

Investigation of Single-Cell and Blood-Brain Barrier Mechanics after Electroporation and
in Primary Brain Cancers

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ABSTRACT

Cell-level and tissue-level mechanical properties are key to healthy biological functions, and many diseases and disorder arise or progress due to altered cell and tissue mechanics. Pulse electric field (PEFs), which employ intense external electric fields to cause electroporation, a phenomenon characterized by increased cell membrane permeability, also can cause significant changes to cell and tissue mechanics. Here, we investigate the mechanics of brain and brain cancer cells, specifically focusing on how PEFs impact cell mechanics and PEF-induced blood-brain barrier disruption. In our first study, we investigate single-cell mechanical disruption of glioblastoma cells after reversible electroporation using Nanonet Force Microscopy (NFM). A precise network of extracellular-matrix mimicking nanofibers enabled cell attachment and contraction, resulting in measurable fiber deflections. Cell contractile forces were shown to be temporarily disrupted after reversible electroporation, in an orientation and field-dependent manner. Furthermore, we found that cell response is often a multi-stage process involving a cell-rounding stage, biphasic stage, and a cell re-spreading stage. Additionally, cell viability post-PEFs was orientation-dependent. In another study, we investigated the mechanical properties of brain cancer for various-grade glioma cells (healthy astrocytes, grade II, grade III, and grade IV (glioblastoma) cells). A microfluidic constriction channel

caused cell deformation as cells, driven by hydrostatic pressure, entered a narrow constriction. Finite element models of cell deformation and a neural network were used to convert experimental results (cell entry time and cell elongation within the channel) into elastic modulus values (kPa). We found that the that low-grade glioma cells showed higher stiffnesses compared to healthy and grade IV glioma cells, which both showed similar values. These results warrant future studies to investigate these trends further. PEFs can induce Blood-brain barrier (BBB) disruption, an effect we studied using a multiplexed, PDMS microdevice. A monolayer of human cerebral endothelial cells on a semi-permeable membrane was used to model the BBB, and permeability was assessed by the diffusion of a fluorescent dye from an upper to lower channel. A custom tapered channel and branching channel design created a linear gradient in the electric field within the device that enabled six electric field strengths to be tested at once against two unexposed (control) channels. Normalization of permeability by the control channels significantly removed experimental noise. We found that after high-frequency bipolar irreversible electroporation (HFIRE) electric pulses, permeability transiently increased within the first hour after electroporation, in a voltage- and pulse-number dependent manner. However, we found significant electrofusion events after pulsing at high voltages, which reduced monolayer permeability below baseline values. This device enables efficient exploration of a wide range of electroporation parameters to identify the optimal conditions for blood-brain barrier disruption. In another blood-brain barrier study, we incorporate dense, polystyrene nanofiber networks to create ultra-thin, ultra-porous basement-membrane-mimics for *In vitro* blood-brain barrier models. Fiber networks are fabricated using the non-

electrospinning Spinneret-based Tunable Engineered Parameters (STEP) technique. Endothelial cells cultured on one side of the fiber network are in close contact with supporting cell types (pericytes) cultured on the backside of the fibers. Contact-orientation co-cultures have been shown to increase blood-brain barrier integrity, and our nanofiber networks increase the physiological realism of basement-membrane mimics for improve modeling. Finally, we investigate how cell viability post-electroporation is impacted by cell morphology. The impact of cell morphology (shape & cytoskeletal structure) on cell survival after electroporation is not well understood. Linking specific morphological characteristics with cell susceptibility to electroporation will enhance fundamental knowledge and will be widely useful for improving electroporation techniques where cell viability is desirable (gene transfection, electrofusion, electrochemotherapy) or where cell viability is undesirable (tumor ablation, cardiac ablation). Precise control of cell shape and orientation enabled by nanofiber scaffolds provides a convenient and expedient platform for investigating a wide variety of factors (morphological and experimental) on cell viability. Altogether, these investigations shed new light on cell mechanical changes due to disease and pulsed electric fields, and suggest opportunities for improving brain cancer therapies.

Investigation of Single-Cell and Blood-Brain Barrier Mechanics after Electroporation and in Primary Brain Cancers

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GENERAL AUDIENCE ABSTRACT

In biology, structure and function are interrelated. Cells and tissue have structures that enable them to perform their proper function. In the case of disease, cell and tissue properties are altered, leading to dysfunction. Alternatively, healthy structures sometime hinder effective treatments, and therefore can be therapeutically disrupted to improve treatments. In this study, we investigate single-cell and multi-cellular mechanical change due to disease or after pulsed electric fields (PEFs), with a specific focus on the brain. Pulsed electric fields (PEFs) use electrodes to deliver short, intense pulses of electrical energy to disrupt cell membranes and change cell mechanics. We studied as single-cell contractility, cancer cell stiffness, and blood-brain barrier (BBB) disruption by PEFs. We found that PEFs cause significant change to cell shape and mechanics, and can disrupt the BBB. By studying several grades of brain cancers, we found that low-grade brain cancer (gliomas) showed increased stiffness compared to healthy and highly diseased (grade IV) cells. To mimic the BBB, we used microfluidic devices to grow specialized brain cells (endothelial cells) on permeable membranes and nanofibers networks and showed that these devices can mimic structures found in animals/humans. Finally, we studied how cell properties (such as shape) determine whether cells will survive PEFs. Taken together,

our investigations improve the understanding of brain mechanics during disease and after PEFs, and suggest the usefulness of PEFs for improved brain cancer therapies.

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Dedication

This dissertation is dedicated to my wife, Kelsa Hope Graybill. Thank you for your continual encouragement and countless sacrifices for me to complete this degree. This dissertation is also dedicated to the thousands of patients suffering from primary brain tumors and neurological disorders.

Attribution

Aniket Jana is a co-first author of Chapter 3. Isaiah Varella and Sydney Overton assisted with data processing on for Chapter 3. Ravi Bollineni developed and performed the computations simulations and neural network used in Chapter 5. John Moomaw and Evan Arents assisted with elements of Chapter 5 such as device design, experimental setup, and data processing. Aniket Jana is a co-first author of Chapter 7.

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1 Chapter 1: Introduction

1.1 Electric fields and biology

Electricity and biology, two subjects that may at first thought seem unrelated or incompatible, have in reality a very complex and important relationship that forms the basis for important bioelectrics research. Endogenous electric fields, established by stationary (electrostatic) or moving (electrodynamic) charges are highly integrated into a plethora of biological functions. For example, as the building blocks of cells, proteins contain binding sites that use 3D structure and electrostatic interacts to bind highly specific targets[1]. Phospholipid bilayers are incorporated into intracellular structures and act as dielectric layers that enable the separation of charges to create electrochemical gradients of ions[2]. The lipid bilayer which encloses the cell, likewise separates intracellular and extracellular charges to establish a transmembrane “resting potential” of $\sim 50\text{mV}$ across the nanometer-thin bilayer (establishing significant local electric fields of $> 10\text{ kV/cm}$) [3]. Transmembrane ion transporter proteins of calcium, sodium, and potassium regulate cytosolic ion concentrations to promote homeostasis. As you read this Introduction and your eyes track across the page, muscle contractions driven by electrical impulses are to thank. Voluntary muscles contraction is initiated by the central nervous system and relayed from the brain to the distant muscle groups by action potentials, or electrical signals travelling at up to 100 m/s [4]. Similarly, involuntary electrical impulses from our sinus node in our heart rhythmically fires electrical impulses to sustain life by heart muscle

contraction[5]. Wound healing, also exploits electric fields to induce cell migration and would closure.[6] Many organisms harness electric fields for even more esoteric functions. Take for instance, the electric eel which produces electrical discharges of hundreds of volts to stun its prey[7]. From eels to proteins, endogenous electric fields are absolutely key to biological function. It follows, therefore, that exogenous electric fields applied to biological tissues may potentiate substantial changes to normal biological function across a wide range of scales.

Exogenous electric fields applied to biological materials can have many uses for biological research and disease treatment. One of the most promising applications of bioelectrics is the use of short, intense electric pulses, known as electroporation. Electroporation pulses can generate large fields across biological membranes that results in membrane disruption and loss of cell homeostasis. The foundational principles of this electroporation are described in the following section.

1.2 Principles of electroporation

Electroporation theory is based on the use of short but intense electrical pulses to induce a voltage across the plasma membrane of cells that drives “pore formation” in the membrane, causing increased membrane permeability. Electroporation can be considered reversible or irreversible (Figure 1.1). Irreversible electroporation uses high electric fields and many pulses to disrupt the cell membrane causing cell death. For reversible electroporation (RE), fewer, lower-magnitude pulses

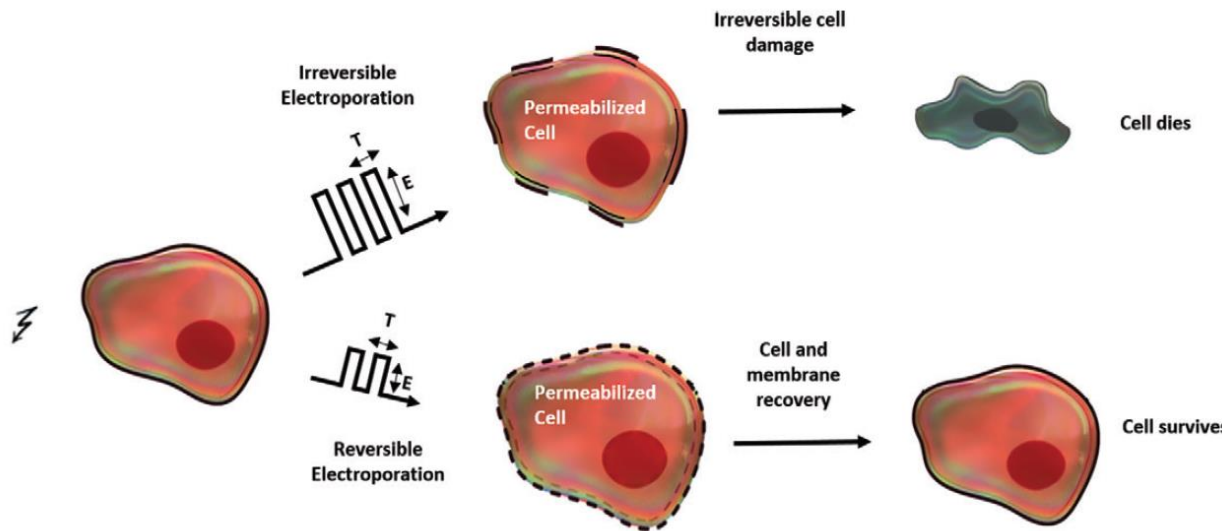


Figure 1.1 Reversible and Irreversible Electroporation

Pulsed electric fields of high electric field and pulse number disrupt the cell membrane causing cell death in Irreversible Electroporation (IRE), but lower magnitude pulses and few pulses only transiently disrupt the cell membrane and enable cell recovery in Reversible Electroporation (RE). Figure from [8].

are used to transiently permeabilize the cell membrane, but still allow for membrane recovery and cell survival. Membrane pores are key for understanding cell response, and pore dynamics are typically divided into four stages: pore initiation, pore expansion, pore stabilization, and pore resealing.

1.2.1 Pore initiation

Standard electroporation theory treats cells as spherical bodies enclosed by a capacitive lipid membrane. For a perfectly spherical cell exposed to a uniform, steady-state electric field, and ignoring electroporative effects, the induced transmembrane voltage can be described by the Schwan equation as:

$$\Delta V = \frac{3}{2} E r \cos \theta$$

Where ΔV is the induced transmembrane voltage (ITV), E is the electric field, r is cell radius, and θ is the angle of the membrane relative to the orientation of the electric field. As can be readily understood from the Schwan equation, exogenous electric fields induce the greatest voltage at the pole of the cells with respect to the electric field. The membrane becomes hyperpolarized at the anode-facing pole of the cell and hypopolarized at the cathode facing side of the cell. Above a “critical” voltage, typically between ~ 0.2 and 1 V/cm[9-15], the ITV rapidly initiates “pores” in the membrane to shunt the electrical current across the membrane. Molecular dynamics simulations show that pores form within nanoseconds of intense electric fields.[16] Water is driven into the hydrophobic interior of the lipid bilayer forming a “water column.” This energetically unstable configuration of the lipids initiates a reorientation of the phospholipid head groups inward to the water creating a hydrophilic pore.

1.2.2 Pore expansion

Hydrophilic membrane pores expand while the electric field is applied. Ultra-short nanosecond electric fields (nsPEFs) create pores with radii on the order of ~ 1 nm.[9] Longer microsecond and millisecond pulses create larger pores with radii of tens of nanometers. In cells, pore expansion is limited to several tens of nanometers.[9] Contrarily, in model systems of cells such as giant unilamellar vesicles (GUVs) composed solely of a plasma membrane, very large micron-diameter pores known as macropores can form.[17] This observation suggests that membrane proteins, such as cytoskeletal proteins limit pore expansion.

1.2.3 Pore Stabilization

Classical electroporation theory describes “meta-stable” membrane pores. Hydrophilic pores are believed to be meta-stable, with an energy barrier stabilizing these structures. This theory, is in part based on experimental results, which reveal that membrane permeability is increased in electroporated cells for several minutes to hours after electroporation. [18-23] More recent molecular dynamics (MD) simulations, however, predict that purely lipidic pores collapse very rapidly after removal of the driving electric field.[9] Thus, while pores have been traditionally considered only a lipid phenomenon, it is becoming clear that pore stabilization on the timescale of minutes is indicative of a more complex pore structure. The lipid membrane composes many transmembrane proteins, and is linked to intracellular structures such as the cell cytoskeleton. One explanation of pore stabilization is that once initiated, purely lipidic pores interact with membrane proteins to form complexes that stabilize the pores.[24]

1.2.4 Pore Resealing

Pore resealing is the process whereby hydrophilic pores are destroyed and the lipid bilayer returns to its original permeability to molecules and ions. Experiments demonstrate that electroporation causes both short-lived and long-lived pores.[25] Short lived pores collapse within nanoseconds to milliseconds after removal of the electric fields. Long-lived pores, however remain present for minutes after electroporation, and the resealing processes have not been well characterized. Cell membrane “healing” processes such as the exocytosis of intracellular membranes may contribute to pore resealing.[26] If pore resealing does not occur rapidly enough, cells will be sufficiently disrupted and cell death will occur. If membrane pores become too large, cell lysis can occur within seconds to minutes after electroporation. In other circumstances, cells can reseal pores, but the disruption and damage from the pulses leads to regulated cell death such as by apoptosis hours to days after treatment. [27-29]

1.2.5 More than pores – Electroporabilization

Membrane pores may not be the only cause of increased membrane permeability. Additional pathways of disruption may occur because of oxidized lipids and altered ion channels. Lipids can be oxidized by the intense electric fields, and form new bonds along the hydrophobic tail groups that alter the tail shape.[9] Altered lipid shapes decrease the packing density of the membrane, and increase the spacing between lipids, enabling increased transport across the cell membrane. Theoretical and experimental evidence show that membrane lipids are chemically altered by high electric fields. Over time, the cell is able to repair the damaged lipids and decrease membrane permeability. However, unlike the rapid resealing of membrane pores, lipid damage

takes significantly longer (on the order of minutes) to complete. Transmembrane ion channels can also be modulated by electric fields. Cells contain transmembrane ion channels that passively or actively regulate the ion concentrations across the membrane. Many of these ion channels, such as those for calcium, are voltage dependent. These proteins can sense the electric potential across the membrane and respond by opening or closing[9]. For calcium, high potentials across the cell membrane can open ion channels. Other ions, such as sodium, also have voltage-gated ion changes. Voltage-gated ion channels are an important part of permeability, as ion flow effects membrane capacitance, and also leads to cellular effects since calcium is a potent regulator of cell function. Voltage-gated ion channels are heavily researched via nanosecond electric pulses, as these pulses are generally too short to induce significant pore formation, but can trigger voltage gated ion channels.

1.3 Electroporation and the cell cytoskeleton

Classical electroporation theory has largely ignored the involvement of the cytoskeleton in cell response to electroporative pulses. However, very recent experimental and computational research has demonstrated the involvement of the cytoskeleton in electroporation therapies. As we briefly described in the previous two subsections, the cytoskeleton likely plays an important role in pore stabilization and resealing. Beyond these effects, the cytoskeleton may play a more central role in cell response to electroporation that previously known. This involvement of the cytoskeleton is covered in-depth in Chapter 2, and is briefly introduced here.

The cytoskeleton is composed of actin, microtubules, intermediate filaments, and septins that collectively provide the cell with mechanical stability.[30] The cytoskeleton enables cell proliferation, differentiation, signaling, migration, and survival. Additionally, the cytoskeleton is responsible for intracellular transport, organelle location, cell contractility, cell shape, cell volume, and cell behavior. [31,32] Importantly, the cell membrane is structurally and functionally linked to the cytoskeleton.[33] Given the key functions of the cytoskeleton, direct alteration of these structures by pulsed electric fields, or disruption of these structures by downstream mechanisms from membrane disruption, cytoskeletal disruption is very important to electroporation outcome (cell behavior, survival/death), and should be considered in electroporation treatments. The profound effects of electroporation on the cell cytoskeleton are described in Chapter 2 and investigated in Chapter 3.

1.4 Applications of Electroporation

Electroporation has found many useful applications in both research and clinical practice (Figure 1.2). For example, reversible electroporation (RE) can transiently permeabilize the cell membrane for the delivery of nucleic acids (DNA and RNA) and other cargo across the cell membrane. [34-36] Increased membrane permeability is exploited in electrochemotherapy (ECT), where electrical pulses are combined with adjuvant chemotherapy to promote the uptake of chemotherapies within tumors. Electrochemotherapy has been shown to increase uptake of chemotherapeutics by up to 100-fold, depending on the drug of interest.[8] Standard ECT procedures use eight, 100 μ s pulses. More recently, Irreversible Electroporation (IRE) has been developed for focal ablation of tumors using monopolar pulses with pulse widths on the order of microseconds. IRE is particularly well-

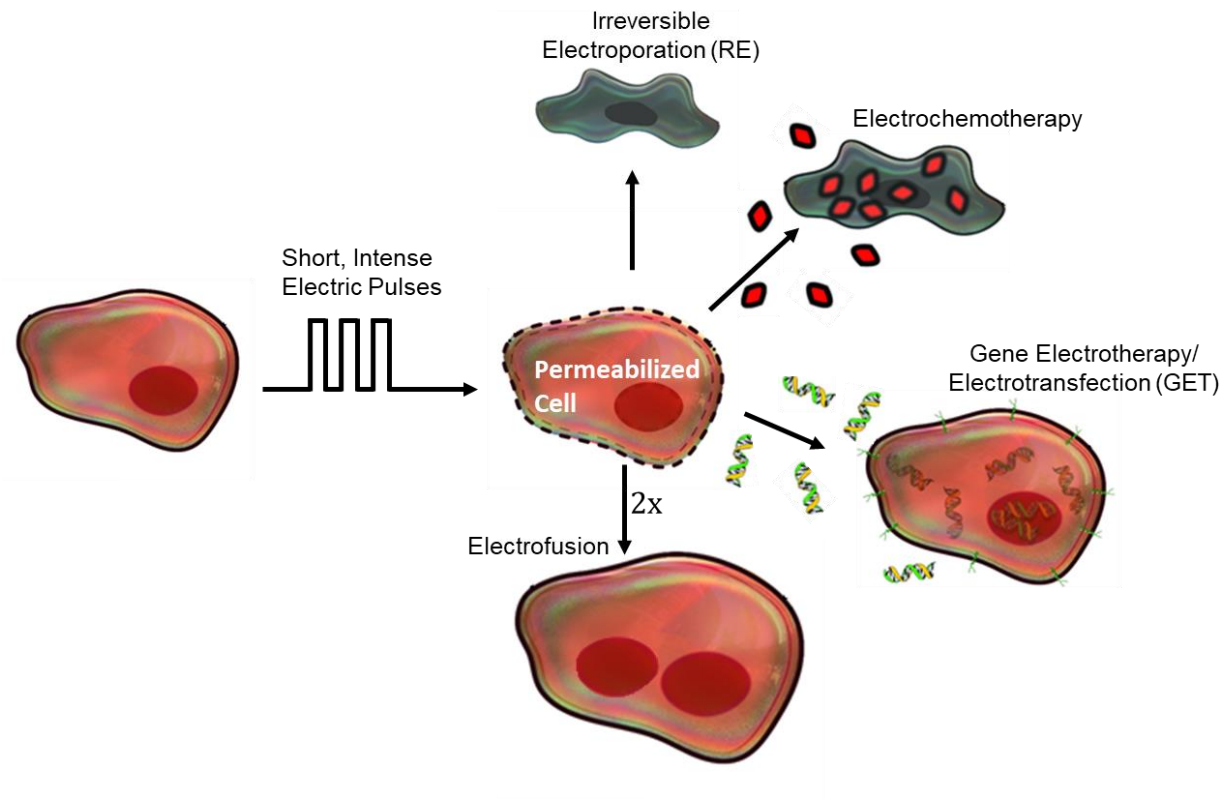


Figure 1.2 Applications of electroporation.

Numerous applications exist for electroporation therapies. Electroporation can cause cell death by Irreversible Electroporation (IRE) alone, or with adjuvant chemotherapy as in electrochemotherapy (ECT). Reversible electroporation applications include Gene electrotherapy (or gene electrotransfection, GET) and cell electrofusion. Figure adapted from [8].

suited for unresectable tumors near critical vasculature, as the non-thermal mechanism of action of IRE preserve these structures. IRE can be administered with one or more needle electrodes in a minimally invasive procedure. Standard IRE procedures use tens-to-hundreds of 100 μ s pulses.

As a refinement of IRE technology, High-Frequency IRE (H-FIRE) employs shorter (1 -10 μ s), biphasic pulses with a short intra-pulse delay. H-FIRE has the distinct advantage of eliminating all or nearly all muscle contractions during procedures because the bi-polar pulses cancel cell action potentials before they can propagate and initiate contraction. IRE has already been used in clinical trials to treat several types of cancer, including locally advanced pancreatic cancer, liver cancer, kidney cancers, and prostate cancer. [37-41]

IRE and HFIRE are also being investigated for treating aggressive brain cancers such as malignant gliomas, and have shown promising results in animal studies. [42-46] In addition to destroying brain tumors cells, IRE and HFIRE show significant potential to disrupt the blood-brain barrier and increase the penetration of chemotherapies to the tumor site.

