collakova, J. Robertson, T. Hawken, C. Aardema, *et al.*, "Assessment of ex vivo perfused liver health by Raman spectroscopy," *Journal of Raman Spectroscopy*, 2015.
- [250] E. Ben-David, L. Appelbaum, J. Sosna, I. Nissenbaum, and S. N. Goldberg, "Characterization of Irreversible Electroporation Ablation in In Vivo Porcine Liver," *American Journal of Roentgenology*, vol. 198, pp. W62-W68, Jan 2012.
- [251] B. A. Boukamp and H. J. M. Bouwmeester, "Interpretation of the Gerischer impedance in solid state ionics," *Solid State Ionics*, vol. 157, pp. 29-33, 2// 2003.

Appendix A

Protocols

Table A-1. SU-8 fabrication steps

Step	Time	Details
Dehydrate wafer	60 min	200 deg C (optional)
Spin SU-8-2035	10 sec	500 rpm
	5 sec	900 rpm
	30 sec	1000 rpm
Soft bake	5 min	65 deg C
	20 min	95 deg C
Expose UV	45 sec	Adjust focus for sharp contrast
Hard bake	3 min	65 deg C
	10 min	95 deg C
Develop	6 min	Only SU8 developer
Wash	Few seconds	In IPA
Final film thickness		105 um

Table A-2. TJ staining of ZO-1 protein for bEnd3 cells

Step	Time	Details
Fixation	1 hour	4% paraformaldehyde (Boston Bioproducts Cat#: BM-155)
Wash	15 min	1xPBS
Permeabilize	30 min	Blocking buffer (BB): 1xPBS with: - Goat serum 10% (Life Technologies, Cat#50062Z) or BSA 2% (Sigma Aldrich, Cat#A7906) - TritonX-100 0.2% (Life Technologies, Cat#HFH10)
Wash	15 min	1xPBS
Primary AB	2 hours	Dilute 2:1000 in BB (EMD Millipore, Cat#MABT339)
Wash	15 min	1xPBS
Secondary AB	1 hour	Dilute 1:100 in BB (EMD Millipore, Cat#AP181F)
Wash	15 min	1xPBS+1 drop/ml NucBlue (Life Technologies, Cat#R37605)

Table A-3. Staining with rhodamine phalloidin and DAPI

Step	Time	Details
Fixation	1 hour	4% paraformaldehyde (Boston Bioproducts Cat#: BM-155)
Wash	15 min	1xPBS
Permeabilize	30 min	Blocking buffer (BB): 1xPBS with: - Goat serum 10% (Life Technologies, Cat#50062Z) or BSA 2% (Sigma Aldrich, Cat#A7906) - TritonX-100 0.2% (Life Technologies, Cat#HFH10)
Wash	15 min	1xPBS
Staining	1 hour	Phalloidin, dilute 1:100 in BB + DAPI, dilute 1:500 in BB
Wash	15 min	1xPBS

Table A-4. Fabrication steps for the double layer microfluidic device

Step	Description	Details
1	Fabricate top and bottom PDMS parts by replication molding Prepare the membrane (ex. cutting from transwell).	
2	Cut and punch the holes in top PDMS parts	
3	Put both PDMS parts in vacuum	1 hr
4	Mix (~20gr) PDMS with curing agent and degas	10:1 w ratio
5	Mix PDMS mixture from step 4 with toluene=>PDMS glue	1:1 w ratio
6	Keep stirring the PDMS glue from step 5 with a magnetic stirrer	Room Temp
7	Spin PDMS glue on a clean glass slide	1000 rpm 1 min
8	Take the PDMS parts from vacuum and immediately put on glue-coated glass slide, face down. Push down slightly to get the air out	Wait 1 min
9	Detach the PDMS parts from glass slide slightly with no lateral movement	
10	Put the membrane on the glue-coated side of the top PDMS part	
11	Put the bottom PDMS part on the assembly of step 10 after proper alignment. Push slightly to get the air out.	
12	Wait	About 15 min
13	Put the assembly on a hot plate, and bake	60 deg C 12 hours
14	Bond the entire assembly to a glass slide to provide mechanical stiffness.	

Table A-5. Fabrication steps for the impedance micro-sensor array

Step	Description		Details
1	Clean silicon wafer with Acetone, IPA, DI water		Spin 100 rpm
2	Adhesion promotion on 4 corners of wafer. Use VM651 (HD microsystems) to wet 4 points on the edge of the wafer. This will keep the polyimide (PI) layer attached during the fabrication steps. Avoid any contact with the rest of the wafer as it will inhibit detachment at the final step		
3	Spin first polyimide layer (PI2525, HD microsystems)		2000 rpm, slow ramp 30 sec,
4	Soft bake PI on hot plate		120 degC for 30 sec 150 degC for 30 sec
5	Cure PI in furnace	Ramp up temp in air	200 degC 4 degC/min
6		Cure in air	30 min
7		Ramp up temp in nitrogen flow 10 lit/min	300 degC 4 degC/min
8		Bake in nitrogen at 300 degC	1 hour
9	Cool down, turn off furnace and let it cool down, with the wafer		Few hours
10	Evaporate Cr/Gold with PVD		30/100 nm thick
11	Pattern Cr/Gold with lithography and chemical wet etching		
12	Spin second polyimide layer		5000 rpm, slow ramp 30 sec
13	Repeat steps 4-9 to cure the second PI layer		
14	Evaporate Ti with PVD		100 nm
15	Pattern Ti after lithography through the second mask with BOE to make a hard mask for etching top PI layer.		
16	Etch PI in oxygen plasma		50 W 30 ml/min O ₂ flow ~80 min
17	Release in BOE; this will remove the Ti mask and also detach the individual probes from the Si wafer		

Appendix B

Fluid flow in the microchannel and shear stress

Some fundamentals of fluid mechanics will be presented in this appendix to show the governing equations of fluid flow in the microfluidic channel and leverage them to control the microenvironment in which the cells are cultured. We will start from the most general case and then will make the equations more specific to microfluidic systems by making proper assumptions. The continuity equation expresses the conservation of mass in classical mechanics. In the most general case consider a compressible fluid where the density ρ is a function of space and time. Considering an arbitrarily shaped, but fixed region Ω in the fluid, the total mass $M(\Omega, t)$ inside Ω can be expressed as

$$M(\Omega, t) = \iiint_{\Omega} \rho(\vec{r}, t) d\vec{r} \quad (\text{A-1})$$

where $d\vec{r}$ is the volume element.

According to Eq. (A-1) the time rate of change of mass inside the control volume is

$$\frac{\partial}{\partial t} M(\Omega, t) = \iiint_{\Omega} d\vec{r} \frac{\partial}{\partial t} \rho(\vec{r}, t) \quad (\text{A-2})$$

On the other hand the mass flow rate density $\vec{J}$ is defined as

$$\vec{J}(\vec{r}, t) = \rho(\vec{r}, t) \vec{v}(\vec{r}, t) = \quad (\text{A-3})$$

where $\vec{v}$ is the Eulerian velocity field. Using Eq. (A-3) the time rate of change of mass inside the control volume could be obtained as

$$\frac{\partial}{\partial t} M(\Omega, t) = - \iint_{\partial\Omega} da \hat{n} \cdot (\rho(\vec{r}, t) \vec{v}(\vec{r}, t)) = - \iiint_{\Omega} d\vec{r} \vec{\nabla} \cdot (\rho(\vec{r}, t) \vec{v}(\vec{r}, t)) \quad (\text{A-4})$$

where da is the unit area element and $\hat{n}$ is the surface unit normal vector. The last expression is obtained by using the Gauss's theorem. By equating Eqs. (A-2) and (A-4) we obtain

$$\iiint_{\Omega} d\vec{r} \left[\frac{\partial}{\partial t} \rho(\vec{r}, t) + \vec{\nabla} \cdot (\rho(\vec{r}, t) \vec{v}(\vec{r}, t)) \right] = 0 \quad (\text{A-5})$$