1.5 Malignant Gliomas and the Blood-Brain Barrier (BBB) as Targets for Electroporation Therapies.

Unfortunately, tumors of the Central Nervous System (CNS) account for a significant percentage of cancer-related deaths in adults and are the most common form of cancer in children.[47] Gliomas are a common form of CNS malignancy that are typically derive from astrocytes, the most abundant cell type in the brain. [48,49] Glioblastoma multiforme (GBM) is the most malignant form of glioma (grade IV) and is a devastating disease with minimally-effective

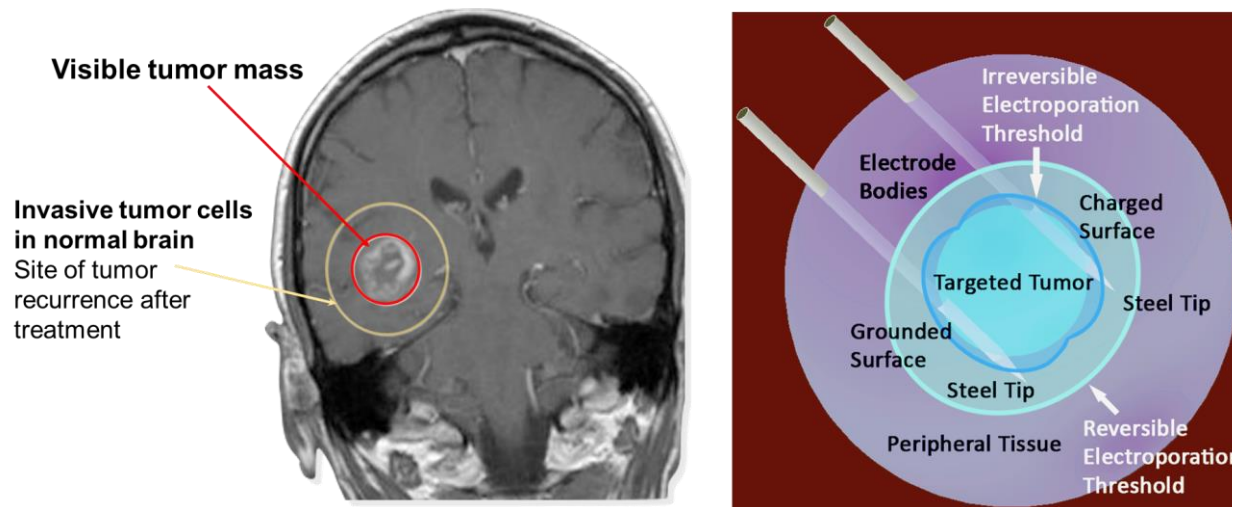


Figure 1.3 Glioblastoma and the Blood-Brain Barrier

GBM cells are highly invasive. Surrounding the tumor bulk is a region of infiltrative tumor cells that lead to therapy failure and tumor recurrence. Adapted from [ref]. IRE and H-FIRE can be applied with one or more electrodes located near the tumor to ablate the bulk tumor and also increase BBB permeability in a large volume surrounding the bulk tumor. Adapted from [50,51].

treatments available. From diagnosis, the average survival of GBM patients is a mere 15 months, with fewer than 5% of patients surviving more than 2 year.^[52] Current therapies for GBM include tumor resection, radiation, and chemotherapy. Unfortunately, these therapies are insufficient for treating GBM for several reasons. Firstly, GBM is highly-invasive. GBM tumor cells rapidly invade the surrounding healthy brain tissue, making complete surgical resection impossible. Likewise, radiation therapy is unable to treat distant, migratory cells due to the risk of damaging critical healthy cells in these regions of the brain. Finally, chemotherapies are largely ineffective at penetrating the brain tissue in clinically-effective doses due to the Blood-Brain

Barrier (BBB). Nearly 100% of all large-molecule drugs and ~98% of small-molecule drugs cannot penetrate the BBB.[53] This leaves few options for glioma treatment, and the low drug success rate makes drug discovery risky and expensive.

1.5.1 The Blood-Brain Barrier (BBB) as a target for cytoskeletal disruption by electroporation.

Specialized cells line the microvessels of the CNS, forming a structure known as the Blood-Brain Barrier (BBB). The BBB strictly regulates the transfer of substances from the blood to the brain tissue in order to maintain a neuroprotective environment. A cross-section of the BBB is schematically presented in Figure 1.4. Cerebral endothelial cells form specialized junctions, known as tight junctions, which strictly regulate the transfer of substances between adjacent cells. Furthermore, cerebral endothelial cells have specialized influx/efflux transporters that regulate the transfer of substances across the cell membrane. Endothelial cells are supported by a thin basement membrane (~100 nm) comprised mostly of laminins and collagen IV.[54,55] Opposite the basement membrane, supporting cell types such as astrocytes, pericytes, and neurons induce BBB function to enhance regulation of substances to promote overall brain health. For example, astrocytes endfeet, which surround the endothelial cells, release soluble factors to regulate endothelial behavior.[56] Taken together, these cells represent the neurovascular unit (NVU). An intact BBB is important for neurological health, and a disrupted BBB is implicated in the progression of many

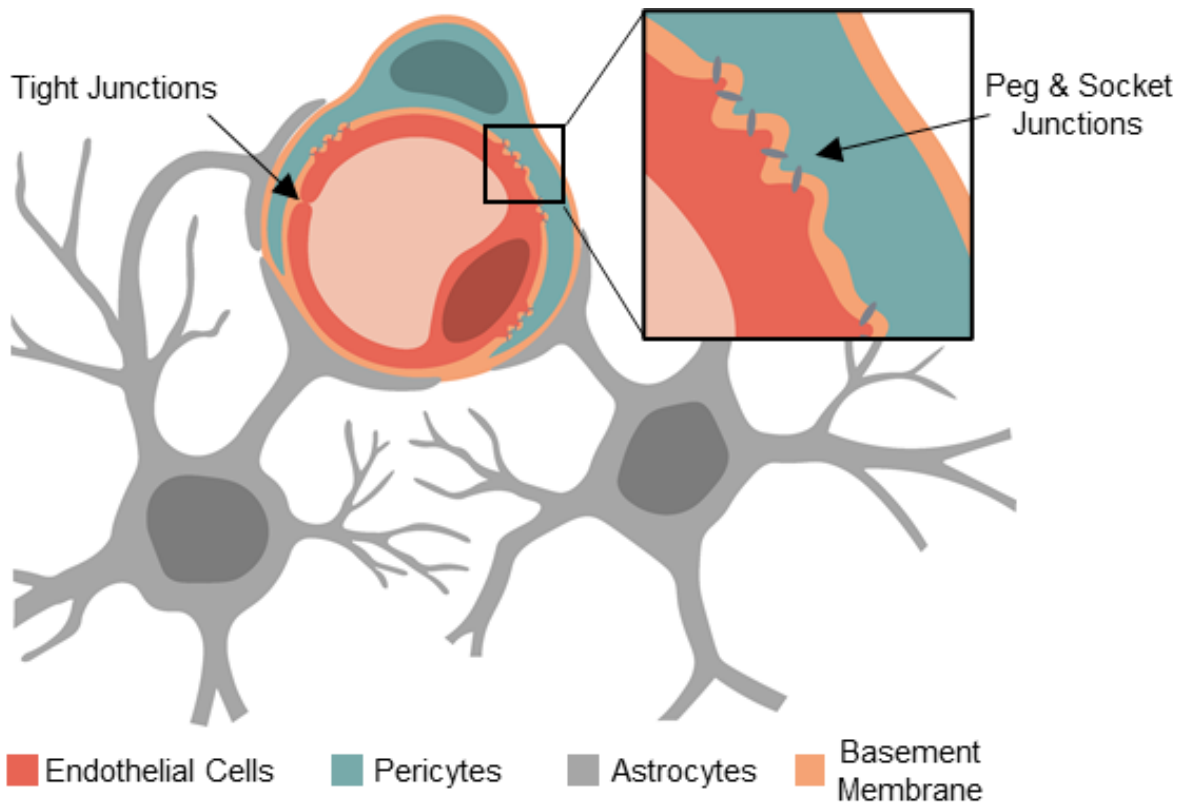


Figure 1.4 Physiology of the Blood-Brain Barrier (BBB)

Cerebral microvessels have a specialized physiology that strictly regulates transport. Tight junctions formed between adjacent endothelial cells regulate transport between cell boundaries, and specialized transporters on brain endothelial cells regulate transport across the cell membranes. Endothelial barrier function is induced by pericyte peg and socket connections and the secretion of soluble factors from astrocyte endfeet. Pericytes and astrocytes are both in close physical contact with endothelial cells.

neurological diseases such as Alzheimer's disease, Parkinson's disease, and others.[57] The integrity of the BBB, while critical to healthy brain function, becomes a significant obstacle for

treating brain conditions with therapeutics. Effective chemotherapy delivery is exceedingly challenging. Novel drugs may help to overcome this barrier, however novel drug delivery methods are needed to improve currently known therapeutics.

Several techniques have been developed to increase BBB permeability for therapeutic purposes. One promising technique is the use of focused ultrasound to open the BBB in a non-invasive and targeted procedure. Low-magnitude focused ultrasounds can mechanically disrupt tight-junction complexes to transiently increase BBB permeability by causing the cavitation of microbubbles that are intravenously injected into the circulation.[58-60] The mechanical stimulation of the microbubbles can transiently open the BBB. This technique is gaining usefulness as stereotactic imaging technology continues to mature, and affords real-time feedback with acoustic emission detection.[61] A limitation of this technique is that small focal areas lead to long treatment times for large treatment volumes. Other stereotactic techniques are under investigation, such as magnetic heating of nanoparticles, laser therapy, stereotactic radiation therapy.[61] Heating of nanoparticles by a low radiofrequency source has been shown to open the BBB by creating local hyperthermia, a condition well-known to open the BBB. Local hyperthermia has also been investigated using superparamagnetic iron oxide nanoparticles under an alternating magnetic field.[62] The safe use of these techniques remain questionable, as improper treatments may severely damage brain tissue. Pulsed lasers offer another means to generate local hyperthermia to open the BBB. Laser interstitial thermal therapy (LITT) uses MRI to guide a laser to the center of a tumor for thermal ablation. This technique has been shown to transiently open the BBB in

patients. Despite significant progress in recent years, current focal techniques to open the BBB have numerous limitations, and established systemic treatments (such as osmotic disruption by mannitol or bradykinin) are associated with adverse systemic side effects. Consequently, new methods of BBB permeability must be explored. Electrical energy, administered in the form of electrical pulses, may offer another mode of BBB opening.

Electroporation has been shown to disrupt the BBB *in vitro* and *in vivo*. [42-46,63] For both IRE and H-FIRE, electric fields of lower magnitude than those used for tumor ablation can reversibly disrupt the BBB. Combining the tumor ablation and BBB-disruption capabilities of IRE and H-FIRE may provide significant improvement to patient outcomes. In regard to neurological diseases and disorders, several electrical regimes are of particular interest, including irreversible electroporation (IRE), high-frequency irreversible electroporation (H-FIRE), tumor treating fields(TTFs), and deep brain stimulation (DBS). Electrical energy, applied as high-magnitude electrical pulses can transiently increase the permeability of cell membranes.

Recent *in vivo* studies have found that H-FIRE pulses can cause BBB disruption in rats.[42,44,64]. H-FIRE pulses (250 V/cm, 300 pulses) administered via intracranial electrodes to the motor cortex of rats revealed significant volumes of BBB disruption by gadolinium-Evans blue albumin tracers, without causing signs of necrosis or apoptosis.[44] Another *in vivo* study in rats found that electroporation of the brain is primarily reversible for electric field magnitudes less than 400V/cm.[64] This same study found evidence of BBB disruption (via Evan Blue and Gadolinium) for electric field magnitudes of 200V/cm to 1000V/cm. A recent *in vivo* study investigated the duration of BBB disruption after HFIRE pulses.[42] HFIRE pulses (600 V/cm, 200 pulses) were

administered to rats, and BBB disruption was measured by gadolinium9m and Evan's blue at 1, 24, 48, 72, and 96 h after treatment. This study found that BBB disruption was maximal at the 1-hour timepoint, but some BBB disruption was apparent until the 72-hour timepoint.

In vitro models of the BBB offer several advantages over *in vivo* experiments for understanding BBB disruption by electroporation. *In vivo* experiments, while results are highly relevant, suffer from high costs per experiment, low-throughput, and potential confounding of results with uncontrolled variables. Furthermore, *in vivo* experiments with animal models often poorly recapitulate the behavior of humans due to species-specific responses.[65,66] This is particularly challenging drug discovery for neurological diseases, where species-specific differences have been shown to be highly significant. Microfluidic devices, however, offer low-cost, high-throughput platforms that can incorporate human cells mimic *in vivo* human properties. Furthermore, microfluidic devices can be designed to precisely control variables so that a single variable can be isolated and tested. Chapter 6 investigates BBB permeability after electrical pulses, and Chapter 7 investigates a novel BBB basement membrane that may improve future *in vitro* BBB studies.

1.6 Research Scope and Aims

The underlying hypothesis of this dissertation is that the quantification of brain cell mechanics and improved blood-brain barrier models will facilitate the development of successful brain cancer therapies such as treatment by pulsed electric fields. We investigate cytoskeletal changes induced by electroporation therapies and stiffness changes induced in brain cancer, both of which have yet

to be well studied. Chapter 2 provides a comprehensive literature review on the current understanding of how the cytoskeleton (including cell-cell junctions, and cell-substrate junctions) are affected by pulsed electric fields, and how this understanding can inform electroporation-based therapies. Chapter 3 describes an original research study that used nanofiber networks to quantify the changes in cell mechanics, specifically cell contractile forces, after applying electrical pulses to glioblastoma cells. This work is directly relevant to reversible electroporation techniques such as electrotransfection, and also for understanding how glioblastoma cells may respond to electroporation *in vivo*. Chapter 4 describes the use of a microfluidic device to compare mechanical properties of glioma cells across several grades to understand if mechanical properties influence the progression of this disease.

Electroporation conditions for optimal BBB disruption have yet to be identified. Chapter 5 describes the use of a microfluidic device to mimic the BBB using a monolayer of human cerebral microvascular endothelial cells (hCMECs) and investigate endothelial monolayer mechanics and permeability. We show how the BBB can be disrupted by both IRE and H-FIRE pulses, using a complex microfluidic device to increase the efficiency of experiments. Existing microfluidic models fail to replicate the physiology and mechanics of the BBB. Chapter 6 introduces a novel basement membrane using dense networks of nanofibers, and investigates their usefulness in BBB models. Finally, Chapter 7 investigates how cell morphology (shape) and cell orientation impact cell viability after electroporation, which until now has not been characterized.

Together, this body of work combines new insights into cytoskeletal disruption by electric fields through investigations on electroporation-induced BBB disruption and astrocytoma

mechanical properties to advance the field of electroporation and brain cancer therapies. Quantification of cell mechanics of key brain cell types (astrocytes, pericytes, endothelial cells) during disease and after pulsed electric fields will facilitate the development of successful brain cancer therapies, such as treatment by pulsed electric fields.

2 Chapter 2: Cytoskeletal disruption after electroporation and its significance to pulsed electric field therapies

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2.1 Abstract

Pulsed electric fields (PEFs) have become clinically important through the success of Irreversible Electroporation (IRE), Electrochemotherapy (ECT), and nanosecond PEFs (nsPEFs) for the treatment of tumors. PEFs increase the permeability of cell membranes, a phenomenon known as electroporation. In addition to well-known membrane effects, PEFs can cause profound

cytoskeletal disruption. In this review, we summarize the current understanding of cytoskeletal disruption after PEFs. Compiling available studies, we describe PEF-induced cytoskeletal disruption and possible mechanisms of disruption. Additionally, we consider how cytoskeletal alterations contribute to cell-cell and cell-substrate disruption. We conclude with a discussion of cytoskeletal disruption-induced anti-vascular effects of PEFs, and consider how a better understanding of cytoskeletal disruption after PEFs may lead to more effective therapies.

Keywords: Pulsed Electric Fields, Electroporation, Cytoskeleton, Actin, Microtubules, Intermediate Filaments, Cell Junctions, nsPEFs, IRE, ECT, Mechanobiology, Vascular lock, Cancer

2.2 Introduction

In recent years, pulsed electric fields (PEFs) have become an important clinical tool for the treatment of tumors by Irreversible Electroporation (IRE)[37-41] and Electrochemotherapy (ECT)[67,68]. Clinical and preclinical studies of nanosecond PEFs (nsPEFs) [27,69], Gene Electroporation (GET)[34-36], and electrofusion[70,71] therapies show significant potential for cancer treatment. Beyond cancer therapy, PEFs are useful for a variety of purposes including bacterial inactivation[72,73], decellularization of tissues [74,75], extraction of biomolecules[76,77], and numerous GET applications[78-83]. Exogenous electric fields applied as short, high-magnitude pulses cause electroporation, a phenomenon characterized by increased cell membrane permeability. Classical electroporation theory describes metastable, lipidic pores formed by PEFs that enable uncontrolled molecular and ionic transport across the cell membrane and cause a loss

of cell homeostasis[84]. Additionally, modulation of voltage-gated ion channels and oxidization of lipids can further increase membrane permeability after PEFs[9]. PEF therapies such as IRE and nsPEFs rely on PEF-induced cell disruption to destroy tumor cells. ECT combines reversible PEF disruption with adjuvant chemotherapy to enhanced drug uptake and causes cell death. Likewise, GET combines reversible PEF disruption with nucleic acids to enhance transfection of cells for therapeutic purposes. Several excellent reviews are available on electroporation theory[9,84] and PEF therapies in clinical/preclinical oncology[8,67,85-87].

In recent years there has been a growing appreciation that the cell cytoskeleton is involved with and affected by PEFs[88]. The cell cytoskeleton, composed of actin, microtubules (MT), intermediate filaments (IFs), and septin, provides structure and mechanical stability to cells, enabling tensional homeostasis with the cell's environment[89,90]. Critical cell functions such as proliferation, differentiation, signaling, migration, and cell survival would not be possible without the cell cytoskeleton[31,32]. These filamentous structures dynamically adapt to control intracellular transport, organelle location, cell contractility, cell shape, cell volume, and cell behavior, among many other functions. Cytoskeletal filaments provide support to the highly fluid, flexible, and extensible plasma membrane through linker proteins, that together enable mechanical interactions with adjacent cells via cell-cell junctions or with the environment via cell-substrate adhesions. Of the studies surveyed in this review, the majority focus on actin and MTs, with few studies considering disruption to IFs and no studies considering disruption to septin (Figure 2.1a).

The number of studies investigating cytoskeletal disruption has increased dramatically in the last decade (Figure 2.1b). In particular, nanosecond PEFs (nsPEFs) have seen a tremendous growth in

the number of studies and now account for over half the studies on PEF-induced cytoskeletal disruption. Studies on cytoskeletal disruption include a broad range of pulse lengths, from nanosecond pulses (nsPEFs) to millisecond PEFs (msPEFs) (Figure 2.1c). Microsecond and millisecond PEFs such as those used for IRE, ECT, and GET are generally applied at electric field strengths between 0.1 and 2 kV/cm. In contrast, nsPEFs generally employ field strengths over 10 kV/cm. (Figure 2.1c). These high field strengths and short pulse lengths of nsPEFs cause smaller pore radii than longer pulses[27,91,92], phosphatidylserine externalization[93,94], elevated intracellular calcium concentration[95-97], depolarization of mitochondria[98-100], DNA damage[95,101,102], altered ion channel activity[103], and apoptosis[27,28]. Results from studies investigating cytoskeletal disruption seem to indicate that most observed features of cytoskeletal disruption are conserved across pulse lengths ranging from nsPEFs to msPEFs. Pulse length and magnitude, however, likely do influence the mechanisms of disruption (see Section 5.6) and are important factors in understanding cell response.

In this review, we seek to provide a comprehensive summary of studies investigating cytoskeletal dynamics after PEF treatment. To this end, we have included a table that summarizes the study parameters and key findings of relevant studies, arranged by pulse length (**Table 2-1**). Beginning our discussion with actin, we briefly review cytoskeletal structure before summarizing descriptions of cytoskeletal changes after PEFs. We then review suggested mechanisms of cytoskeletal disruption, and discuss how cytoskeletal disruption impacts cell-cell and cell-substrate interactions. Finally, we discuss the significance of cytoskeletal disruption to electroporation therapies, focusing on

anti-vascular effects, and end our review with an outlook on how understanding cytoskeletal disruption may improve PEF therapies.

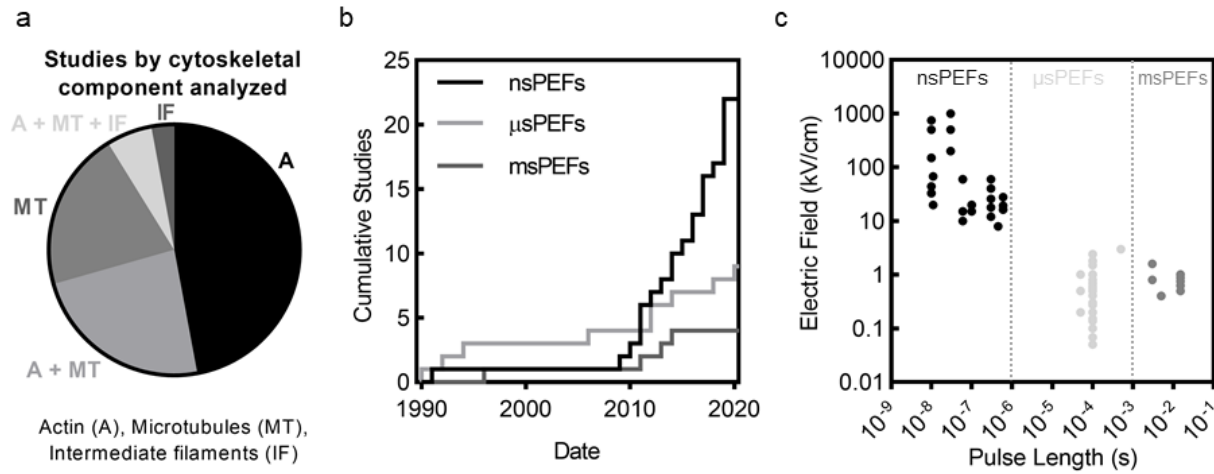


Figure 2.1 An analysis of published studies since 1990 on cytoskeletal disruption by PEFs.

(a) Actin disruption is the cytoskeletal component most frequently investigated by studies. Many studies also consider MT disruption. Few studies, however, consider disruption to intermediate filaments (IFs) and no studies consider septin disruption. (b) Since 2010, there has been significant interest in nanosecond PEF (nsPEFs), which now account for over half of all studies on PEF-induced cytoskeletal disruption. Microsecond PEFs (μ sPEFs) and millisecond PEFs (msPEFs) have also seen an increase in studies. (c) Studies cover a wide range of pulse lengths and field magnitudes. nsPEFs are applied at high field strengths (generally >10 kV/cm), while μ sPEFs and msPEFs are applied at lower (0.1-2 kV/cm) field strengths. Data points show field strengths tested in these studies.

2.3 Actin and PEF-induced Actin Disruption

Evolutionarily conserved and highly abundant in cells, actin is a key component of the cell cytoskeleton. Actin monomers polymerize spontaneously to form thin (~ 7 nm-diameter), flexible, helical filaments composed of two strands of actin subunits[104]. Actin polymerization preferentially occurs at the plus end of an actin filament, and filament dynamics are controlled by a variety of regulatory proteins that nucleate new filaments, elongate existing filaments, terminate filaments, sever filaments, crosslink filaments, and sequester unpolymerized actin monomers. Hydrolysis of actin-bound ATP occurs randomly along filaments and leads to depolymerization.

Actin fibers generally interact to form networks or bundles, that regulate cell shape, locomotion, intracellular transport, enable cell-cell and cell-matrix interactions, and define the cell's mechanical properties. In a cell, normal actin features include stress fibers, lamellipodia, filopodia, microvilli, and retraction fibers (Figure 2.2a)[31]. Importantly, actin forms the cell cortex, a dense network of crosslinked filaments beneath the lipid bilayer. Cortical actin connects to the plasma membrane via linker proteins to provide mechanical support to the otherwise highly extensible and compliant lipid bilayer. To create contractile forces, aligned and bundled actin filaments form stress fibers and use ATP-driven myosin motors to contract. Compared to muscle cells, actin contractility is more dynamic in non-muscle cells and enables cells to quickly adapt their structure to changing conditions. Additionally, actin links to cell-cell junctions such as adherens and tight junctions, as well as to cell-substrate adhesion complexes such as focal adhesions. The numerous, dynamic roles of actin allow PEF-induced disruption to take on many forms.

2.3.1 Actin Disruption

Visual manifestations of actin disruption after PEFs (Figure 2.2a-c) vary significantly between studies. For example, actin filaments have been reported to become shorter[19], thinner[22,105], fragmented[22,106], less densely spaced[105,106], misaligned[19,106], depolymerized[18,106,107], and show decreased fluorescence[19,102] after PEFs. Some studies report actin localization to the cell periphery[105] and honeycomb-like f-actin patterns[20,22] after PEFs. Actin contraction and detachment of transvacuolar actin bundles in plant cells were also observed[107]. Several studies report diminished f-actin features concomitant with an increase in background fluorescence, presumably indicating more oligomeric actin[19]. After electroporation, cells have been reported to have ruffled membranes[18,22,102], microvilli[108-110], form podosomes[105], show bright actin spots (foci) on the surface of cells[102,111], or show a partial loss of the actin cortex[111,112]. Some studies report a loss of membrane bright spots and homogenous actin[113]. Cell rounding and shape change is also frequently reported[18,19,102,105,111,113,114]. Unique features, such as pseudopod-like blebs, have also been demonstrated after PEFs[115]. Yet, at least one study reported no changes to actin after PEFs[21].

Factors that cause these cytoskeletal alterations are discussed in Section 5, but the diversity of disruption characteristics reported by studies is at least in part due to cell type, pulsing media, and pulse type (duration, magnitude). For a complete list of studies evaluating actin disruption after PEFs, see Table 2-1. Table 2-1 includes information such as cell type, cell attachment (adherent, suspension, monolayer), pulsing parameters, and key cytoskeletal findings from each study, and

is arranged by pulse length. We briefly highlight a few studies indicating actin disruption in the following paragraphs.

In a study by Stacey et al., actin-stained human cervical cancer (HeLa) cells showed membrane ruffling and reduced staining intensity within one minute after a single 60ns pulse (60 kV/cm) (Figure 2.2c) [102]. By 3-4 minutes, cells rounded and showed membrane speckling (bright spots). Human T lymphocytes (Jurkat cells) were more susceptible to PEF treatment than HeLa cells, and showed speckled membrane morphology within seconds but did not recover.

Chopinet et al. showed that PEF-treated (5ms, 0.4 kV/cm, 8 pulses (p)) Chinese hamster ovary (CHO wild type) cells had membrane rippling, fewer membrane extensions, and a smoother, more homogeneous membrane 3-15 minutes after PEFs[18]. Stress fibers were disrupted and were not visible between 15-23 minutes. By 23 minutes after pulsing, cells re-spread and no longer showed membrane rippling. Membrane permeability was reduced to baseline levels within 10 minutes of pulsing, demonstrating that cytoskeletal disruption continued after membrane resealing. Cell swelling was observed. This study used atomic force microscopy (AFM) and found that cell stiffness decreased after PEFs, a topic that is discussed in the Section 2.1.1.

Application of 100ns pulses (20 kV/cm, 20p) by Steuer et al. to rat liver epithelial (WB-F344) cells caused actin reorganization within 5 minutes[19]. In contrast to well aligned actin stress fibers spanning the cell before PEF treatment, 5 minutes after PEFs actin fibers appeared shorter and less aligned. After 15 minutes, few well-defined fibers were visible, but instead an increase in diffuse background intensity throughout the cell was seen, indicating oligomeric actin in the cytosol. Actin filaments began to reform by 30 minutes, and a partial recovery was achieved by 60 minutes. The

study found that the recovery timescale of the actin cytoskeleton did not correlate with temporal changes in Young's modulus (YM).

Meulenberg et al. studied actin disruption in a human dermal microvascular endothelial cell (HMEC-1) monolayer after PEFs and ECT treatment (100 μ s, 0.07-0.7 kV/cm, 8p) over a range of field strengths (Figure 2.2b)[22]. Cells showed f-actin fibers spanning the cells, which formed an intact monolayer before pulsing. Ten minutes after low-magnitude PEFs, f-actin remained intact but granules and spots formed on the outer membrane. At the same timepoint, high fields resulted in less-rigid and fragmented actin, cell swelling, and peripheral actin in a honeycomb-like appearance. Two hours after PEFs, cells continued to show swelling and f-actin was significantly recruited to the cell periphery. ECT decreased cell turgidity and caused a spindle-like morphology. Monolayer recovery was achieved for low-level PEFs by 24 hours, but high-magnitude PEF and ECT treated cells did not recover.

In another study by Kanthou et al. with a monolayer of human umbilical vein endothelial cells (HUVECs), actin fibers dissolved immediately (<5 minutes), and staining showed diffuse background fluorescence after 100 μ s PEFs(0.05-0.2 kV/cm, 3p)[20]. Fine processes extended away from the cell periphery, and actin acquired a honeycomb-like appearance. Actin regained its structure within 1 to 2 hours. Western blotting indicated that phosphorylated myosin light chain (pMLC), which mediates actomyosin contraction, decreased immediately after PEFs but then dramatically increased in a burst at 30 and 60 minutes. The total level of actin was unaffected for a least 16 hours.

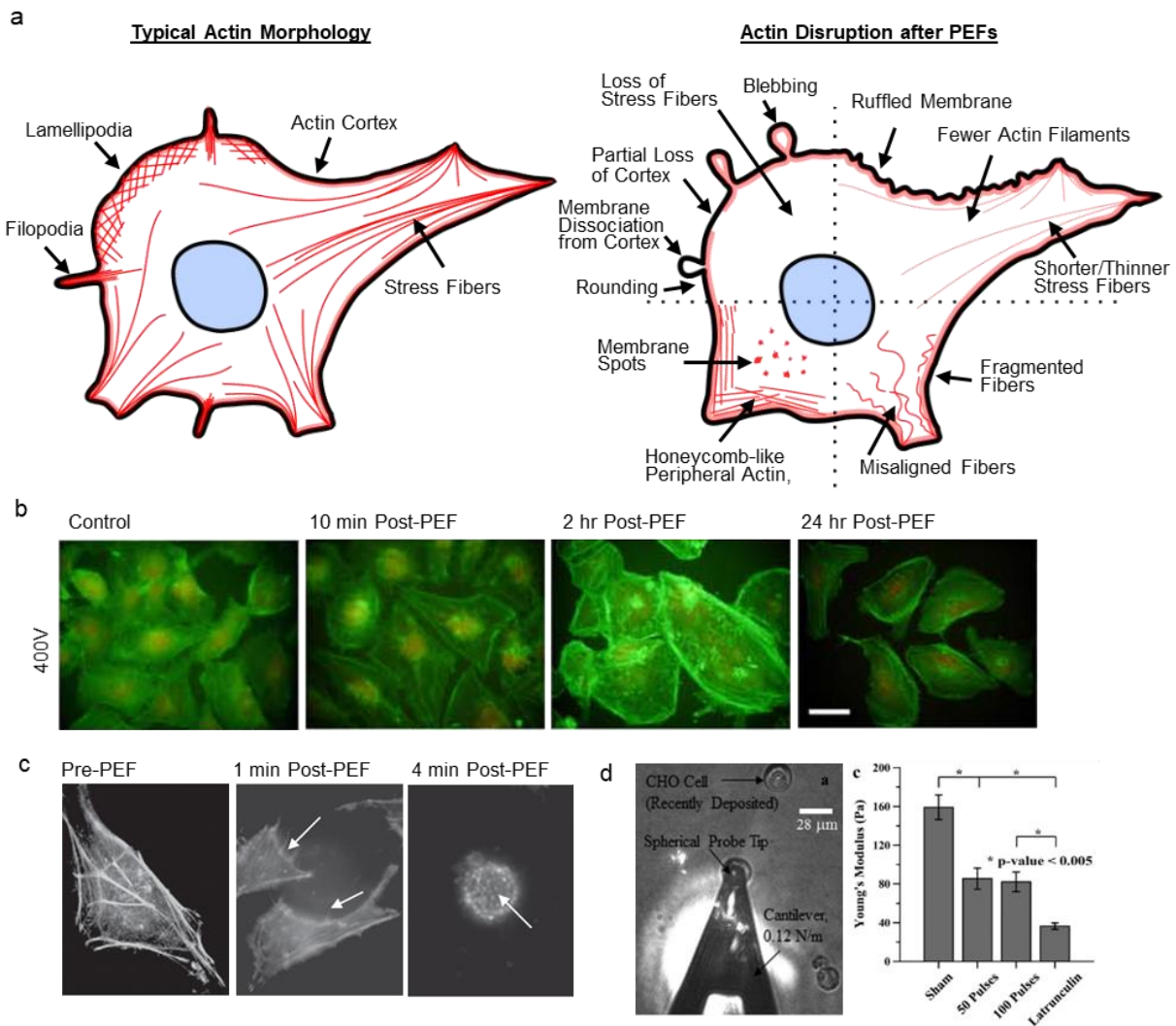


Figure 2.2 PEF-induced actin disruption.

(a) Normal actin structures include stress fibers, filopodia, lamellipodia, and cortical actin (left). PEF-induced cytoskeletal disruption takes on many forms, such as a loss of f-actin, cell rounding, membrane ruffling, actin spots/foci/podosomes, dissociation of the cortex from membrane, and blebbing. (right) (b) Actin disruption of a human dermal microvascular endothelial cell (HMEC-1) monolayer after eight, 100 μ s PEFs. Scale bar 50 μ m. Adapted from [22]. (c) Human cervical cancer (HeLa) cells after a single 60ns PEF showed membrane ruffling

(center), cell rounding (right), and actin spots on the membrane (right). Adapted with permission from [102]. **(d)** Atomic force microscopy (AFM) measurements show cell elasticity decreases after PEFs. The Young's modulus (YM) of Chinese hamster ovary (CHO-K1) cells decreased ~50% after 10ns PEFs (50 and 100 pulses). Adapted with permission from [112].

Table 2-1 Summary of studies on cytoskeletal disruption by pulsed electric fields

Study (Year)	Cell Type (A: adherent; S: suspension; M: monolayer)	Pulse Length	Field Strength (kV/cm)	Pulse # (freq)	Pulsation Buffer (with(+) or without(-) Ca^{+2})	Cytoskeletal Agent	Focus	Outcomes
Harkin et al. (1996)[21]	Chick embryo corneal fibroblasts (A)	10-20ms ¹	0.5, 0.625, 0.75, 0.875, 1.0	1 (N/A)	Basal Media (+ Ca^{+2}); Buffers (+/- Ca^{+2})		Actin MT IF	Media as pulsation buffer inhibited migration for 2 h, caused MT loss after 10 minutes, but showed MT recovery in 3-4hrs; Some buffers preserved migration and MTs, excepted with high concentrations of CaCl. Extracellular calcium adversely affects cell migration due to MT disruption. Staining showed no impact to actin. Perinuclear collapse of IFs, with recovery in 3-4hrs.
Chopinet et al. (2013)[18]	CHO wild type (A)	5ms	0.4	8 (1Hz)	Buffer (- Ca^{+2})		Actin	AFM measurements showed YM decreased 40% after PEFs; YM more spatially homogeneous within 1 min; YM similar for electrode-facing regions and perpendicular-facing regions; Membrane rippling, loss of actin fibers 3-15 min; YM not correlated with cell resealing time; Cell swelling present; Cells re-spread by 23 min.
Chopinet et al. (2014)[116]	CHO wild type (A)	5ms	0.4	8 (1Hz)	Buffer (- Ca^{+2})	LatB LatA	Actin	AFM showed YM of CHO cells decreased 30% by LatB, and recovered in 35 min after drug removal; Magnitude and duration of YM response are similar between PEF treatment and recovery from LatB; Cells do not recover from LatA and PEFs. PEFs before LatB treatment showed additive effects.
Hohenberger et al. (2011)[117]	BY-2 ² (S)	3ms; 10ns	0.8, 1.6; 33	1-10, (1Hz); 1-20, (NR)	Buffers (+/- Ca^{+2})		Actin MT	Genetically modified BY-2 cells with increased actin bundling showed less PI uptake; Actin bundling stabilized the cell membrane against permeabilization after msPEFs and nsPEFs.
Downey et al. (1990)[118]	Human Neutrophils (S)	NR ³	NR	2 (NR)	Buffer (+ Ca^{+2})		Actin	Influx of extracellular calcium post-PEFs caused depolymerization of f-actin.

Perrier et al. (2019)[119]	Actin-GUV; Empty-GUV	500µs	0.1-3, 3-10	1-30, 2-4 (0.017 Hz)	Buffer (-Ca ⁺²)		Actin	Actin-GUVs had increased and prolonged dye uptake compared to empty-GUVs; Actin-GUVs had reduced electrodeformation; Actin cortex fluorescence decreased after PEFs; Electrophoretic effects on actin calculated to be 4x greater than electrodeformation effects.
Rols et al. (1991)[110]	CHO-WTT (A)	100µs	1.5	10 (1Hz)	Buffer (-Ca ⁺²)	CytB, COL, ATP, GTP	Actin MT	Pretreatment with COL decreased resealing time and electrofusion rate post-PEFs; CytB had no significant change on electrofusion rates.
Rols et al. (1992)[108]	CHO-WTT (A) RBC (S)	100µs	1.8, 2.4	10 (1Hz)	Buffer (-Ca ⁺²)	CytB, COL, ATP, GTP	Actin MT	COL-treated cells resealed 3x faster; ATP/GTP in buffer did not affect resealing time; Pore resealing, but not pore formation, affected by cytoskeleton; Microvilli density increased post-PEFs.
Teissie et al. (1994)[109]	CHO-WTT (A) RBC (S)	100µs	1.8, 2.4	10 (1Hz)	Buffer (-Ca ⁺²)	CytB, COL, ATP, GTP	Actin MT	COL-treated cells resealed 3x faster; Microvilli density increased post-PEFs; Extracellular ATP increased microvilli length; Resealing rate was dependent on MTs.
Kanthou et al. (2006)[20]	HUVEC (M)	100µs	0.05, 0.1, 0.15, 0.2	3 (1Hz)	Basal Media (+Ca ⁺²)		Actin MT IF	Actin and MTs depolymerized in 5 min; Actin became honeycomb-like; MTs fragmented; Burst of pMLC at 30 and 60 min; Cytoskeletal recovery 1-2 hrs; IFs relatively unchanged, except at cell periphery.
Meulenber g et al. (2012)[22]	HMEC-1 (M)	100µs	0.068, 0.137, 0.274, 0.411, 0.548, 0.685	8 (1Hz)	Buffer (-Ca ⁺²)		Actin MT	Actin stress fibers thinned, fragmented, and took on a honeycomb-like organization; ECT caused cell shrinkage; MTs became densely packed, less extended, and fragmented; Partial monolayer recovery at 24 hrs for PEFs, but no recovery for ECT; Cell swelling by 10 min; Cell edges ruffled at 2hrs; ECT caused more rapid increase in membrane permeability.
Szewczyk et al. (2018)[120]	C2C12 (A, S) RD (A, S)	100µs	0.6, 0.8, 1	8 (1Hz)	Buffers (+/- Ca ⁺²)		Actin	Ca ⁺² in buffer increased zyxin expression and actin stress fiber tension in normal C2C12 cells, but decreased zyxin expression and depolymerized actin in cancerous RD cells; Zyxin changes indicated altered cell-cell and cell-substrate connections; Adherent cells showed higher viability after PEFs than suspended cells.

Kim et al. (2020)[121]	NCI-H640 (A) MCR-5 (A)	100μs	0.3, 0.5, 0.7, 1	8 (10Hz)	Basal Media (+Ca ⁺²)	CytD	Actin	CytD pretreatment decreased PI uptake after PEFs compared to PEFs alone; Annexin V-FITC signal decreased with low concentrations of CytD and low field strengths.
Pehlivanov a et al. (2012)[105]	MDA-MB-231 (A); MCF-7 (A); NIH/3T3 (A)	Bipolar: 50-20- 50μs	0.2, 0.5, 1.0	8 (1Hz)	Basal Media (+Ca ⁺²)		Actin	Adhesion post-PEFs was cell-type and field-strength dependent; More cytoskeletal disruption in cancerous cells than fibroblasts; Stress fibers were thinner, fewer, and at high fields located peripherally; Podosomes formed; Actin recovered in 24-48 hrs, except at high fields.
Pakhomov et al. (2014)[113]	CHO-K1 (A)	600ns	1.92	1,4 (2Hz)	Buffer (-Ca ⁺²)		Actin	Mitigating cell swelling prevented actin disruption post-PEFs. Without mitigating swelling, cells showed increased fluorescence of diffuse actin, reduced bright spots, and reduced overall actin fluorescence.
Thompson et al. (2014)[122]	CHO-K1 (A)	600ns	16.2	1, 20 (NR)	Buffers (+/- Ca ⁺²)	PTX	MT	Ca ⁺² in buffer caused MT disruption and halted lysosome transport; MT disruption occurred despite mitigating blebbing and swelling; PTX stabilized MTs against depolymerization after PEFs.
Thompson et al. (2016)[123]	CHO-K1 (A)	600ns; 10ns	27.7; 150	1, 5, 10, 20 (1Hz)	Buffers (+/- Ca ⁺²)		IF	Localization of cortical lamin within the nucleus after PEFs; Disruption of lamin cortex correlated with nuclear permeabilization.
Tolstykh et al. (2017)[124]	CHO-K1 (A)	600ns	16.2	1, 20; (5 Hz)	Buffer (+Ca ⁺²)		Actin	PIP ₂ depletion and PLC activity led to cell swelling and blebbing; Edelfosine to block PLC activity inhibited blebbing.
Xiao et al. (2011)[125]	HepG2 (A)	450ns	8	30 (1Hz)	NR	CytB	Actin	CytB treatment before PEFs decreased necrotic and apoptotic cells; CytB alone did not decrease viability compared to controls.
Ford et al. (2010)[28]	B16-F10 (S)	300ns	12, 18, 26, 40, 60	1, 3, 10 (NR)	Buffer (-Ca ⁺²)		Actin	Caspase activity and cytoskeletal integrity mutually exclusive; ATP decreased after nsPEFs
Steuer et al. (2016)[106]	WB-F344 (M)	100ns	15,20	20 (NR)	Complete Media (+Ca ⁺²)		Actin	F-actin fragmented, less organized, and depolymerized after PEFs; cell morphology generally unchanged; Partial actin recovery by 60 min.
Steuer et al. (2017)[19]	WB-F344 (M); WB-Ras (M) ⁴	100ns	20	20 (NR)	Complete Media (+Ca ⁺²)		Actin	AFM showed >30% decrease in YM after 8 mins; Actin fibers shorter, less aligned at 5 min; increased diffuse fluorescence at 15 min; YM recovered to control values at 13 -28 min; Partial recovery 30-60 min; PEFs did not induce tumorigenic behavior.

Stacey et al. (2011)[102]	Jurkat (S) HeLa (A) SV40 (A)	60ns	60	1 (N/A)	Buffer (NR)	CytB	Actin	Adherent cells had ruffled membranes and rounded up with speckled actin spots; Jurkat cells showed actin speckling; Decreased viability in HeLa and SV40 cells after pretreatment with CytB.
Rassokhin et al. (2011)[115]	U-937 (A)	60ns	10	>1000 (10- 20Hz)	Buffer (-Ca ⁺²)	CytD	Actin	Pseudopod-like bleb (PLB) growth toward the anode during PEFs; CytD prevented PLBs; Actin caused unique shape; Inhibiting cell swelling prevented PLBs; Not replicated in CHO, Jurkat, or GH3 cells.
Dutta et al. (2015)[111]	Jurkat Clone E6-1 (S)	60ns	15, 60	1 (N/A)	Complete Media (+Ca ⁺²)		Actin	AFM showed 53% decrease in YM after 15 kV/cm PEFs and minimal actin/morphological changed; At 60 kV/cm, YM decreased 85%; Cell shape change, peripheral actin became more diffuse, and actin foci formed.
Marracino et al. (2019)[126]	N/A	30ns	200, 500, 1000	1 (N/A)	N/A		MT	MD simulations showed tubulin dipole moment increased 50% at 200 kV/cm and 300% at 1 MV/cm; No unfolding of structural motifs, but C-terminus tail pulled away from tubulin body.
Průša et al. (2019)[127]	N/A	30ns	1000	1 (N/A)	N/A		MT	MD simulations of kinesin-I docked to a tubulin heterodimer indicated altered kinesin dipole properties, altered contact surface area between kinesin and tubulin, and altered structures including MT binding motifs and nucleotide hydrolysis sites.
Chafai et al. (2019)[128]	N/A	11ns	20	100, 200, 400, 800 (1Hz)	Buffer (-Ca ⁺²)		MT	Purified tubulin showed decreased polymerization after PEFs; Autofluorescence measurements suggested conformational changes after PEFs; Altered zeta potential of tubulin after PEFs; AFM showed altered tubulin structures after PEFs; Immunoblots showed no damage to tubulin.
Havelka et al. (2019)[129]	RBL-2H3 (A)	11ns	~ 67.5	4000 (100Hz)	Buffer (+Ca ⁺²)		MT	GFP-tagged MT end-tracking protein EB3 showed decreased fluorescence and size after PEFs.
Thomson et al. (2013)[130]	CHO-K1 (A) U-937 (S) Jurkat Clone E6-1 (S)	10ns	150	100 (NR)	Complete Media (+Ca ⁺²)	PTX, JAS, LatA, NOC	Actin MT	LatA pretreatment decreased CHO-K1 elasticity to levels of Jurkat cells, however CHO-K1 cells had higher viability after PEFs; MT disruption by NOC decreased PI uptake and Annexin V-FITC fluorescence; LatA pretreatment increased PI uptake and Annexin V-FITC; JAS and PTX pretreatment did not change membrane damage.