To have the integral equal to zero for any choice of region Ω the integrand should be zero, which yields the continuity equation:

$$\frac{\partial \rho}{\partial t} + \vec{\nabla} \cdot (\rho \vec{v}) = 0 \quad (\text{A-6})$$

For the special case of incompressible flow the continuity equation reduces to

$$\vec{\nabla} \cdot \vec{v} = 0 \quad (\text{A-7})$$

Newton's second law for fluid systems is called the Navier-Stokes equations which gives the constitutive equations for the Eulerian velocity field $\vec{v}(\vec{r}, t)$. The Newton's second law gives

$$\rho D_t \vec{v} = \sum_j \vec{f}_j \quad (\text{A-8})$$

Where $\vec{f}_j$ represents the force densities and D_t is the material time-derivative:

$$D_t = \frac{\partial}{\partial t} + (\vec{v} \cdot \vec{\nabla}) \quad (\text{A-9})$$

Therefore, we will have

$$\rho D_t \vec{v}(\vec{r}, t) \equiv \rho \frac{d}{dt} \vec{v}(\vec{r}(t), t) = \rho \left[\frac{\partial}{\partial t} \vec{v}(\vec{r}, t) + (\vec{v} \cdot \vec{\nabla}) \vec{v}(\vec{r}, t) \right] \quad (\text{A-10})$$

Which is the left side of Eq. (A-8). Different forces could be applied to the fluid. Here we consider the most important ones: pressure $\vec{F}_p$, viscous force $\vec{F}_v$, and gravity $\vec{F}_g$, with $\vec{f}_p$, $\vec{f}_v$ and $\vec{f}_g$ representing the corresponding force densities. The gravity force density is

$$\vec{f}_g = \rho \vec{g} \quad (\text{A-11})$$

The pressure force density is obtained as follows.

$$\vec{F}_p = \int_{\partial\Omega} da \, \hat{n}(-p) = \int_{\Omega} d\vec{r} \, (-\vec{\nabla} p) \quad (\text{A-12})$$

The integrand of the volume integral represents the force density.

$$\vec{f}_p = -\vec{\nabla} p \quad (\text{A-13})$$

The viscous force can be written as a surface integral of the applied shear stress to the volume element.

$$\vec{F}_v = \int_{\partial\Omega} \vec{\tau} \cdot \hat{n} da = \int_{\Omega} \vec{\nabla} \cdot \vec{\tau} d\vec{r} \quad (\text{A-14})$$

where $\vec{\tau}$ is the shear stress tensor. Again, the integrand of the volume integral represents the force density.

$$\vec{f}_v = \vec{\nabla} \cdot \vec{\tau} \quad (\text{A-15})$$

The constitutive relations are needed to state the shear stress as a function of velocity field. Here we will assume that the fluid is Newtonian, meaning that its viscosity is independent of the strain rate. For Newtonian fluid the shear stress tensor and the viscous component of the strain rate tensor are related according to

$$\vec{\tau} = 2\eta\vec{\epsilon} = \eta\left(\left(\vec{\nabla}\vec{v}\right) + \left(\vec{\nabla}\vec{v}\right)^T\right) \quad (\text{A-16})$$

Substituting into Eq. **Error! Reference source not found.** we get

$$\vec{f}_v = \vec{\nabla} \cdot \vec{\tau} = \vec{\nabla} \cdot \eta\left(\left(\vec{\nabla}\vec{v}\right) + \left(\vec{\nabla}\vec{v}\right)^T\right) \quad (\text{A-17})$$

For an incompressible fluid that simplifies to

$$\vec{f}_v = \eta\nabla^2\vec{v} \quad (\text{A-18})$$

with all other terms vanishing due to the continuity equation. Therefore the complete Navier-Stokes equations for Newtonian incompressible fluid is

$$\rho \left[\frac{\partial}{\partial t} \vec{v} + (\vec{v} \cdot \vec{\nabla}) \vec{v} \right] = \rho \vec{g} - \vec{\nabla} p + \eta \nabla^2 \vec{v} \quad (\text{A-19})5$$

We will now make proper assumptions to make the Navier-Stokes equations simplified and more specific for analyzing the fluid flow inside the microfluidic channels. It is assumed that the gravity force is negligible compared to the viscous force due to the small size of the channel.