Berghöfer et al. (2009)[107]	BY-2 (S)	10ns	33	1 (N/A)	Buffer (-Ca ⁺²)	PHD	Actin MT	Depolymerization of cortical actin; Detachment of transvacuolar actin bundles from cell periphery; Actin contraction toward the nucleus; PHD pretreatment decreased uptake of trypan blue and suppressed actin detachment from cell periphery; MTs affected within 1 min, and maximally disordered by 3 min.
Thomson et al. (2014)[112]	CHO-K1 (A)	10ns	150	50, 100 (1Hz)	Complete Media (+Ca ⁺²)	LatA	Actin	AFM showed that YM of newly-adherent cells decreased ~50% after PEFs and caused partial loss of the actin cortex; LatA caused ~80% decrease in YM and fully disrupted the actin cortex; LatA treatment before PEFs increased PI uptake and decreased viability.
Carr et al. (2017)[100]	U-87 MG (A)	10ns	44	100 (10Hz)	Buffers (+/-Ca ⁺²)		MT	MTs showed buckling, breaking, depolymerization; MT end-tracking protein EB3 showed altered dynamics post-PEFs. Decreased tubulin and EB3 comet fluorescence after PEFs; Decreased number of EB3 comets, but comet length increased; MT disruption independent of intra/extracellular calcium; MT disruption temporally linked with mitochondria depolarization.
Timmons et al. (2018)[131]	N/A	10ns	50-750	1 (N/A)	N/A		MT	MD simulations indicated conformational changes to charged and flexible regions of sidechains and loops of tubulin such as α :H1-B2 loop, β :M-loop, and c-termini. Intradimer curvature increased in simulations after PEFs.

Abbreviations: MT Microtubules, IF intermediate filaments, PI Propidium Iodide, CytB Cytochalasin B, CytD Cytochalasin D, LatA Latrunculin A, LatB Latrunculin B, PHD Phalloidin, JAS Jasplakinolide, PTX Paclitaxel, ATP Adenosine Triphosphate, GTP Guanosine Triphosphate, COL Colchicine, NOC Nocodazole, PIP2 Phosphatidylinositol 4,5-bisphosphate, PLC phospholipase C, AFM atomic force microscopy, pMLC phosphorylated myosin light chain, GUV giant unilamellar vesicle, freq Frequency, MD Molecular Dynamics

1 Exponentially decaying pulse (125-960 uF), Time constant 10-20ms.

Exponentially decaying pulse (25uF) Time constant not reported.

3 WB-Ras derived from WB-F344 with H-Ras oncogene. Genetically engineered to overexpress actin-binding domain 2 of plant fimbrin or with inducible expression of actin-bundling WLIM1 protein

2.3.2 Actin-induced cell elasticity changes

Actin has been shown to be the primary cytoskeletal component contributing to cell elasticity, or Young's modulus (YM) [132-134]. Thus, changes to the actin cytoskeleton can be investigated by atomic force microscopy (AFM). Several studies show that the YM significantly decreases after PEFs. In a study by Chopinet et al., the YM of adherent CHO (wild type) cells decreased across the cell within one minute, and by 8 minutes was only 60% of pre-PEF values after millisecond PEFs (5ms, 0.4 kV/cm, 8p)[18]. During this time, cell elasticity became homogenous across the cell. Recovery of heterogeneous elasticity (areas of high stiffness) began by 17 minutes, and YM slowly returned to pre-pulse values by 35 minutes. Nanosecond PEFs also decrease YM. Steuer et al. found that the elasticity of rat liver epithelial (WB-F344) cells decreased by approximately one third across the cell periphery and 60% above the cell nucleus at 8 minutes after 100ns PEFs (20 kV/cm, 20p)[19]. Recovery of elasticity occurred between 13-28 minutes, despite incomplete f-actin recovery at these timepoints. In this study, cells did not uptake propidium iodide (PI) and did not show signs of swelling. In a study by Dutta et al., a single 60ns PEF at 15 kV/cm caused little morphological changes in human T lymphocytes (Jurkat Clone E6-1), however YM decreased over 50% when these cells were fixed immediately after PEF treatment[111]. At 60 kV/cm, cell shape was altered, peripheral actin became more diffuse, foci appeared, and YM decreased 85%. In another study by Thompson et al., even very short 10ns PEFs (150 kV/cm, 100p) decreased YM 50% on relatively round, newly-adherent Chinese hamster ovary (CHO-K1) cells 90 minutes after PEFs and imaging revealed a partial loss of the actin cortex (Figure 2.2d)[112].

Due to the short indentation depths used in some of these studies, decreased YM points to dissociation of the plasma membrane from the actin cortex due to a loss of lipid anchoring[18]. These studies also show partial loss of the actin cortex and loss of f-actin fibers, which may contribute to decreases in YM. Inhibiting actin polymerization with chemical agents decreases YM similarly to PEF treatment[116].

2.3.3 Actin, membrane permeability, and cell viability

There is strong evidence that cytoskeletal proteins or associated proteins effect membrane permeability, particularly pore expansion and resealing. For example, PEF treatment of giant unilaminar vesicles (GUVs) results in micron-diameter macropores. Macropores, however, do not form on PEF-treated cells or actin-encapsulated GUVs[119]. The lack of macropores in cells suggests pore expansion is limited by the cytoskeleton and associated proteins [135]. Secondly, experiments with cells demonstrate long-lived permeability after PEFs (minutes - hours)[18,25,108,136,137], a result that contradicts molecular dynamics simulations[24,138-140] and lipid vesicle experiments[141,142] that show purely lipidic pores reseal rapidly (nanoseconds - milliseconds). Compared to empty GUVs, actin-encapsulated GUVs show significantly prolonged permeability[119]. Likewise, agar-encapsulated GUVs show extended permeability compared to fluid-filled GUVs because the impingement of the membrane on the agar structure stabilizes pores.[17] Electroporabilized regions of the cell are also not laterally mobile[143], suggesting cytoskeletal involvement.

To understand how actin structure impacts cell permeability and viability post-PEFs, several studies pretreated cells with actin-disrupting agents prior to PEF treatment. **Table 2-2** lists agents

used to disrupt actin and the studies using these agents. Use of these agents requires careful attention to concentration and exposure because these agents can be toxic, and thus deleterious to cell viability and permeability on their own. Given the reported data, it is unclear whether inhibition of actin polymerization is deleterious or protective against PEFs.

Table 2-2 Agents used to disrupt the cytoskeleton

ACTIN	Inhibit Polymerization	Stabilize Polymerization	Studies Used
Cytochalasin B or D (CytB/CytD)	X		[102,108,109,115,121,125]
Latrunculin A or B (LatA/LatB)	X		[112,116,130]
Phalloidin (PHD)	X		[107]
Jasplakinolide (JAS)		X	[130]
ATP		X	[108,109]
MICROTUBULES	Inhibit Polymerization	Stabilize Polymerization	Studies Used
Colchicine (COL)	X		[108,109]
Nocodazole (NOC)	X		[130]
Paclitaxel (PTX)		X	[122,130]
GTP		X	[108,109]

Several studies show that exposure to actin-inhibiting drugs before PEFs increases membrane permeability and decreased cell survival after PEF treatment. In a study by Rols et al., permeability of Chinese hamster ovary (CHO-WTT clone) cells exposed to cytochalasin B (2, 20, 60μM; 30 min incubation) caused extended permeability and significant cell death after PEF treatment (100μs, 1.8 kV/cm, 10p)[108]. Likewise, in a study Stacy et al., two cell lines pretreated with cytochalasin B (10μM; 30-40 min) had decreased viability after a single 60ns PEF (60 kV/cm)

compared to PEF treatment alone[102]. Thompson et al. showed a similar result in two studies using 10ns PEFs (150kV/cm), finding that latrunculin A pretreatment (1.2 μ M; 1 hour) increased PI uptake and Annexin V-FITC signal after PEFs, and decreased cell viability of CHO-K1 cells compared to PEF treatment alone[112,130]. These studies suggest disruption of the actin cortex leads to extended permeability and decreased post-PEF viability.

Other studies, however, report protective effects of actin disruption before PEF treatment. In a recent study by Kim et al., pretreatment with cytochalasin D (0.2-2 μ M; 1 hour) significantly decreased PI uptake in human lung carcinoma cells (NCI-H460) and normal human lung fibroblasts (MRC-5) after 100 μ s PEFs (0.3-1 kV/cm, 8p) [121]. Pretreatment with low levels of cytochalasin D (0.2-0.4 μ M) followed by low magnitude PEFs also decreased Annexin V-FITC signal. In another study by Xiao et al., disruption of actin in human hepatocellular carcinoma (HepG2) cells with cytochalasin B (2 μ M; 30 min) prior to PEFs (450ns, 8 kV/cm, 30p) led to fewer apoptotic and necrotic cells (higher overall viability) than undisrupted cells treated with PEFs[125]. In this study, early apoptotic cells decreased from $17 \pm 3\%$ to $5 \pm 3\%$ as indicated by Annexin+/PI- cells, and necrotic cells decreased from $21 \pm 3\%$ to $14 \pm 3\%$. Cytochalasin B inhibited the loss of mitochondrial membrane potential after nsPEFs, suggesting that actin disruption may prevent signal transduction from the membrane to the mitochondria and thus hinder apoptosis. Finally, Berghofer et al. showed that in a tobacco cell line (BY-2), pretreatment with phalloidin (1 μ M; 30 min) before 10ns PEFs (33 kV/cm, 1p) suppressed actin detachment from the cell periphery and reduced trypan blue uptake[107].

Additional studies are needed to elucidate the factors that contribute to the differential responses of cells to PEF treatment after actin disruption. Cell type, drug type, and drug exposure (concentration and duration) likely have a significant influence on study outcomes. Pulse length and pulse magnitude also may be partly responsible for the differential outcomes.

Actin stabilizing drugs have shown little effect on cell response to PEFs. Thomson et al. found that chemically stabilizing actin by jasplakinolide (10nM; 1 hour) before PEF treatment did not change the PI and Annexin V-FITC fluorescence of CHO-K1 cells compared to PEF treatment alone (10ns, 150 kV/cm, 100p) [130]. In other investigations by Rols et al. and Teissie et al., the addition of ATP and GTP (1mM) to the pulsation buffer did not change membrane permeability or resealing times of CHO-WTT clone cells after 100 μ s PEF treatment (1.8 kV/cm, 10p)[108,109].

2.4 Microtubules and PEF-induced Microtubule Disruption

Microtubules (MTs) are composed of α - and β -tubulin heterodimers that stack together, forming 13 protofilaments that create a hollow, cylindrical tube-like structure of approximately 25 nm-diameter[32,144]. Unlike actin, MTs are relatively stiff[145,146]. MTs nucleate on the centrosome (in animal cells) near the nucleus and radiate outward toward the cell periphery (Figure 2.3a)[147]. MTs exhibit dynamic instability, rapidly switching between polymerization and depolymerization at their plus ends[148]. Growing MTs assemble GTP-bound tubulin at their tips creating a cap, that if depleted by GTP hydrolysis causes catastrophic depolymerization as protofilaments peel away from the walls[149]. Many MT-associated proteins regulate tubulin polymerization[150].

MTs play an important role in mitosis, meiosis, intracellular transport, and cell mobility[32]. Motor proteins kinesins and dyneins power intracellular transport with ATP[151], and control the positions of intracellular organelles[152].

2.4.1 Microtubules disruption

MT disruption after PEF treatment has been less studied than actin, but MTs have been reported to fragment[20,22,100], buckle[100], become less extended[22], become densely packed[22], be depolymerized[21], and change polymerization dynamics[100] (Figure 2.3a). The timescale of MT recovery is similar to that of actin, with recovery in a few hours [20,21]. Table 2-1 lists studies investigating MT disruption along with key findings of these studies. A few of these studies are highlighted below, while others are discussed in Section 5 on the mechanisms of cell disruption.

Harkin et al. showed that chick embryo corneal fibroblasts treated with 10-20ms PEFs (0.5-1.0 kV/cm, 1p) in serum-free media lost their dense network of radiating MTs, with few MTs remaining after 10 minutes [21]. Short MTs were present after 1 hour, and a complete recovery of MTs occurred in within 3-4 hours.

In a study by Kanthou et al., 100 μ s PEFs (0.05-0.2 kV/cm, 3p) delivered to a monolayer of human umbilical vein endothelial cells (HUVECs) caused MT disruption in the form of MT fragmentation and depolymerization[20]. At sufficient voltages, complete loss of MTs occurred. Recovery of MT structure occurred in 1-2 hours post-PEFs. Total β -tubulin concentration remained constant for up to 16 hours after PEFs.

In another study by Thompson et al., cells treated with 600ns PEFs (16.2 kV/cm, 20p) showed MT depolymerization within seconds when the pulsation buffer contained calcium (Figure 2.3b), even in buffer supplemented with polyethylene glycol to mitigate swelling and blebbing[122]. Pulsation buffer with calcium resulted in inhibited lysosomal transport as MT structures depolymerized, whereas lysosomes were mobile and MT disruption was less pervasive in calcium-free media.

A recent study by Carr et al. showed that 10ns pulses (44 kV/cm, 100p) delivered to human glioblastoma (U-87 MG) cells caused MT buckling, breakage, depolymerization, and altered MT dynamics as indicated by GFP-labelled end-tracking protein EB3 (Figure 2.3c) [100]. After PEFs, the number of EB3 comets decreased, and comet length increased. Tubulin and EB3-GFP accumulated near the plasma membrane and decreased in fluorescence. These observations suggest a reduced number of nucleating/growing MTs, but a faster growth rate. In this study, MT disruption was temporally associated with loss of mitochondrial membrane potential, and EB3 changes were found to be independent of calcium and cell swelling, suggesting a direct breakdown of interphase MTs. A subsequent study by Havelka et al. using 11ns pulses (~67.5 kV/cm, 4000p) also showed significant changes to EB3 dynamics in rat basophilic (RBL-2H3) cells[129].

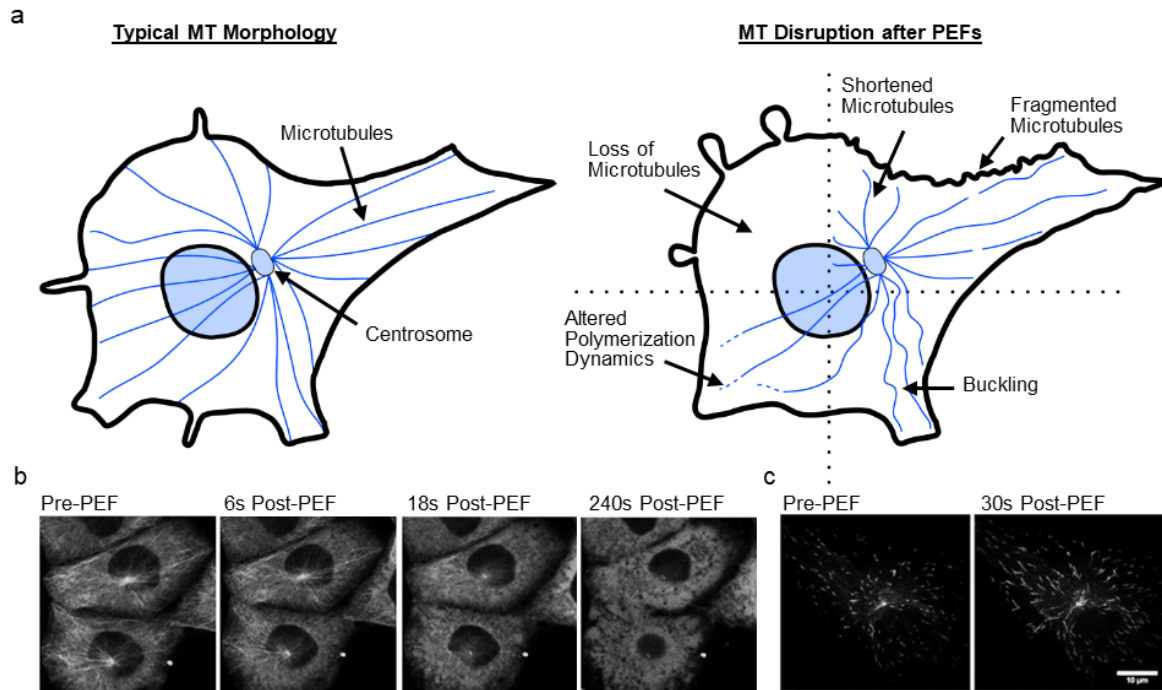


Figure 2.3 PEF-induced microtubule (MT) disruption.

(a) MTs nucleate from the centrosome in mammals, and radiate (polymerize) outward under normal conditions (left). After PEFs, MTs may show buckling, fragmenting, altered growth, or be depolymerized (right). (b) Chinese hamster ovary (CHO-K1) cells show depolymerization of MTs after 600ns PEFs in calcium-containing media. Adapted from [122]. (c) MT end-tracking protein EB3 demonstrates altered MT dynamics in human glioblastoma (U-87 MG) cells after 10ns PEFs. Both the rate of polymerization and number of polymerizing MTs change. Adapted from [100].

2.4.2 Microtubules, membrane permeability, and cell viability

Inhibiting MTs may decrease membrane permeability to provide protective effects from PEFs, according to some studies. Table 2-2 lists MT-disrupting agents and the studies using these agents. In two similar studies by Rols et al. and Teissie et al., half of CHO-WTT clone cells treated with 100 μ s PEFs (1.8 kV/cm, 10p) resealed within 6 minutes, but inhibiting MTs by pretreatment with colchicine (6.3 μ M, 30 min) decreased this resealing time to within 2 minutes[108,109]. In another study by Rols et al., CHO-WTT clone cells that were pretreated with colchicine (6.3 μ M, 30 min) before 100 μ s PEFs (1.5kV/cm, 1p) showed decreased electrofusion events, suggesting MT involvement with membrane dynamics[110]. In a study with 10ns PEFs (150kV/cm, 100p) by Thompson et al., CHO-K1 cells treated with nocodazole (10 μ M; 1 hour) had less PI uptake and reduced Annexin V-FITC fluorescence, suggesting that chemically disrupting MTs might interfere with cell signaling and prevent additional membrane damage[130].

As with actin stabilization, MT stabilizing drugs have shown little effect on cell response to PEFs. Thompson et al. found that stabilization of MTs by paclitaxel (7.5nM; 1 hour) did not change the amount of damage to the cell membrane after 10ns PEFs (150 kV/cm, 100p) in CHO-K1 cells[130]. Additional studies, however, are needed to fully characterize the effects of MT agents on cell response to PEFs.

2.5 Intermediate Filaments and Septins

Intermediate filaments (IFs) assemble from fibrous subunits in a coil-coiled configuration to create rope-like filaments of high tensile strength. These filaments are dynamic and flexible, with an average diameter of about 10nm[153]. IFs resist mechanical stresses by crosslinking other filaments

within the cell and anchoring to desmosomes on the plasma membrane. IFs are composed of a family of related proteins having a common structure and can be broadly grouped into four categories: keratin filaments, vimentin and vimentin-related filaments, neurofilaments, and nuclear lamins[154].

Disruption of IFs after PEFs has not been well-studied, however studies show IFs can be disrupted by PEFs. Harkin et al. found a perinuclear collapse of vimentin intermediate filaments following 10-20ms PEFs (0.5-1.0 kV/cm, 1p) in chick embryo corneal fibroblasts[21]. Recovery of vimentin fibers paralleled MT recovery, with full recovery in about 3-4 hours. In a study by Kanthou et al., 100 μ s PEFs (0.05-0.2 kV/cm, 3p) caused minimal vimentin disruption to a confluent monolayer of HUVECs, despite significant actin and MT disruption[20]. However, some vimentin disruption was seen at the periphery of cells, and recovered within 2 hours. Finally, Thompson et al. showed cortical lamin localized within the nucleus during PEF treatment (600ns, 27.7 kV/cm) in CHO-K1 cells[123]. Disruption of the lamin cortex correlated with nuclear permeabilization. These studies show IF disruption can occur, but more research is needed to fully evaluate PEF-induced effects.

Septins are considered the forth component of the cytoskeleton. These GTP-binding proteins can form into filaments and rings[155]. To date, no study has investigated septin disruption after PEFs. However, low-magnitude (<2.5 V/cm) fields applied at 100-300kHz, known as tumor treating fields (TTFields), have been shown to interfere with septin localization during mitosis[156].

2.6 Mechanisms of Cytoskeletal Disruption

A complete understanding of the mechanisms of PEF-induced cytoskeletal disruption is still lacking. However, numerous mechanisms of disruption have substantial experimental and computational support. Direct mechanisms of disruption via interactions between the electric field and cytoskeletal proteins (or associated proteins) may include conformation changes, electrophoresis, and electromechanical effects. Secondary, downstream mechanisms may also lead to cytoskeletal disruption through cell swelling, elevated cytosolic calcium levels, ATP depletion, cell signaling, or other pathways. In the following section we discuss support for various mechanisms of cytoskeletal disruption.

2.6.1 Actin – direct mechanisms

Experiments with actin-encapsulated GUVs by Parrier et al. suggest that the actin cortex can be affected by electric fields through direct mechanisms such as electrophoresis and electromechanical stress[119]. Delivering consecutive pulses (500 μ s, 0.1-10 kV/cm, 1-30p) of increasing field strength to actin-encapsulated GUVs resulted in reduced fluorescence of the actin cortex, but not the diffuse fluorescence within the GUVs (Figure 2.4a). The decreased fluorescence of the cortex, which suggests a breakdown of actin, occurred over tens of seconds after the pulse and was slightly greater at the poles. Since biological processes can be excluded in GUVs, direct effects on actin are suggested. The study compared the approximate mechanical and electrophoretic forces experienced by an average actin filament and found that electrophoretic forces were likely 4 times larger than mechanical forces. Mechanical forces on the membrane are derived from Maxwell-stress induced bending and stretching of the lipid membrane. Due to these forces, empty GUVs take on prolate, oblate, or spherocylindrical shapes during PEF treatment and

cause membrane bending and stretching[141,157]. This shape change, although significantly attenuated in cells and actin-encapsulated GUVs, may contribute to cortical actin disruption. In this study, however, the calculated force of mechanical disruption was below reported values for filament rupture or depolymerization, suggesting that electrophoretic forces had the major role on the actin cortex. Continued research with biomimetic GUVs of increasing complexity will further elucidate how these structures are involved in PEF treatments[158]. Alteration of actin-associated proteins by PEFs may also result in direct changes to actin dynamics, however this has yet to be investigated.

2.6.2 Microtubules – direct mechanisms

Tubulin is a highly polar molecule and is highly negatively charged, especially at the c-terminus tail. Compared to all proteins of known structure, tubulin has a 4-5 times higher electrical charge (-22e average per monomer) and dipole moment (2,166 Debye) than average[126]. These unique electrical properties make tubulin a target for direct modulation by external electric and magnetic fields. Numerous studies have experimentally demonstrated that purified MTs can migrate and align under electric fields[159-165]. Electric fields have also been shown to disrupt MT polymerization, which is exploited as the mechanism of action of tumor treating fields (TTFields) that use low magnitude (<2.5 V/cm) fields applied at 100-300kHz[166]. Molecular dynamics simulations also indicate that electric fields of GHz frequencies can disrupt tubulin and tubulin associated proteins[167-169]. Adding to the existing literature on MT dynamics under electric fields, recent molecular dynamics studies and experimental studies now demonstrate that PEFs can directly disrupt MTs.

Molecular dynamics simulations by Timmons et al. indicate conformational changes to tubulin after nsPEFs[131]. Simulation of a single 10ns PEF (750 kV/cm) indicated conformational changes to charged and flexible regions of sidechains and loops of tubulin such as α :H1-B2 loop, β :M-loop, and c-termi (Figure 2.4b). Fields as low as 50 kV/cm caused rearrangement of the α :H1-B2 loop in simulations. Since loop-loop interactions govern binding of adjacent heterodimers in MTs, changes to these regions could promote MT catastrophe (depolymerization). Conformational changes to tubulin may also affect a MTs ability to resist buckling. Additionally, intradimer curvature increased in simulations of PEF treatment. Intradimer curvature increases during depolymerization as protofilaments "peel" away from the MTs, so increased intradimer curvature after PEFs suggests PEFs-reduced MT stability. In another molecular dynamics study of tubulin by Marracino et al., simulation of a 30ns PEF showed that fields of 200 kV/cm increased the dipole moment by 50%, and fields of 1 MV/cm increased the moment by three times[126]. Increased dipole moment may make the MT lattice unstable. This study did not show unfolding to tubulin's secondary structure motifs up to 1 MV/cm, however electrostatic forces did pull the c-terminus tail away from the tubulin body. As the c-terminus is required for tubulin-associated protein interactions such as with motor proteins, this deformation may change MT dynamics after PEFs.

Experimental studies also indicate that PEFs can directly modulate MT dynamics. In a study by Chafai et al. with purified tubulin, MTs showed altered polymerization dynamics after PEFs (11ns, 20 kV/cm, 100-800p)[128]. Tubulin treated with 400 and 800 pulses did not polymerize to the same levels as untreated controls. At high fields, autofluorescence decreased following depolymerization of the MT, reflecting conformational changes. The zeta potential of tubulin

(highly influenced by the c-terminus tail) decreased for 400 and 800 pulses before polymerization, but returned to control values after depolymerization. Immunoblotting did not reveal any damage to α -tubulin. MT structure, as measured by AFM, indicated changes after PEFs (Figure 2.4c). After 200 pulses, MTs showed decreased height indicating collapsed or open structures, while 800 pulses resulted in MTs of even lower height, suggesting open structures.

Modulation of MT-associated proteins/structures such as MT motor proteins, MT severing enzymes, MT-associated MAP proteins, and altered MT-membrane interactions may also alter MT dynamics. For example, molecular dynamics simulations by Průša et al. of the motor protein kinesin-I docked on a single tubulin heterodimer indicated that a 30ns PEF (1 MV/cm) can alter the kinesin dipole moment (magnitude and angle), affect the contact surface area between kinesin and tubulin, and alter important structures such as MT binding motifs and nucleotide hydrolysis sites[127]. These nsPEF-induced changes to kinesin may result in altered MT dynamics. Furthermore, nsPEFs have been shown to generate acoustic shock waves[170] that may disrupt MT in a similar manner as ultrasound[100,171,172]. However, more research is needed to evaluate these pathways of disruption.

2.6.3 Swelling/Volume Change

Cell swelling is a common response to PEFs, typically manifested by cell blebbing and rounding[18,22,112,113,115,116,122,124]. PEFs permeabilize the cell membrane to small solutes but not to larger solutes, creating colloid-osmotic pressure that drives water into the cell. To block cell swelling, sucrose or other large molecules such as polyethylene glycol can be added to the pulsation buffer as these large molecules cannot enter the permeabilized cell and can balance

colloid-osmotic forces. The cytoskeleton is structurally and functionally linked with various membrane transporters, which together actively regulate cell volume[173]. Given the interdependent relationship between cell volume and the cytoskeleton, swelling after PEFs can cause cytoskeletal changes.

An example of swelling-induced cytoskeletal breakdown was reported by Pakhomov et al. after 600ns PEFs (1.92 kV/cm, 4p) were applied to CHO-K1 cells (Figure 2.4d)[113]. In buffer that did not inhibit PEF-induced swelling, nsPEFs caused cell rounding and led to partial disassembly of actin fibers and non-filamentous actin "patches". However, buffer with added sucrose to block colloid-osmotic swelling prevented the disassembly of actin structures, implicating swelling as the cause of actin disruption. As another example, Rassokhin et al. showed that during PEF treatment (60ns, 10 kV/cm, >1000p) of human monocytes (U-937 cells), inhibiting colloid-osmotic swelling via sucrose inhibited pseudopod-like bleb formation, implicating water influx in their formation[115].

Despite strong evidence that swelling drives cytoskeletal disruption, several other studies indicate cytoskeletal disruption in the absence of cell swelling[19,100], suggesting additional mechanisms are involved.

2.6.4 Cytosolic calcium concentration

The concentration of free calcium in the cytosol is around 10^{-4} mM, more than 10,000 times less than the extracellular concentration under normal conditions ($\sim 1-2$ mM)[174]. Low cytosolic calcium is maintained by calcium pumps that use ATP to pump calcium outside the cell or inside

the endoplasmic reticulum (ER). When PEFs disrupts the cells membrane, extracellular calcium can enter the cell by diffusion through membrane pores or passage across voltage-gated calcium channels. Even in the absence of extracellular calcium, cytosolic calcium levels can increase after PEFs due to permeabilization of the ER, which contains calcium concentrations typically at 0.1-0.8 mM [175]. Calcium is a potent signaling molecule, and modulates the cytoskeleton including both actin and MTs. Table 2-1 indicates whether studies on PEF-induced cytoskeletal disruption included calcium in the pulsation buffer.

High cytosolic levels of calcium have been shown to depolymerize MTs and to affect actin filaments [176-178]. For example, Downey et al. showed that electropermeabilized neutrophils had a breakdown of f-actin in buffers with calcium concentrations greater than normal intracellular levels (~100 nM) [118]. Similarly, Harkin et al. found that pulsation buffer containing calcium chloride levels above 100 μ M caused MT disruption in chick embryo corneal fibroblasts after PEFs (10-20ms, 0.5-1.0 kV/cm, 1p)[21]. Serum-free media (calcium concentration ~1mM) used as the pulsation buffer caused MT disruption and inhibited migration in cells. Contrarily, cells displayed normal migration and MT structure after PEFs in buffer containing low amounts of calcium (1 μ M). In another study, Thompson et al. found that cells treated with 600ns PEFs (16.2 kV/cm, 20p) showed MTs depolymerization when the pulsation buffer contained calcium, even when cell swelling was mitigated (Figure 2.4e)[122]. MT disruption was less pervasive in calcium-free media. As a final example, Titushkin et al. found that even low-level electric fields (2 V/cm) caused cytoskeletal disruption such as decreased YM and activated-ERM proteins that were likely caused by calcium influx [179].

Calcium independent MT disruption, however, is also reported. For example, Carr et al. showed that nsPEFs (10ns, 44 kV/cm, 100p) applied to human glioblastoma (U-87 GM) cells caused a breakdown of MTs without increased cytosolic calcium levels, as monitored by Fluo-4 AM fluorescence[100]. Thus, calcium appears to be one of many pathways for cytoskeletal disruption. Calcium modulates many pathways, and thus calcium influx may lead to a diversity of cell responses that together disrupt the cell cytoskeleton.

2.6.5 ATP Depletion

Restoration of ion concentrations (such as calcium) after PEF disruption is an energetically expensive process that is accomplished by ATP-powered pumps such as Na^+/K^+ -ATPase and Ca^{2+} ATPase. ATP consumption by ion pumps combined with metabolite leakage through membrane pores can result in severe ATP depletion [28,180,181]. ATP depletion has been shown to alter cytoskeletal dynamics[182,183] (although it may have little effect on cell mechanical properties[184]), and thus PEF-induced ATP depletion may be a factor in cytoskeletal response. In one study by Titushkin et al., non-electroporating direct current (2 V/cm, 60min) applied to osteoblasts increased the tether length of the cell membrane similar to cells with chemically-depleted ATP [179]. ATP depletion inhibited linker proteins and caused membrane separation from the cytoskeleton. However, as mentioned previously, Rols et al. showed that pre-incubation of cells with ATP and GTP did not affect cell membrane resealing[109]. Additional studies are needed to determine the contributions of ATP depletion to cytoskeletal disruption.

2.6.6 Additional Mechanisms

A recent paper by Tolstykh et al. implicates the depletion and hydrolysis of the lipid signaling molecule phosphatidylinositol-4-5-bisphosphate (PIP₂) as a root cause of cell swelling and blebbing after nsPEFs[124]. PIP₂ is important for a number of signaling pathways, and regulates ion channels, modulates cell volume, and binds to many actin regulatory proteins that control actin dynamics. After 600ns PEFs (16.2 kV/cm, 20p), PIP₂ depletion was observable 2 seconds after pulsing. The poles of the cells showed the most PIP₂ depletion, the same regions that showed the most pronounced blebbing. Dimming of the actin cortex occurred and blebbing began by 9 seconds, the same time that peak phospholipase C (PLC) activity was detected in a previous study[185]. Pretreatment of cells with edelfosine to block PLC activity and prevent PIP₂ hydrolysis significantly reduced cell perimeter changes and eliminated blebbing for treatment of a single pulse (Figure 2.4f). These results suggest PIP₂ depletion and PLC activation initiate a pathway that causes membrane dissociation from the actin cortex and leads to cell swelling and blebbing.

In addition to the numerous mechanisms outlined above, other mechanisms may also contribute to cytoskeletal disruption including intracellular protein release[186], pH changes[114], generation of reactive oxygen species, activation of membrane-bound receptors, stretch-activated cation channels, or possibly many others. Caspase activity after PEFs causes cytoskeletal breakdown, however caspase activity indicates apoptosis[28]. Significant opportunities still exist for identifying the complete pathways of cytoskeletal disruption.

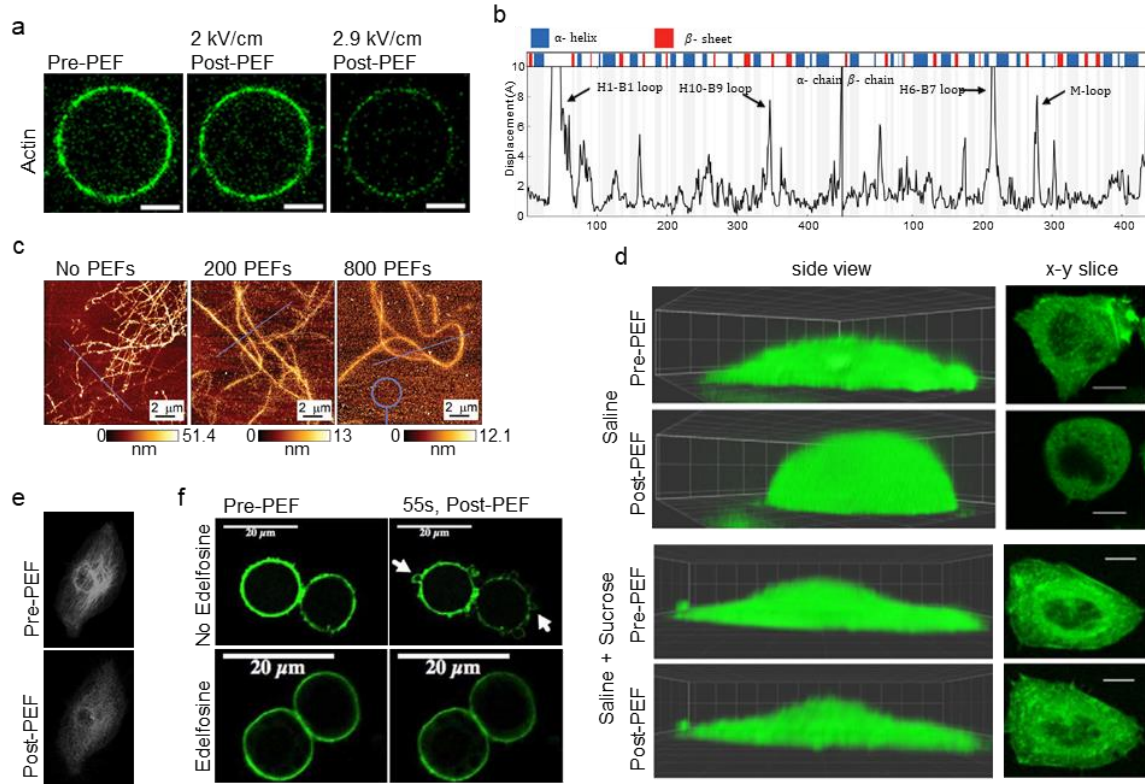


Figure 2.4 Mechanisms of cytoskeletal disruption.

(a) Actin-encapsulated GUVs show decreased cortical actin fluorescence after PEF treatment, suggesting direct disruption of actin by electric fields. Scale bars 5 μm . Adapted from [119]. (b) Molecular dynamics simulations of tubulin show conformational changes to loops and side chains after nsPEF. Adapted from [131]. (c) AFM imaging of polymerized, purified MTs after PEF treatment showed a loss of MT cylindrical structure as demonstrated by MT height. Adapted with permission from [128]. (d) Inhibiting swelling of Chinese hamster ovary (CHO-K1) cells maintained actin features after 600ns PEFs (bottom). However, swelling caused a loss of actin structures and led to more homogenous actin

structure (top). Scale bars 10 μm . Adapted with permission from [113]. **(e)** Loss of MTs in CHO-K1 cells occurred after 600 ns PEFs when the pulsation buffer contained calcium, even when swelling was mitigated. Adapted from [122]. **(f)** Treatment of CHO-K1 cells with edelfosine to inhibit PLC activity and PIP_2 hydrolysis prevented blebbing after PEFs. Adapted with permission from [124].

2.6.7 Disruption mechanisms and pulse length

While most observed features of cytoskeletal disruption seem to be conserved across pulse lengths ranging from nsPEFs to msPEFs, it seems likely that unique disruption characteristics may arise from particular pulsing parameters (length and magnitude). Given the available studies, however, it is difficult to make a proper assessment of the differences in cytoskeletal disruption between PEFs of various pulse lengths (nsPEFs, μsPEFs , and msPEFs). A few studies investigate more than one pulse length[117,123], however essentially no studies have provided a comprehensive analysis of cytoskeletal response across a wide range of pulse lengths. Several studies do investigate cytoskeletal disruption using the same cell type (CHO-K1, for example), however direct comparison is complicated by varying experimental conditions and analysis methods between studies. Future studies that maintain constant experimental conditions (e.g. same cell type, same pulsing media, same analysis method) and test a range of pulse lengths may more clearly identify unique characteristics of disruption based on pulse length.

Despite the current lack of studies comparing differences in cytoskeletal responses across pulse lengths, our current understanding of the mechanisms of disruption suggests that some mechanisms may be unique to (or more prominent at) certain pulse lengths and magnitudes. For example, direct modulation of cytoskeletal (or cytoskeletal-associated) proteins by conformational changes likely are limited to PEFs of very high field strengths such as those used during nsPEFs ($>10\text{kV/cm}$). Longer μsPEFs and msPEFs , however, may more readily enable cytoskeletal breakdown by electrophoresis and electrodeformation due to the longer duration of the applied field. (Millisecond PEFs are widely used for gene transfection due to their electrophoretic effects[187].) Longer pulses also generate larger pores than nsPEFs[27,91,92], which may enable the leakage of larger cytoplasmic molecules. Leakage of cytoplasmic molecules (proteins, ATP, GTP, etc.) may lead to cytoskeletal disruption. Other mechanisms of disruption, however, such as calcium influx and colloid-osmotic swelling, likely occur across pulse lengths ranging from nsPEFs to msPEFs . PEFs ranging from nanoseconds to milliseconds can disrupt the lipid membrane and ion channels that together alter intracellular ion concentrations to cause cytoskeletal disruption. Likewise, membrane and ion disruption can lead to volume changes that alter the cytoskeleton for various pulse lengths. Beyond these insights on how pulse length may affect cytoskeletal disruption, additional studies are needed to more precisely determine the predominant mechanism(s) for various pulse lengths.

2.7 Cell-Matrix and Cell-Cell Junction Disruption

A critical function of cytoskeletal filaments is to interact mechanically and biochemically with a cell's environment via transmembrane adhesion complexes[188]. Adhesion complexes, such as

focal adhesions, link the cytoskeleton to the cell's environment, which *in vivo* is the extracellular matrix. Cell-to-substrate interactions are responsible for cell shape, migration, signaling, differentiation, and cell function[31,189]. Likewise, cell-to-cell junctions such as adherens junctions and tight junctions establish mechanical stability, enable cell-cell signaling, and limit molecular transport between adjacent cells (paracellular transport)[190,191]. Both cell-to-cell and cell-to-substrate interactions are disrupted by PEFs.

2.7.1 Cell-Matrix Disruption

Cell-substrate disruption is commonly reported after PEF treatment in the form of cell rounding and shape change[18,19,102,105,113,114]. Cell rounding may occur to accommodate changes due to cell swelling, or be due to breakdown of adhesion sites. Loss of cell adhesion is temporary after PEF treatment (except for cell death), and PEFs do not induce tumorigenic characteristics in cells such as anchorage-independent growth[19]. Adherent cells typically remain adherent after PEF treatment despite some rounding, however in one study complete detachment of cells was reported[102].

Adhesion after PEFs is likely cell-type dependent. Pehlivanova et al. used a crystal violet assay to show that the adhesive behavior of two human breast cancer cell lines (MDA-MB-231, MCF-7) and a mouse fibroblast cell line (NIH/3T3) is cell-type and field-strength dependent after biphasic PEFs (50 μ s + 50 μ s, 0.2-1 kV/cm, 8p)[105]. A similar finding of cell-type dependent adhesion was indicated in another study by Szewczyk et al. Zyxin, a protein involved with focal adhesions, showed increased expression in healthy mouse myoblasts (C2C12 cells) after 100 μ s PEFs (1 kV/cm, 8p), but decreased expression in human rhabdomyosarcoma (RD) cells, indicating altered

cell-substrate connections[120]. Harkin et al., found that chick embryo corneal fibroblast migration, which requires cell-substrate interactions, did not occur in the first 2 hours after millisecond PEFs (10-20ms, 0.5-1.0 kV/cm, 1p) in serum-free media[21].

Some studies have suggested that adherent cells with highly developed cytoskeletons may be more resistant to PEFs than cells in suspension[102,192], however more studies are needed to support or refute this hypothesis.

2.7.2 Cell-Cell Junction Disruption

Cell-to-cell disruption is commonly studied *in vitro* with cell monolayers, as cell-cell junctions limit monolayer permeability by connecting adjacent cells. Adherens junctions and tight junctions link the actomyosin contractile systems of adjacent cells via linker proteins to transmembrane proteins, such as vascular endothelial cadherin (VE-cadherin)[193,194]. Several studies show that the permeability of endothelial monolayers increases after electroporation. Kanthou et al. showed that VE-cadherin fluorescence decreased in a human umbilical vein endothelial cell (HUVEC) monolayer after PEFs (100 μ s, 0.05-0.2 kV/cm, 3p)[20]. Cytoskeletal and junctional disruption resulted in increased permeability during the first 30 minutes after PEFs. Likewise, Meulenberg et al. showed increased permeability of a human dermal microvascular endothelial cell (HMEC-1) monolayer after PEFs and ECT[22]. ECT induced more rapid cell-to-cell disruption than PEFs alone, and decreased cell turgidity while increasing cell-cell gaps. Numerous other *in vitro* studies demonstrate increased monolayer permeability and cell-cell junction disruption after PEFs[63,106,195-199]. Electrofusion of adjacent cells [22,200] can also occur during PEF treatment, and the cytoskeleton likely plays a key role in this process [201,202].

2.8 Considerations for Electroporation Therapies.

Cytoskeletal disruption is principally manifested as anti-vascular effects after *in vivo* PEF therapies. Healthy microvascular is maintained by a balance of intracellular forces generated by the cytoskeleton of endothelial cells, and extracellular forces transferred cell-cell and cell-matrix across adhesion complexes[203]. Alteration of actin and MT dynamics, therefore, can cause significant endothelial barrier dysregulation. Studies show that inhibition of actin or MT polymerization results in increased barrier permeability[204-207] and capillary collapse[208]. Furthermore, increases in endothelial contractility via actomyosin stress fibers results in enlarged cell-cell gaps and leaky vessels [209,210].

PEFs ranging from nanoseconds to milliseconds cause significant decrease of blood flow to treated tissues, a phenomenon known as the vascular lock effect[23,211]. This effect has been well studied in 100 μ s ECT therapies. After 100 μ s PEFs, blood flow reduces to near zero flow within seconds. This rapid, but transient reduction of blood flow is attributed to vasoconstriction of afferent arterioles, mediated by the sympathetic nervous system in response to electroporation of muscle and/or vascular endothelial cells [23]. Within minutes, a second mode of disruption becomes prominent: cytoskeletal and cell-cell junction disruption. As has been already described, significant alteration of the cell cytoskeleton occurs within minutes after PEFs treatment. Endothelial cells may be particularly susceptible to PEFs and ECT[212,213], thus enhancing barrier dysregulation. Loss of barrier integrity leads to extravasation of fluids, increased interstitial fluid pressure, and decreased intravascular pressure that together result in decreased perfusion. In addition, endothelial cell swelling physically obstructs vessels leading to increased vascular

resistance and decreased perfusion[213]. Without the addition of chemotherapy, effects of reversible electroporation can last up to 24 hours. ECT treatment, however, causes a permanent loss of blood flow due to endothelial cell death.

nsPEFs also cause profound and prolonged blood flow disruption[69,214-217]. A study by Bardet et al. showed that nsPEFs as short as 10ns transiently decreased capillary diameter, caused capillary collapse, and decreased perfusion[215]. Other studies show nsPEFs destroy the capillaries feeding tumors within about one day, and cause extended loss of perfusion for greater than two weeks[69,214].