Pressure-driven steady state flow in channel: Poiseuille flow

In microfluidic systems the fluid is driven through a long, straight and rigid channel by applying a pressure difference. In this case only the longitudinal (x-axis) component of the velocity is nonzero.

$$\vec{v}(\vec{r}) = v_x(y, z) \hat{e}_x \quad (\text{A-20})$$

Therefore the Navier-Stokes equation reduces to

$$\eta \nabla^2 [v_x(y, z) \hat{e}_x] - \vec{\nabla} p = 0 \quad (\text{A-21})$$

This equation could be further simplified knowing the unidirectional nature of the flow.

$$\eta \left(\frac{\partial^2}{\partial y^2} + \frac{\partial^2}{\partial z^2} \right) v_x(y, z) = \frac{\partial}{\partial x} p(x) \quad (\text{A-22})$$

The fact that the left side of Eq. (A-22) is a function of (y, z) and the right side is only a function of x , implies that both sides should be equal to the same constant number. Solving for the right side first yields

$$p(x) = \frac{\Delta p}{L} (L - x) + p_0 \quad (\text{A-23})$$

in which we have assumed a pressure drop Δp over the length L of the channel. With this we finally arrive at the second order partial differential equation for the velocity field in the channel along with the no-slip boundary condition.

$$\left(\frac{\partial^2}{\partial y^2} + \frac{\partial^2}{\partial z^2} \right) v_x(y, z) = -\frac{\Delta p}{\eta L} \quad (A-24)$$

$$v_x(y, z) = 0 \quad \text{for } (y, z) \text{ on the channel boundary}$$

Hydraulic resistance

Solving the governing partial differential equation (A-24) for different channel cross sections reveals that in the pressure-driven steady state flow through a straight channel, a constant pressure drop Δp results in a constant flow rate Q , which are related with the hydraulic resistance R_{hyd} .

$$\Delta P = R_{hyd} Q \quad (A-25)$$

Common channel cross sections in microfluidic systems include circular and rectangular. The hydraulic resistances for these sections are

$$R_{rec} = \frac{12\eta L}{1 - 0.63(h/w)} \frac{1}{h^3 w} \quad L : \text{length}, h : \text{height}, w : \text{width} \quad (A-26)$$

$$R_{cir} = \frac{8\eta L}{\pi R^4} \quad R : \text{radius}$$

These equations are useful when calculating the flow rates or pressure drops in microfluidic channels.

Wall shear stress

Shear stress is an important parameter of the microfluidic cell culture systems. Some cells are sensitive to shear stress and their viability could be compromised if exposed to excessive shear stress, while some other cells such as endothelial cells, should be exposed to flow-induced shear in order to express their physiological phenotype. Cells are usually cultured on the walls of the microfluidic channels. Therefore, knowing the wall shear stress τ_w is critical. Considering a

rectangular cross section with $h \ll w$, the differential equation (A-24) could be solved to give the velocity field in the channel.

$$h \ll w \rightarrow \frac{\partial v_x}{\partial z} = 0$$

$$\frac{\partial^2 v_x}{\partial y^2} = -\frac{\Delta p}{\eta L} \xrightarrow{\int} \frac{\partial v_x}{\partial y} = -\frac{\Delta p}{\eta L} y + C_1 \xrightarrow{\int} v_x = \frac{1}{2} \left(-\frac{\Delta p}{\eta L} \right) y^2 + C_1 y + C_2$$

B.C :

$$\left. \frac{\partial v_x}{\partial y} \right|_{y=0} = 0 \rightarrow C_1 = 0 \quad (A-27)$$

$$v_x \Big|_{y=h/2} = 0 \rightarrow C_2 = \frac{\Delta p}{\eta L} \frac{h^2}{8}$$

$$\tau_w = \eta \left. \frac{\partial v_x}{\partial y} \right|_{y=h/2} = \frac{h \Delta p}{2L}$$