The anti-vascular effects of ECT are clinically useful, as the vascular lock prevents convection of adjuvant drugs (calcium, chemotherapy) away from the treatment area. ECT is also effective for eliminating bleeding from of bleeding tumors[23] and needle insertion sites. Reduced blood flow also induces localized hypoxia in the treatment area, which can be key for promoting tumor cell death.

PEF-induced vascular hyperpermeability may also be exploited for the treatment of brain tumors. The blood-brain barrier (BBB) creates a neuroprotective environment for the brain by strictly regulating the transport of substances into the brain through a variety of specialized transporters and cell-cell tight junctions. Many chemotherapies show limited ability to cross this barrier in clinically-effective doses[218,219]. PEF-induced BBB disruption should, therefore, provide a therapeutic benefit when applied in conjunction with chemotherapies. *In vivo* studies show that PEF application to the brain can disrupt the BBB [42-46]. BBB disruption may even occur at sub-electroporation thresholds[63], suggesting that PEF treatment of brain tumors may enable

irreversible electroporation of the tumor bulk within a large region of reversible BBB disruption. Clinical studies will be required to assess the use of PEFs therapies for the treatment of brain cancers, but preclinical studies show significant promise.

2.8.1 Cytoskeletal targets for improved PEF therapies.

Malignant cells show distinct cytoskeletal alterations from healthy cells, and an improved understanding of how the cytoskeleton is involved with PEF therapies may lead to new opportunities to exploit differences between healthy and cancerous cells. Many PEF studies show differential responses between healthy cells and cancerous cells [220-223], and cytoskeletal differences may be a contributing factor of this response. For example, Pehlivanova et al. showed that under the same PEF treatment, human breast cancer cells and mouse fibroblasts had different cytoskeletal responses – structure was conserved in fibroblasts while cancer cells showed a loss of their cytoskeleton [105]. Malignant cells adapt their cytoskeleton for proliferation and infiltration and generally have reduced stiffness[224,225]. Given the differences between healthy and cancerous cells, future studies may show ways to preferentially target malignant cells based on their cytoskeleton.

Many anti-cancer drugs, such as paclitaxel, target MTs since these structures are critical for cell division. PEFs may find synergy with taxane-based cancer therapies to modulate MT dynamics to alter drug binding to tubulin[126]. Some cancer cells show altered γ -tubulin levels, which modulate MT nucleation. Thus, modulating MT nucleation by PEFs may open new therapeutic opportunities[129]. Electromechanical models of mitotic spindle vibration by Havelka et al. suggest nsPEF-driven electro-acoustic behavior of mitotic spindles may have a disrupting effect on

kinetochore-microtubule binding or chromatid separation with implications for cancer treatment[226]. Actin disruption can cause mitochondrial dysfunction, and thus PEF-induced actin disruption may prove useful for inhibiting mitochondrial function for cancer therapies[227]. Further research may reveal additional cytoskeletal targets for cancer therapy.

2.9 Conclusions

Considering the cellular response to PEFs from a cytoskeletal perspective reveals a complex, multi-factor process of cytoskeletal disruption. Given the available data, cytoskeletal response to PEFs is undoubtedly influenced by cell type, pulse parameters (pulse length, pulse magnitude, pulse number), and pulsation media. Direct effects on the cytoskeleton, such as protein conformation changes, electrophoretic effects, and electromechanical effects are accompanied by downstream effects to produce observed cell morphology and behavior. Downstream mechanisms include swelling, calcium influx, ATP depletion, PIP₂ depletion, and likely others. Additional research is required to determine which mechanisms are predominant. Cytoskeletal disruption is chiefly manifested in PEF therapies by anti-vascular effects of treated tissues. Further research is needed to evaluate the possibility of new targets based on cytoskeletal dynamics for improved PEF therapies. Insight into the exact cause of cytoskeletal disruption and the mechanisms of cytoskeletal recovery may prove useful for improving tumor cell death and PEF treatment selectivity.

Abbreviations:

PEFs Pulsed Electric Fields, IRE Irreversible Electroporation, ECT Electrochemotherapy, YM Young's Modulus, MT Microtubules, IF intermediate filaments, PI Propidium Iodide, PIP2 Phosphatidylinositol 4,5-bisphosphate, PLC phospholipase C, AFM atomic force microscopy, pMLC phosphorylated myosin light chain, GUV giant unilamellar vesicle, CHO Chinese Hamster Ovary cells; HUVEC Human umbilical vein endothelial cells, BY-2 Tobacco cell, B16f10 Mouse Melanoma Cells, SV40 normal fibroblast, U937 human monocytes, WB-F344 rat liver epithelial cells, WB-Ras derived from WB-F344 with H-ras oncogene, U87 Human Glioblastoma, HepG2 Human liver cancer, MDA-MB-231 Human Breast Cancer, MCF-7 Human Breast Cancer, 3T3 Fibroblast, HMEC-1 human microvascular endothelial cells, CAF-87350B1 primary human Fibroblasts from normal tissue at margin of infiltrating ductal carcinoma, RBL-2H3 Rat basophil, C1C12 normal murine muscle cell line, RD human rhabdomyosarcoma cell line, H640 human lung carcinoma, MRC5 – human lung fibroblast.

3 Chapter 3: Single Cell Forces after Electroporation

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KEYWORDS electroporation, nanofibers, cytoskeleton, forces, mechanobiology

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Attribution: Both authors contributed equally. PMG, AJ, ASN, and RVD conceived of the experiments. PMG and AJ performed the experiments and analysis. RKK and ASN developed the module to calculate cell forces. PMG created the finite element models. PMG wrote the paper and AJ, ASN, RVD, and RKK edited and revised the manuscript.

3.1 Abstract

Exogenous high-voltage pulses increase cell membrane permeability through a phenomenon known as electroporation. This process may also disrupt the cell cytoskeleton causing changes in cell contractility; however, the contractile signature of cell force after electroporation remains unknown. Here, single-cell forces post-electroporation are measured using suspended extracellular matrix-mimicking nanofibers that act as force sensors. Ten, 100 μ s pulses are delivered at three voltage magnitudes (500 V, 1000 V, and 1500 V) and two directions (parallel and perpendicular to cell orientation), exposing glioblastoma cells to electric fields between 441 V cm^{-1} and 1366 V cm^{-1} . Cytoskeletal-driven force loss and recovery post-electroporation involves three distinct stages. Low electric field magnitudes do not cause disruption, but higher fields nearly eliminate contractility 2-10 minutes post-electroporation as cells round following calcium-mediated retraction (Stage 1). Following rounding, a majority of analyzed cells enter an unusual and unexpected biphasic stage (Stage 2) characterized by increased contractility tens of minutes post-electroporation followed by force relaxation. The biphasic stage is concurrent with actin disruption-driven blebbing. Finally, cells elongate and regain their pre-electroporation morphology and contractility in 1-3 hours (Stage 3). With increasing voltages applied perpendicular to cell orientation, we observe a significant drop in cell viability. Experiments with multiple healthy and cancerous cell lines demonstrate that contractile force is a more dynamic and sensitive metric than cell shape to electroporation. A mechanobiological understanding of cell contractility post-electroporation will deepen our understanding of the mechanisms that

drive recovery and may have implications for molecular medicine, genetic engineering, and cellular biophysics.

KEYWORDS electroporation, nanofibers, cytoskeleton, forces, mechanobiology, Pulsed electric fields, actin

3.2 Introduction

Exogenous electric fields, applied as short, high-voltage pulses can disrupt the cell through a process known as electroporation. Electroporation increases membrane permeability, enabling molecular and ionic diffusion across the cell membrane.[9,84] Electroporation techniques such as gene transfection,[83] electrofusion,[70] and electrochemotherapy[67] exploit a transient increase in membrane permeability, while irreversible electroporation induces cell death through a loss of homeostasis for nonthermal tumor ablation.[8,37,39]

Developed over five decades, the classical theory on electroporation focuses on the mechanisms and timescales of membrane disruption.[14,84,228,229] While it is now widely accepted that hydrophilic pores, electrically-altered lipids, and modulated voltage-gated ion channels increase membrane permeability within microseconds of intense electric field application, the mechanisms and timescale (minutes to hours) of membrane resealing remain only partially understood.[9,24,25,230] Recently, there is a growing appreciation that pulsed electric fields cause disruption to the cell cytoskeleton.[19-22,88,100,102,105,107-109,112,113,116,117,119,125,130,227,231] The cytoskeleton generates contractile forces that maintain tensional homeostasis required for survival while also dynamically connecting to the cell

membrane. Studies show that chemically- or physically-disrupting the cytoskeleton alters membrane permeability after electroporation[109,232,233] and affects cell viability.[102,108,125,130] Electric fields may also decrease the elastic modulus of cells.[18,112,116] However, the dynamic changes in cell contractility and tensional homeostasis after electroporation have not been quantified yet.

Here, we use a custom-built device to enclose suspended extracellular matrix-mimicking fibers that act as force sensors (Nanonet Force Microscopy, NFM[234-236]), enabling us to causally link cell contractility, shape, and viability of single cells undergoing electroporation. We describe a biphasic mechanical response and remodeling of the cytoskeleton post-electroporation. Our analysis of multiple cell types, reveals three distinct stages during the recovery process: an initial loss in contractility immediately post-electroporation (Stage 1), a biphasic force response (Stage 2), and a final force recovery stage to pre-electroporation contractility (Stage 3). Cytoskeletal staining reveals that the biphasic response is concurrent with actin-mediated bleb retraction and is most pronounced when the electric field direction is parallel to the axis of cell elongation, which interestingly also results in higher cell viability. Altogether, our data suggest targeted opportunities to optimize electroporation for genetic engineering, gene therapy, and molecular medicine.

3.3 Results

3.3.1 Microfluidic chip integrates force-sensing nanofibers.

We fabricated a custom microfluidic device that integrates the nanofiber scaffold between two needle electrodes spaced 1 cm apart (Figure 3.1a). Using the previously reported non-electrospinning STEP technique,[237,238] we generated suspended polystyrene nanofiber networks: 250 nm-diameter nanofibers spaced 15 μm apart were fused to larger, 2 μm -diameter orthogonal fibers spaced 275 μm apart. Rotation of the nanofiber scaffold 90 degrees reoriented the electric field from a “parallel” configuration that aligned the electric field direction from being parallel to the nanofibers to a “perpendicular” orientation that placed the nanofibers perpendicular to the field (Figure 3.1b).

We refer to these orientations simply as the parallel ($\parallel$) and perpendicular ($\perp$) orientations. Cells elongate between adjacent nanofibers and deflect them according to the cell's inherent contractility (Figure 3.1c). In this study, we subjected human glioblastoma cells (U251, Sigma-Aldrich) to ten, 100 μs square-wave pulses at 1 Hz delivered at 500 V, 1000 V, or 1500 V, generating approximately uniform electric fields across the scaffold region (Figure 3.1d, Figure 3.7c-f). At applied voltages of 500 V, 1000 V, and 1500 V, our models indicated that the scaffold region-of-interest (cut-out region of scaffold, see Figure 3.1d) experienced electric field magnitudes of $441 \pm 12 \text{ V cm}^{-1}$, $882 \pm 23 \text{ V cm}^{-1}$, and $1323 \pm 35 \text{ V cm}^{-1}$ respectively for the parallel orientation, and $455 \pm 12 \text{ V cm}^{-1}$, $911 \pm 24 \text{ V cm}^{-1}$, and $1366 \pm 36 \text{ V cm}^{-1}$ respectively

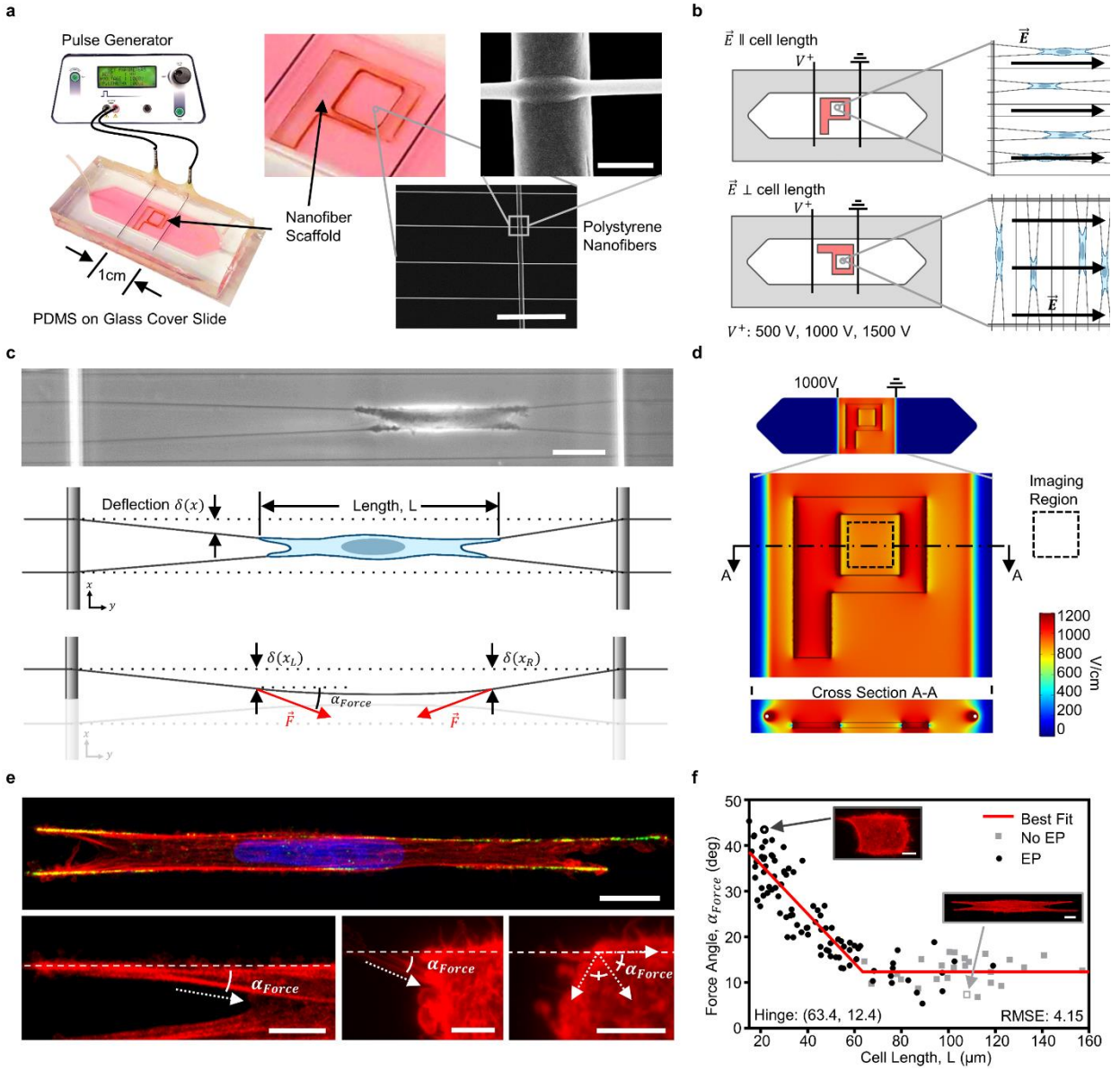


Figure 3.1 Nanofiber force sensing.

a) A microfluidic PDMS device encloses a nanofiber network spun on a hollow substrate. The cells are electroporated *via* a high-voltage pulse generator connected to two needle electrodes. Precisely-controlled nanofiber networks are fabricated using the STEP technique: $\phi 250$ nm

fibers (15 μm spacing) are deposited orthogonal to $\varnothing 2\text{ }\mu\text{m}$ fibers (275 μm spacing) and fused at their intersections. Scale bar 20 μm (lower), and 1 μm (upper). b) *Parallel orientation*, cell long-axis aligned with the electric field; *Perpendicular orientation*, cell long-axis perpendicular to the electric field. c) Single cells attach to parallel nanofibers, causing visible fiber deflection. Scale bar 25 μm . The deflecting fiber is modeled as a fixed-fixed beam with two point-loads, $\vec{F}$, located at the attachment points of the cells and at an angle α_{Force} . d) A finite element model indicates that the electric field is approximately uniform within the region of interest at the center of the scaffold. e) An elongated cell shows f-actin stress fibers (red) and focal adhesions (green) along the fibers. Contractile force is applied along the f-actin stress fibers (e, bottom left), retraction fibers (e, bottom center), or at the bisection of the membrane angle (e, bottom right). The dashed lines indicate fiber location. Scale bars 10 μm (top) and 5 μm (bottom). f) The angle α_{Force} is well approximated by a two-segment line when plotted against cell length. For cells longer than 63.4 μm , α_{Force} is constant at 12.4°. For cells shorter than 63.4 μm , α_{Force} is approximated as a linear function of cell length. Inset images show a round and elongated cell with corresponding data points. Scale bars 5 μm . EP, electroporation.

for the perpendicular orientation. The contractile force of a cell was calculated by the deflection of the flexible nanofibers.[234,239] To calculate the contractile force from fiber deflection, we model a deflecting nanofiber as a fixed-fixed beam with two point-source loads, $\vec{F}$, acting at the two primary adhesion sites, one at either end of the cell.[234,240-242] The direction of the force application, α_{Force} , was determined from the orientation of the actin stress fibers, actin-rich retraction fibers, or as specified in the Methods (Figure 3.1e). In general, α_{Force} is

approximately linearly- dependent on the cell length (L) for $L < 63.4 \mu\text{m}$, but constant ($\sim 12.4^\circ$) for $L \geq 63.4 \mu\text{m}$ (Figure 3.1f).

3.3.2 Contractility as a biophysical metric of electroporation

Almost immediately after electroporation, cell elongation decreased and membrane blebs formed. In the parallel orientation, cell rounding became appreciable with increasing field strengths (Figure 3.2a). Interestingly, in the perpendicular orientation, cells largely retained their elongated morphology (Figure 3.2b) unless the field strength was high (1500 V), which resulted in high levels of blebbing and cell rounding. Membrane blebbing decreased over a span of several minutes, after which the cells re-spread along the fibers. We quantified the temporal dynamics of the cell length, average fiber deflection (measured at cell attachment points), and contractile

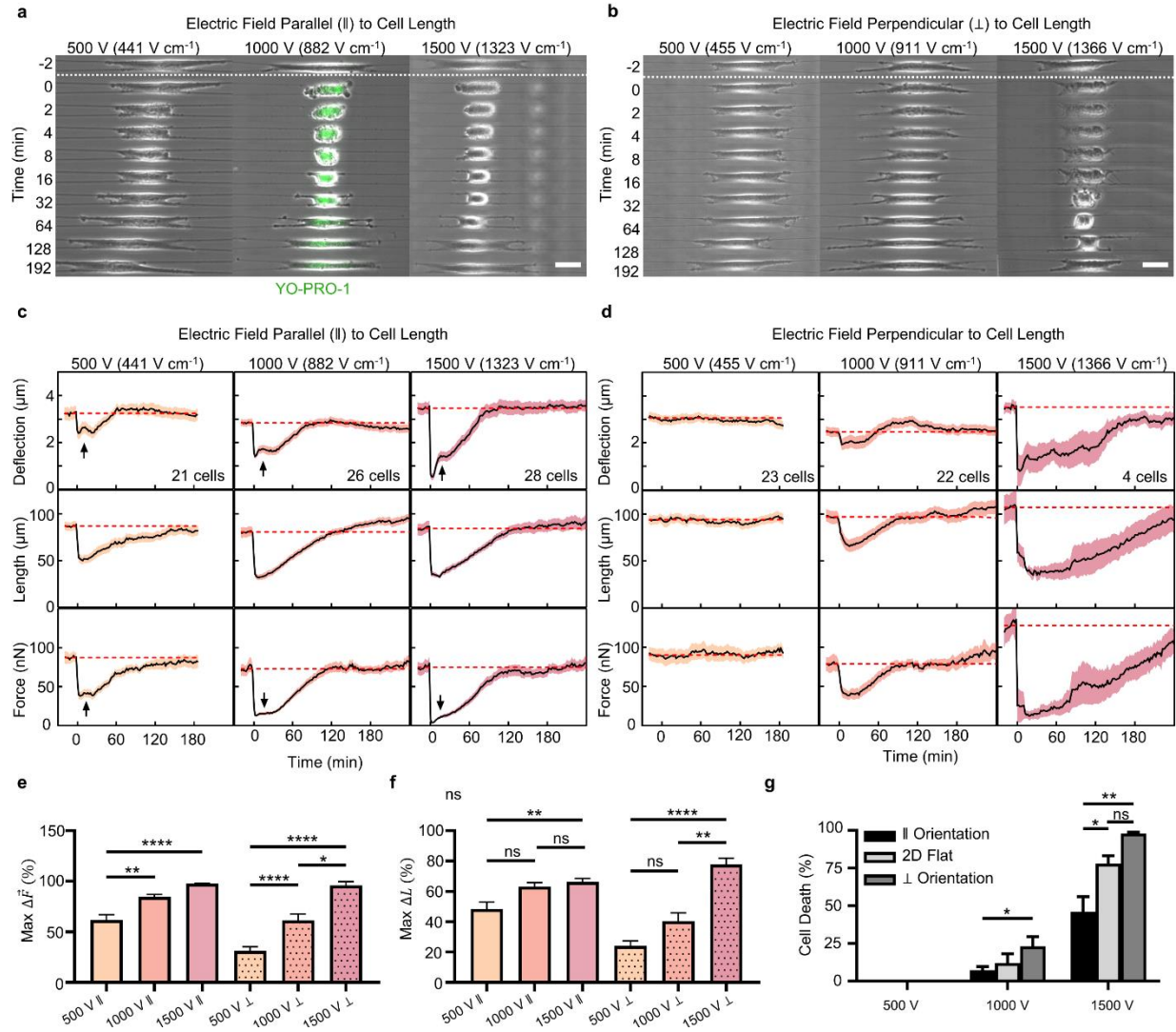


Figure 3.2 Cell shape and contractile force response to electroporation.

a-b) Cell responses to electroporation at 500 V, 1000 V, 1500 V in the (a) parallel orientation (441 V cm⁻¹, 882 V cm⁻¹, and 1323 V cm⁻¹ respectively) and (b) perpendicular orientation (455 V cm⁻¹, 911 V cm⁻¹, and 1366 V cm⁻¹ respectively). The onset of membrane disruption by electroporation is visualized for 1000 V $\parallel$ by the inclusion of YO-PRO-1 (green, background subtraction and thresholding used to remove background signal, see Methods). Cells show

rounding, membrane blebbing, and an eventual return to a characteristic elongated state in a voltage- and orientation-dependent manner. Scale bars are 25 μm . c-d) Average fiber deflection, cell length, and contractile force post-EP for cells in the parallel (c) and perpendicular orientation (d). Cells in the parallel orientation show rapidly decreased length and contractility, and recovery within 1-2 hours. For the perpendicular orientation, no response occurs at 500 V, a moderate response occurs at 1000 V, and an extreme response is seen at 1500 V. Unexpectedly, fiber deflection and force plots show a transitory increase shortly after electroporation (see arrows on plots) indicating multiple stages in the recovery process. Error bands show standard error. e-f) Maximum percent decrease in force and cell length is greatest at high voltages. g) Electroporation at high electric fields causes significant cell death, particularly in the perpendicular orientation.

force response for different field strengths both in the parallel (Figure 3.2c) and perpendicular orientations (Figure 3.2d).