Δp could be stated as a function of hydraulic resistance and the flow rate:

$$\Delta p = R_{rec} Q$$

$$= \frac{12\eta L}{h^3 w} Q \quad (A-28)$$

which yields the wall shear stress at the top or bottom of the channel $y = \pm h/2$

$$\tau_w = \frac{6\eta Q}{h^2 w} \quad (A-29)$$

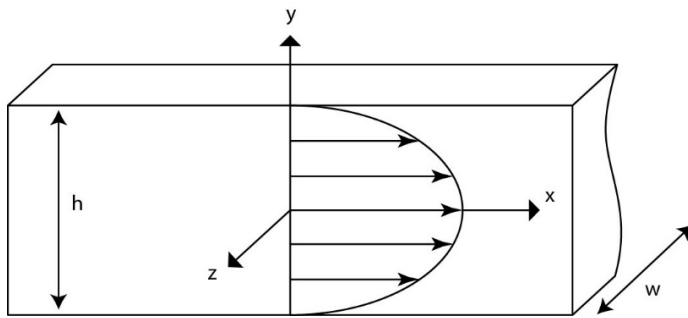


Figure 2. Pressure driven flow in rectangular channel

It should be noted that the above solution is only valid for channels with a small height compared to channel width. Another empirical relation is suggested in the literature for the channels with comparable width and height dimensions:

$$\tau_w = \frac{2\eta Q}{h^2 w} \left(\frac{m+1}{m} \right) (n+1) \quad (A-30)$$

$$m = 1.7 + 0.5\alpha^{-1.4}, n = 2 \quad \text{for } \alpha = \frac{h}{w} < \frac{1}{3}$$

Appendix C

Impedance Multiplexer

The impedance analyzer that we use for our applications (Gamry Reference 600) provides a 4-bit control signal that we used to control the multiplexer. 16 states could be selected with these 4 bits using a demultiplexer chip.

The circuit is designed in two separate levels which are electrically connected together. The bottom circuit incorporates a demultiplexer chip (CD74HC4514EN) which translates the 4 bit control input to 16 bits. Each of the 16 bits is connected to a reed relay on the top circuit through a transistor which turn the relays on and off. The input of the relays are connected to the samples to be measured and their output is connected to one of the two output ports of the circuit to be connected to the impedance analyzer. Depending on the application, different relay configurations could be used in the top circuit. Here we designed and fabricated two different boards. In the first board, one of the ports is always connected to a fixed pin and is considered for the counter electrode. The other port could be switched between 16 inputs using the relays. At each time depending on the control signal only one relay could be on. This circuit could be used for impedance measurements using sensors which feature a counter electrode. In the second circuit the relays are divided between the two ports, i.e., each port could be connected to 8 different samples. However, in this circuit, after each relay is triggered by the control signal, it stays on by using a memory feature that is enabled by a second channel in each of the relays. Briefly, after the relay is triggered on by the control signal, one of the two channels in the relay close the circuit to provide power for itself, keeping the relay on even when the control signal turns off. That feature enables multiple relays to be switched on, therefore facilitating impedance measurement between any combination of the

inputs. The electrical circuits for a relay unit in each of the multiplexer designs are shown in Figure B-1.