In the parallel orientation, all applied voltages immediately reduced the average contractile force, and showed complete recovery within 1-2 hours (Figure 3.2c). Increasing the applied voltage caused a larger decrease in the cell force (Figure 3.2e) and length (Figure 3.2f). Unexpectedly, we discovered a transitory biphasic rebound in the cell contractile force immediately after the initial decrease in force post-electroporation, as indicated by the black arrows in Figure 3.2c. Analysis of individual force responses in the parallel orientation reveals that a majority of the cells (50 of 75 of sampled cells, 67%) underwent a multi-stage force response, discussed in detail later in this paper.

In the perpendicular orientation, higher electric fields caused a greater decrease in force (Figure 3.2e) and length (Figure 3.2f) after electroporation, however cell response was quite different from the parallel orientation. Contractile force and cell length did not change after electroporation of 500 V, similar to steady-state cell behavior observed in control (sham) experiments (Figure 3.8f). Application of 1000 V $\perp$ decreased contractile force and elongation, but this decrease was smaller (Figure 3.2e, Figure 3.8a-b), more gradual, and recovery occurred sooner than 1000 V $\parallel$. Few cells survived 1500 V $\perp$, but surviving cells showed higher-than-average pre-pulse contractility (Figure 3.9a), had dramatically reduced force and lengths after treatment, and had a long recovery to near pre-electroporation conditions. Interestingly, cell death (Figure 3.9b) was higher for cells treated in the perpendicular orientation than in the parallel orientation (Figure 3.2g, Table 3-4). At 1500 V, the percent cell death increased dramatically from $46 \pm 10\%$ ($\parallel$ orientation) to $98 \pm 1\%$ ($\perp$ orientation). At the end of data collection post-electroporation (3-4 hours), we did not find any significant differences in cell length or contractile force compared to pre-electroporation values for either cell orientation (Figure 3.8c,d).

3.3.3 Contractility reveals a multi-stage cell recovery post-electroporation

The recovery process can be divided into the following stages (Figure 3.3a-b, Movie S1): a cell-rounding stage (Stage 1) of decreasing force and cell rounding, a biphasic stage (Stage 2) characterized by a transient rebound of force and subsequent relaxation, and a cell-spreading stage (Stage 3) during which the cell recovers its pre-electroporation force and length. In Figure

73

3.3c, we show the multi-stage dynamics of contractile force and fiber deflection for a single cell in each treatment condition. We found that this response occurs more frequently in the parallel orientation (for example, 20 of 26 cells for 1000 V $\parallel$ (77%) *versus* 8 of 22 cells for 1000 V $\perp$ (36%)). See Table 3-3 for all percentages.

Stage 1: Recovery begins with a cell-rounding stage that typically occurs within the first 5 minutes (longer for 1500 V $\perp$) after pulsing and is characterized by a rapid reduction of force. Cell extremities retract along the fibers (*via* actin retraction fibers (Figure 3.1e)) causing rounding and membrane blebbing (Figure 3.3b, $t=0-2$ min). At 500 V and 1000 V, cell length decreases significantly more in the parallel orientation than the perpendicular orientation during the first two minutes post-electroporation (Figure 3.3d). Since calcium is known to breakdown cell focal adhesions[243] and is also an important ion regulating cell response to electroporation,[180] we repeated experiments in calcium-free DMEM (see Methods). For electroporation of 1000 V $\parallel$ in serum-free media with no extracellular calcium, cells universally remained elongated on the fibers shortly after electroporation (Figure 3.3g), in stark contrast to the ubiquitous rounding that occurred after the same electroporation conditions in standard cell culture media.

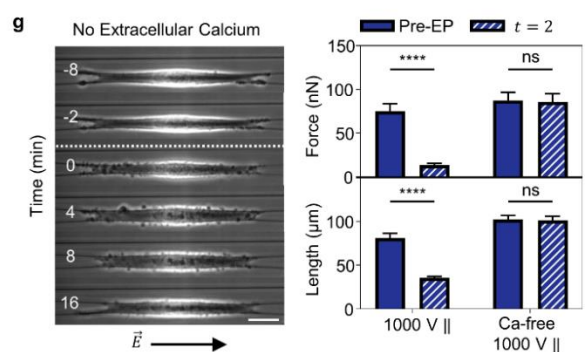
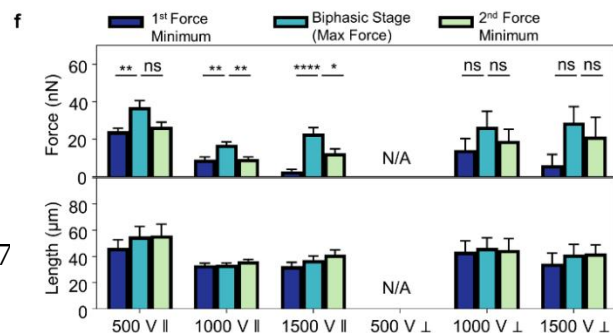
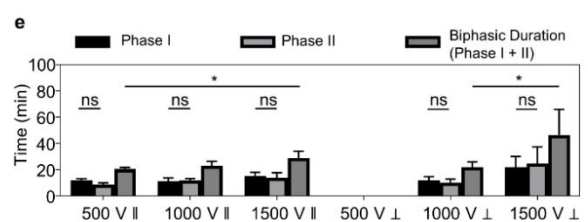
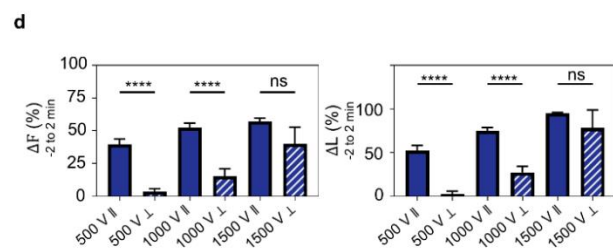
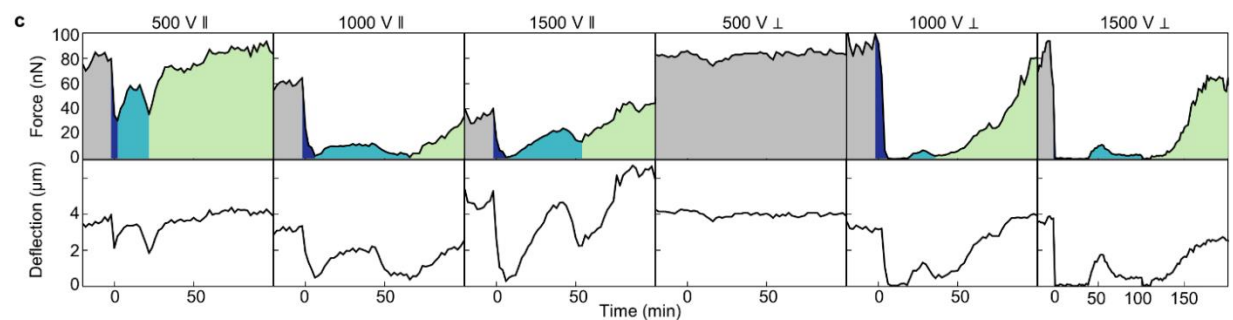
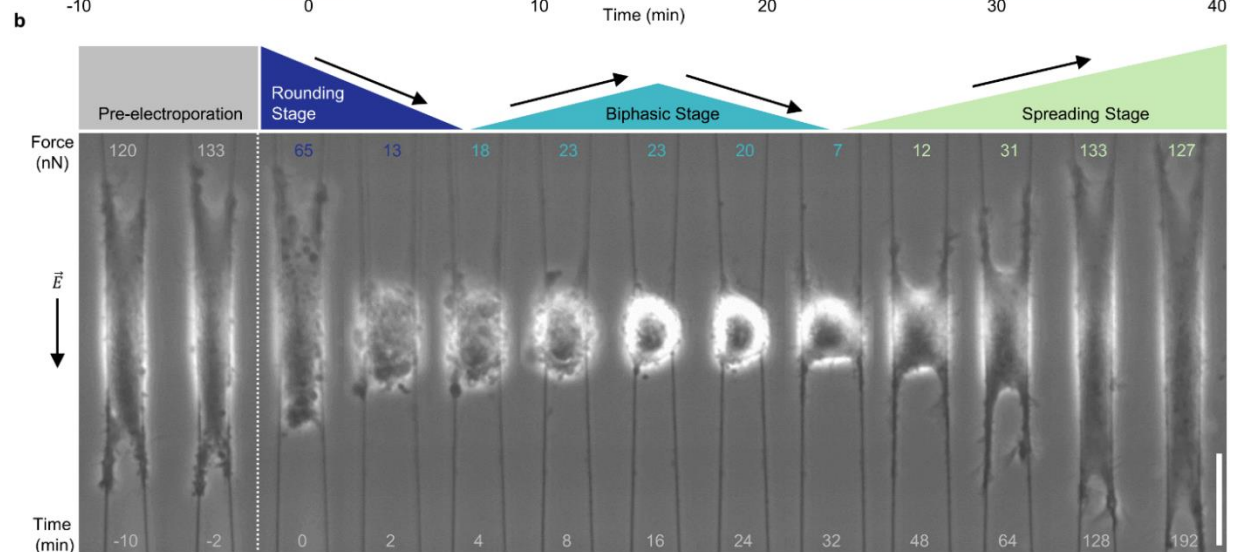
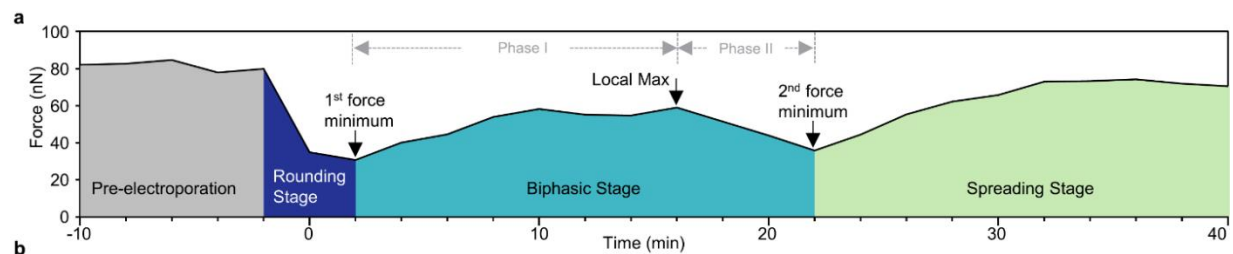


Figure 3.3 Electroporated cells show a biphasic force recovery.

a) A sample contractile force profile for a cell treated at 500V $\parallel$. The cell-rounding stage (Stage 1) begins after electroporation and ends at the first force minimum. Contractile forces increase during phase I of the biphasic stage (Stage 2) before decreasing again in phase II. The cell-spreading stage (Stage 3) begins after the second force minimum during which the cell recovers its pre-electroporation contractility. b) Images of a cell showing a multi-stage response (1000 V $\parallel$). Corresponding force values are presented for each image and the stages of recovery are schematically represented above. Scale bar 25 μ m. c) Contractile force and deflection for a representative cell from each treatment demonstrating a multi-stage response. Note a minimal response for 500 V $\perp$. d) Cells in the perpendicular orientation have significantly less change in force and length in the first two minutes after electroporation, except at very high voltages (1500 V). e) The biphasic stage lasts 20-30 minutes (longer for 1500 V $\perp$) with higher voltages causing slightly longer durations. The duration of phase I is nearly equivalent to phase II. f) Force significantly increases during the biphasic response. The first and second minimum values are not significantly different. During the biphasic stage, cell length does not change significantly. g) Image sequence of a representative cell electroporated (1000 V $\parallel$) in calcium-free media. Scale bar 25 μ m. Cell force and length remain unaffected in calcium-free DMEM during the first two minutes after electroporation (1000 V $\parallel$).

Stage 2: The biphasic stage begins at a force minimum following the cell rounding stage, in a state of high membrane blebbing. A significant increase of contractile force (Figure 3.3f) occurs during the first half of this stage (phase I), averaging 11 ± 1 nN or 14% of the average

pre-electroporation value. Some cells show force increases up to 40 nN or 48% of the average pre-electroporation force value. The maximum force during this stage is reached when nearly all membrane blebs have disappeared (Figure 3.3b, $t = 16-24$ min). Following the local force maximum, contractility decreases (Figure 3.3b, $t = 16-32$ min) to a local minimum value that is not statistically different from first minimum (phase II) (Figure 3.3f). The duration of phase I and phase II are nearly equal (Figure 3.3e). During the entire biphasic stage, cell length remained almost unchanged (Figure 3.3f). The total duration of the biphasic stage increased with increasing field strength (Figure 3.3e) in both the parallel orientation (500 V: 21 ± 1 min; 1000 V: 25 ± 3 min; 1500 V: 32 ± 4 min) and in the perpendicular orientation (500 V: no response; 1000 V: 22 ± 1 min; 1500 V: 47 ± 19 min).

Stage 3: The cell spreading phase is characterized by a gradual recovery of contractility as the cell re-spreads along the fibers (Figure 3.3b, $t = 32-216$ min). Cells eventually recover their pre-electroporation elongation as well as their pre-electroporation contractile force.

3.3.4 Cytoskeletal reorganization drives cell contractile response post-electroporation

The cytoskeleton plays a major role in regulating the contractile response of cells. We performed immunofluorescent staining for the major cytoskeletal components, actin (Figure 3.4) and microtubules (Figure 3.10), on fixed cells at various timepoints post-electroporation (1000 V $\parallel$; 0.5, 2, 8, 16, 32, 128 min and 24 hrs) and compared cytoskeletal structure with non-electroporated cells. Non-electroporated cells show well-developed actin stress fibers and continuous microtubules aligned with cell elongation (Figure 3.4a-b, Figure 3.10). During the cell-rounding stage (Figure 3.3b, Figure 3.4a-b, 0.5 and 2 min timepoints) as cells begin to detach from the fibers and decrease in length, the aligned actin fibers and microtubules get disrupted and blebs form on the cell membrane. By 8 minutes, the f-actin stress fibers are nearly non-existent, and cells take on a rounded shape with the actin primarily localized at the cortex or on actin-rich retraction fibers originating from prior adhesion sites along the nanofibers. Throughout the cell-rounding stage and at the early phases of the biphasic force response stage (2, 8 min post-electroporation), we observed high levels of membrane blebbing (Figure 3.3b) with very large individual blebs (up to 20-30 μm^2 projected area). Using two metrics for blebbing, bleb size^[244] and membrane “roughness” (see Methods for details), we found blebbing to be maximal at two minutes post-electroporation, with significant decreases by 16 minutes, and baseline values by 32 minutes (Figure 3.4c-f). Also, at 32 minutes, we observed the formation of actin stress fibers, indicating the start of Stage 3 (cell-spreading stage) of force recovery. At 128

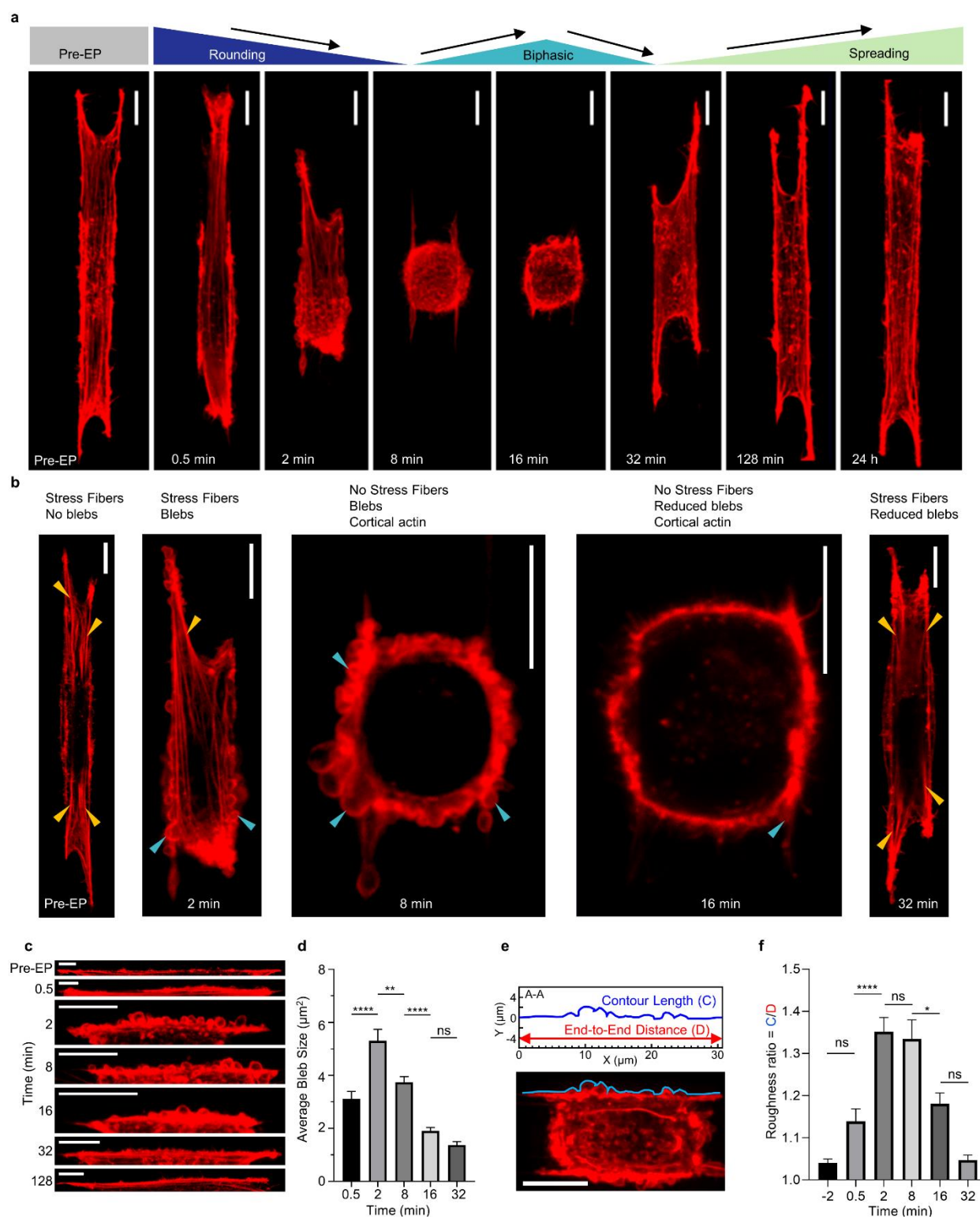


Figure 3.4 Electroporation disrupts the cytoskeleton.

a) Maximum intensity projections of cells stained for actin before electroporation and during various stages of recovery post-electroporation (1000 V ||). b) A single confocal z-slice taken in the nanofiber plane. Pre-EP, actin cytoskeleton consists primarily of well-defined stress fibers (yellow markers). During the cell-rounding stage (2,8 min), both stress fibers and blebs (blue markers) are present. During the biphasic stage (8,16 min), cells are rounded with cortical actin and no stress fibers. Eventually, stress fibers begin to reform (32 min) and blebbing is reduced as the cell enters the cell-spreading stage and regains its characteristic cytoskeletal structure. c,d) Bleb size analysis. Large blebs form immediately after electroporation (maximum at 2 min), but are reduced to baseline values within 32 minutes. e,f) Membrane roughness is quantified by the ratio of the contour length of the membrane along the nanofiber by the cell length end-to-end distance. Membrane roughness is at its maximum at 2 minutes after electroporation and returns to pre-EP values after 32 minutes. All scale bars 10 μm . EP, electroporation.

min, electroporated cells show a fully recovered cytoskeletal structure with well-defined actin stress

fibers and aligned, continuous microtubules. Blebbing dynamics in calcium-free media followed similar trends, but the bleb recovery was faster (Figure 3.11a,b).

3.3.5 Cell membrane permeability aligns with electric field orientation

Given our observations that the electric field orientation with respect to cell shape altered cell responses in contractile force and cell length, we wanted to determine how orientation affected membrane permeability. Using propidium iodide (PI) as a marker of membrane permeability, we visualized the temporal dynamics of PI uptake during and after the application of ten, 1000 V pulses delivered at 1 Hz. We found that PI entered cells at both the anodic (hyperpolarized) and cathodic (depolarized) sides of the cells (with respect to electric field): near the cell's endpoints (protrusions) in the parallel orientation and along the cell's width in the perpendicular orientation (Figure 3.5, Movie S2). Incidentally, we observed that PI uptake was biased toward the cathodic side of the cell (Figure 3.5c) with the initial rate of fluorescence intensity change (a.u./s) nearly two times greater compared to the anodic side in the parallel orientation (22.9 ± 3.7 vs 10.7 ± 1.1 a.u./s), while the perpendicular orientation showed a smaller but still significant difference between the cathodic and anodic side (26.1 ± 5.7 vs 18.5 ± 3.7 a.u./s). To investigate if PI uptake was dependent upon calcium in the buffer, we repeated experiments in calcium-free media at 1000 V. We found that the temporal uptake of PI in calcium-free media at 1000 V followed similar trends as in regular media at 1000 V (Figure 3.11c,d) with no significant difference in PI uptake 60 seconds after electroporation (Figure 3.11e).