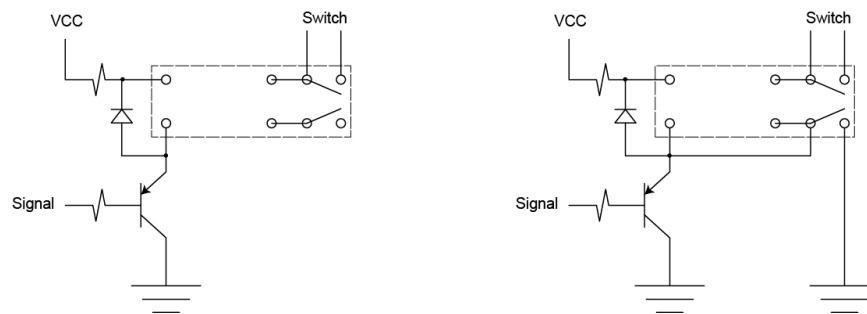
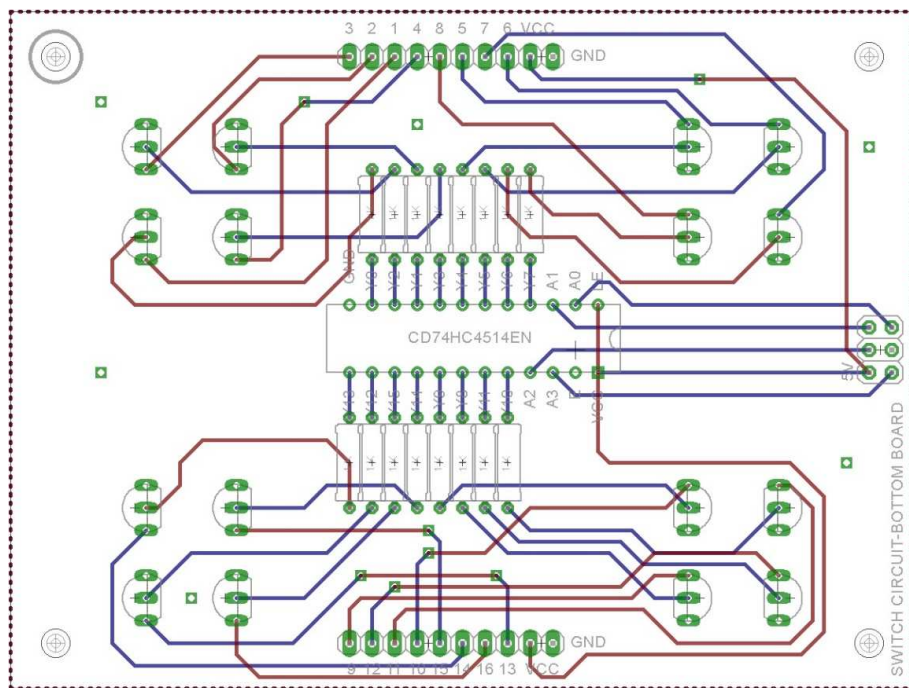


Figure B-1. Control circuit for each relay unit

The electrical circuits were designed in the Eagle software and the gerber files were extracted. Figure B-2 **Error! Reference source not found.** shows the designed circuit for the relay boards and Figure B-3 shows the circuit for the bottom board. The gerber files were sent to a vendor to be printed on the circuit boards. (Advanced circuits, Aurora, Co). Figure B-4 shows the final assembled multiplexer.



Appendix D

Impedance models of diffusion

Several empirical models have been proposed to account for the diffusion impedance at the electrode-electrolyte interface. Assuming an infinite diffusion layer the infinite Warburg impedance is defined as

$$Z_D = \frac{A_w}{\sqrt{j\omega}}(1-j) \quad (C-1)$$

Where A_w is the Warburg coefficient. The infinite Warburg impedance is actually the CPE with a frequency independent phase of 45° . The porous bounded Warburg or the “O” circuit element is defined as:

$$Z_D = \frac{\sqrt{2}A_w}{\sqrt{j\omega}} \tanh(B\sqrt{j\omega}) \quad (C-2)$$

The B parameter in the bounded Warburg model is called the Warburg time constant and is related to the Nernst diffusion layer thickness, δ , and the diffusion coefficient of ions according to:

$$B = \frac{\delta}{\sqrt{D}} \quad (C-3)$$

The Gerischer impedance is used to model the diffusion phenomenon coupled to a chemical reaction and is expressed as [251]:

$$Z_G = \frac{Z_0}{\sqrt{k+j\omega}} \quad (C-4)$$