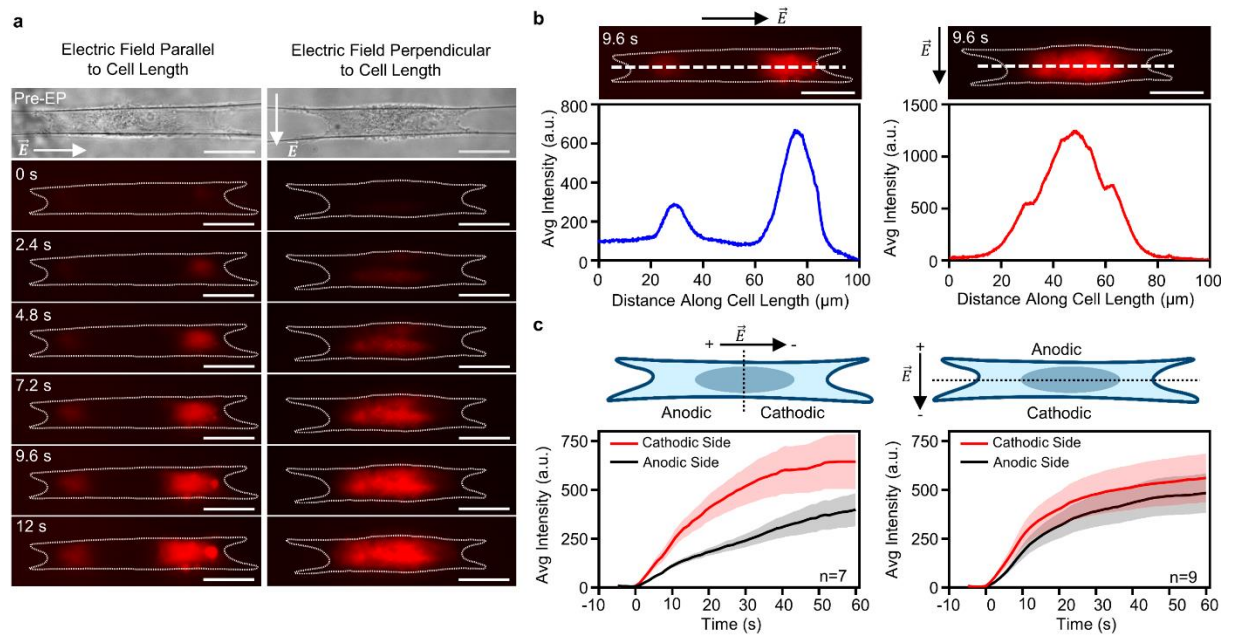


Figure 3.5 Cell orientation affects permeability after electroporation.

a) Cells show uptake of propidium iodide (PI) during and after the application of ten, 1000 V pulses ($\parallel$: 882 V cm^{-1} ; $\perp$: 911 V cm^{-1}) delivered at 1 Hz. Time $t = 0$ indicates the end of the first pulse. PI uptake reveals distinct spatial distributions of membrane permeability for the parallel and perpendicular orientations. In the parallel orientation (a, left column), PI uptake is located near the cell attachment points. However, in the perpendicular orientation (a, right column), uptake is near the central sidewalls. White dashed lines show cell boundary pre-electroporation. The time series indicates that membrane permeability and the influx of PI precedes cell rounding. See Movie S2. Scale bars 20 μm b) Representative intensity plots taken along the cell length (large white dashed lines) demonstrate the distinct spatial distributions of membrane

permeability in the parallel and perpendicular orientations. c) PI uptake is biased toward the cathodic (depolarized) side of the cell. Error bands show standard error. (Figure 5 c).

3.3.6 Cell contractility and not necessarily cell shape is sensitive to pulsed electric fields

We wanted to inquire if our findings of cell shape and force recovery were generalizable to other cell types, so we investigated electroporation effects to five other cell lines: VAMT cells (human mesothelioma), human thyroid cancer cells, CHO-K1 cells (Chinese hamster ovary), C2C12 cells (mouse myoblasts), and HeLa cells (human cervical cancer). We performed electroporation experiments with cells in the parallel orientation (Figure 3.6a, b). Although the magnitude of contractile force pre-electroporation varied between cell lines, all cell lines demonstrated a rapid and significant loss in force after electroporation, followed by an eventual recovery of force in 1 to 2 hours. Importantly, experiments with all cell types showed instances of cells undergoing biphasic force responses (Stage 2) during recovery (Figure 3.6c). Furthermore, we found that contractile force is a much more sensitive indicator of cell recovery compared to cell shape (length) alone, as HeLa and CHO cells showed minimal rounding, but a significant loss of contractile force after electroporation. Overall, our experiments with additional cell types, healthy and cancerous, demonstrate that the loss of contractile force after electroporation was cell-line independent, while a severe loss of cell shape was cell-line dependent.

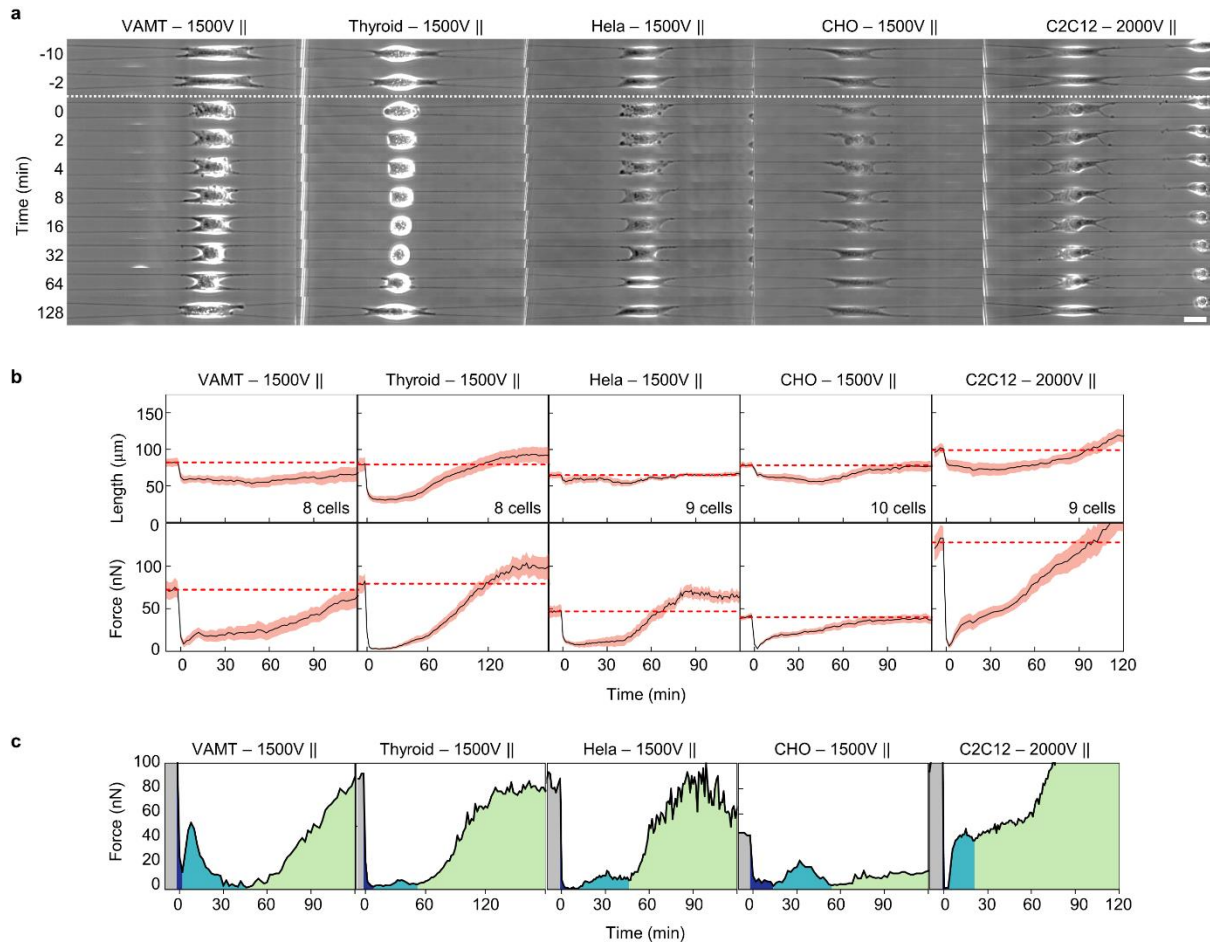


Figure 3.6 Electroporation experiments on five additional cells lines conducted at 1500 V or 2000 V in the parallel orientation.

a) Brightfield images of cell responses to electroporation. Scale bar is 25 μm . b) Cell length and contractile force responses for five cell types after electroporation. Note that the cell contractile force is more dynamic than the cell shape. All cell types demonstrate a robust loss and recovery of forces post-electroporation, but do not necessarily show any significant changes in the cell shape. c) An example of a multi-stage response for each additional cell type.

3.4 Discussion

Despite the extensive development and use of electroporation technologies in various biological applications, the dynamics of mechanical recovery to tensional homeostasis in cells post-electroporation remain poorly understood. Complementary to membrane-centric approaches to electroporation reported in literature, here we provide a mechanobiological investigation of electroporated cells by measuring the contractile forces of single cells adherent to extracellular matrix-mimicking suspended fibers. Our network of suspended nanofibers constrains cells to be elongated, in high-aspect ratio shapes with inherent mechanical, biological, and spatial anisotropy, properties that we investigate with two electric field directions (Figure 3.1). We

demonstrate that both electric field magnitude and direction significantly impact the contractile response of cells, which exhibits three distinct stages (Figure 3.2): an initial loss in contractility immediately post-electroporation (Stage 1), a biphasic force response (Stage 2), and force recovery to pre-pulse contractility (Stage 3).

Cells attached to our parallel network of fibers form clusters of focal adhesions at their extremities, and upon electroporation, the adhesions are broken, leading to cell rounding (Stage 1). Our observations of minimal cell rounding in the absence of extracellular calcium (Figure 3.3g) implicates extracellular Ca^{2+} -induced focal adhesion disassembly as a possible contributing factor to the cell-rounding stage. It is well established that electroporation disrupts the transmembrane Ca^{2+} gradient[180,181] and causes an influx of extracellular calcium inside the

cell, and that locally-elevated intracellular calcium levels can lead to disassembly of focal adhesions within minutes.[243,245-247] This conclusion is reinforced by our observation that significant PI uptake occurs prior to cell rounding (Figure 3.5 and Movie S2). To disambiguate the role of serum and calcium in cell rounding, we performed experiments in both serum-free and calcium-free media. Our findings that cell rounding was observed after electroporation in calcium-containing DMEM (1.8mM calcium, no serum), and minimal rounding in calcium-free media further confirm the role of calcium in cell rounding (Figure 3.11f). Cell rounding after electroporation has been previously attributed to colloid-osmotic swelling,^[18,113,248,249] and our results suggest that extracellular calcium is an additional factor driving the loss of cell adhesion. Incidentally, we found some evidence that cells with higher levels of pre-pulse contractility undergo a faster cell-rounding stage, suggesting a ‘slingshot effect’ caused by the contractile actin stress fibers that accelerate the rounding process (Figure 3.8e). Independent of the magnitude of pre-pulse contractility, we observe a rapid loss of actin stress fibers and microtubule structure following the electric field treatment, as prior studies have also noted.[18,21,106,107] Cytoskeletal disruption was concomitant with the formation of blebs, with peak bleb size observed at 2 minutes post-electroporation (1000 V $\parallel$) corresponding closely with maximal loss of cell contractility. Bleb elimination by 32 min (1000 V $\parallel$) post-electroporation is in reasonable agreement with the timescales of membrane resealing previously reported.[109,229,230,250,251]

Membrane disruption during electroporation is driven by an induced transmembrane potential (ITP), causing pore formation if ITP values exceed a “critical” threshold (~ 0.2 -1 V).[9-15] Our simple ITP models (Figure 3.12, steady-state, constant membrane conductivity) predict

pore formation ($ITP \geq 1.0$ V) for all conditions except 500 V perpendicular, in good agreement with our experimental results demonstrating loss of contractility and cell length for these conditions. However, a maximal ITP value of 0.5 V for cells treated at 500 V in the perpendicular orientation indicates minimal (or no) electroporation, a result consistent with minimal changes to contractile force or length and no YO-PRO-1 uptake observed (data not shown). Incidentally, our experiments showed that PI uptake was asymmetric with a bias toward the cathodic (depolarized) side of the cell, a finding consistent with previous studies but not explained by our ITP models or electroporation theory.[252,253] Our models indicate that cells in the parallel orientation should have greater overall disruption compared to the perpendicular orientation due to higher ITP values. However, our experiments revealed higher cell viability for cells electroporated in the parallel orientation. Modifying our model to include dynamic membrane conductivity,[200,254] [255] electric field disruption near the nanofibers, pore characteristics (number and radii),[255] and/or total mass transport across the membrane[256,257] might reconcile the differences between experimental viability results and model predictions. Cell shape-change after pulsing, which is more rapid and prominent after electroporation in the parallel orientation, may also contribute to increased viability in this orientation, as rounding may promote cell recovery by reducing membrane tension or by reducing the cell's surface area, thereby reducing transport across the disrupted membrane.

As cells become rounded at the end of the cell-rounding stage (Stage 1), the contractility measurements demonstrate a biphasic response with a significant rise in forces but with a minimal change in cell length during the first half of this stage (Stage 2). Although the exact mechanisms responsible for the biphasic force response remain unknown, we suggest three likely

contributing factors. First, during the initial phase of force recovery, contractility increases as blebbing decreases. Bleb reduction by contraction of cortical actin likely contributes to the increased contractility we observe.[258,259] Second, cell volume may decrease slightly after electroporation (Figure 3.9c-d) likely due to cytoskeleton tension-mediated water efflux,[260,261] thus leading to an increase in force. Third, increased levels of intracellular Ca^{2+} have been shown to cause aggregation of endoplasmic reticulum Ca^{2+} sensor STIM1, which then leads to the recruitment of the force-bearing focal adhesion protein, talin, and could thereby account for the increased cell contractility in the first phase of this biphasic stage.[262] The reduced contractility seen in phase II is most likely indicative of a reorganization of actin, possibly from a bleb-reduction configuration to a spreading configuration, yet the specific mechanisms are unknown.

Irrespective of the electric field strength and orientation, cells that were viable post-electroporation (see Methods for viability criteria) had fully-restored contractility typically within the first 1-3 hours post-electroporation (Stage 3). Such timescales for cell contractile recovery fall within the reported range of cytoskeletal recovery times (minutes to hours).[18-23] Cell force recovery has been previously investigated in the context of rapid mechanical stretching events,[263-268] and not for electroporation, which we show here. While mechanical stretching and electric field treatment are fundamentally different cues, the cellular contractility response to perturbation bear similarities. Studies show that rapid mechanical stretching disrupts tensional homeostasis leading to rapid disruption of the actin cytoskeleton, followed by recovery of cell contractility either in single or multi-stages. For example, Nekouzadeh *et al.* demonstrated that rapid stretching (20% strain at 800%/s) induced a multi-stage recovery process: a stage of rapid force increase and relaxation (>1 min), a force plateau stage, and finally a gradual active

force recovery stage that lasted around 20 minutes.[268] Loss and recovery of cell force was attributed to cytoskeletal fluidization (depolymerization of the actin cytoskeleton) and cytoskeletal re-solidification (actin stress fiber recovery) respectively. While these force response patterns resemble the multi-stage force dynamics reported by us, we note the importance of membrane disruption linked with integrity of cytoskeletal networks in electroporation. The large-scale bleb formation and recession observed in our studies post-electroporation is synchronized with disruption and formation of actin fibers (Figure 3.4), which might explain why the timescales of electroporation-mediated biphasic force recovery (few minutes) differ from the stretch-mediated shedding of forces occurring typically within a few seconds. Additionally, while mechanical stretching induces a loss in contractility without affecting the overall cell shape, electroporation-mediated disruption can have a loss of cell shape (rounding) followed by recovery (spreading). Such substantial cell shape recoveries may further explain the longer timescales of force recovery (2-3 hours) compared with mechanical stretching (~30 minutes).[265,268]

3.5 Conclusion

In conclusion, the prevailing paradigm in electroporation emphasizes membrane disruption and resealing as the key metrics in cell recovery, yet from our study across multiple cell types, it is increasingly clear that cytoskeletal dynamics play a significant role in cell shape and force recovery. Our ECM-mimicking nanofiber-based platform demonstrates that mechanical force recovery is a robust metric to evaluate cell recovery after electroporation. We

anticipate our mechanobiological approach to cell recovery will yield insights clarifying the relationship between membrane dynamics (permeability, tension, composition) and cytoskeletal dynamics (structure, contractility), and show how these properties impact cell survival.

Fundamental knowledge on the relationship between cell mechanics and membrane permeability may link membrane resealing times with the force response, knowledge that may reveal the mechanisms of pore resealing and enable optimizations for electroporation technologies such as gene transfection. We expect that a better understanding of the mechanical response to electroporation will present target-opportunities in reversible electroporation applications such as genetic engineering, gene therapy, and molecular medicine.

3.6 Methods

3.6.1 Device Fabrication and Fiber Deposition

Scaffold bases consisting of a hollow square region (See Figure 3.1. Outer scaffold dimensions: 8 x 8 mm; inner region: 3 x 3 mm) were laser cut from ~250 μm -thick polystyrene coverslips (Fisher Scientific). Using our previously reported non-electrospinning Spinneret based Tunable Engineered Parameters (STEP) technique, we fabricated force-sensing nanofiber networks consisting of a horizontal array of densely-spaced (inter-fiber spacing: $14.5 \pm 0.7 \mu\text{m}$) small diameter (~250 nm, $233 \pm 4 \text{ nm}$) nanofibers deposited on a vertical array of widely-spaced (inter-fiber spacing: $279 \pm 9 \mu\text{m}$) large diameter (diameter ~2 μm , $1.92 \pm 0.11 \mu\text{m}$) support fibers. Fiber networks were fused at their junctions using a custom fusing chamber. The 250 nm-diameter fibers were prepared using a 7 wt% solution of polystyrene (MW: 2,000,000 g/mol; 90

Category No. 829; Scientific Polymer Products, Ontario, NY, USA) in *p*-xylene (X5-500; Thermo Fisher Scientific, Waltham, MA, USA), while a 2 wt% solution (Polystyrene MW: 15,000,000 g/mol; Category No. PL2014-9001, Agilent Technologies, Santa Clara, CA, USA) was used for the large, 2 μ m support fibers. Individual fiber diameter and inter-fiber spacing were confirmed *via* Scanning Electron Microscopy (SEM) images. Mechanical properties of the fibers are listed in Table 3-5.

A single channel in polydimethylsiloxane (PDMS) was bonded to a glass coverslip to contain a plastic scaffold placed between two electrodes spaced 1-cm apart. A master mold for the PDMS channel was fabricated on a glass slide using acrylic. A 1/16" acrylic sheet was laser-cut to form the channel region (30 x 10 x 1.6 mm), and epoxied onto the glass slide. Acrylic walls surrounding the channel region were laser cut from 3/16" acrylic and epoxied in place. PDMS was mixed in 10:1 (wt/wt) base to cross-linker, degassed, and cast-molded using the master mold. After curing at 80 °C for 2-3 hours, the PDMS was removed from the mold and inlet and outlet holes were punched with a 0.75 mm biopsy punch. Before bonding to a glass cover slip, high-vacuum grease (Dow Corning, Midland, MI) was used to tack the scaffold in place on the glass slide. Bonding of the PDMS to the cover slip was achieved using a plasma cleaner (Harrick Plasma). To complete the device assembly, stainless steel acupuncture needles (diameter 0.18 mm) were carefully inserted through the device at a spacing of 1 cm and epoxied in place. The assembled device was placed under vacuum until use.

3.6.2 Cell Culture and Experimental Procedure

The glioblastoma cell line U251 (Sigma-Aldrich) was cultured according to standard practices in growth media consisting of Dulbecco's Modified Eagle Media (DMEM) with L-Glutamine, 4.5 g/L Glucose and Sodium Pyruvate (Corning) supplemented with 10% Fetal Bovine Serum (FBS, R&D Systems) and 1% Penicillin Streptomycin (Gibco). Growth media had a conductivity of 12.8 mS/cm, an osmolarity of 355 mOsm, and contained 1.95 mM calcium. U251 cells tested negative for mycoplasma. Cells were passaged at 70-90% confluency. Both C2C12 mouse myoblasts (ATCC) and HeLa cells[269] were cultured in growth media consisting of DMEM with L-Glutamine, 4.5 g/L Glucose and Sodium Pyruvate (Corning) supplemented with 10% Fetal Bovine Serum (FBS, R&D Systems). VAMT cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS, R&D Systems). Chinese hamster ovary (CHO-K1, ATCC) cells were cultured in growth media consisting of DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, Thermo Fischer Scientific) supplemented with 10% Fetal Bovine Serum (FBS, R&D Systems). Thyroid cancer cells[270] (isolated from young mouse tumors) were cultured in F12 medium (Ham's F-12 Nutrient Mixture, Thermo Fischer Scientific) supplemented with 10% Fetal Bovine Serum (FBS, R&D Systems).

To prepare a device, the device was sterilized with ethanol, washed with PBS, and incubated for 45 minutes with 4 $\mu\text{g/ml}$ Fibronectin in PBS. Cells were trypsinized, centrifuged at 150 x g for 5 minutes, and resuspended in media at 0.1×10^6 cells/ml. Cells in suspension were added to the device and incubated at 37 °C and 5% CO₂ for 20-40 minutes. The device was then

transferred to the microscope and incubated for 2 hours at 30 °C and 5% CO₂ before data collection. Contractile force experiments were performed on a Zeiss microscope (Zeiss AxioObserver Z1) with an incubation chamber maintaining a 30 °C and 5% CO₂ environment throughout the entirety of the experiments. The incubation temperature was decreased from standard 37 °C incubation to 30 °C to remove the potential of thermal damage caused by Joule heating during pulsing (Figure 3.9e-f, See Mitigation of Thermal Effects subsection of the Methods). Brightfield data was captured at 20x (phase objective, 0.8 NA) at intervals of 2 minutes. For each experiment, approximately 25 to 50 locations on the fiber network were imaged every 2 minutes. Imaging locations frequently contained more than one cell. For U251 experiments, twenty minutes of baseline data were collected before electroporation and cells were maintained in growth media for the duration of the experiment. For consistency with U251 experiments, experiments with other cell types were all performed in DMEM growth media (devices flushed ~20 minutes pre-experiment for cells not cultured in DMEM growth media). Ten minutes of baseline data were collected before pulsing.

To prepare cells for treatment in calcium-free DMEM, cells were seeded in complete growth media and allowed to adhere and spread on the fibers for at least 2 hours under incubation at 37°C and 5% CO₂. After incubation, devices were removed from the incubator and flushed thoroughly (2x, each flush with 2x device volume) with calcium-free DMEM (DMEM with 4.5g/L Glucose and without L-Glutamine, Sodium Pyruvate, Calcium Chloride. Gibco, 21068-028). The free calcium concentration was measured to be approximately 50 µM (Cell Biolabs Inc., MET-5121), and free calcium was not chelated to prevent alterations in cell morphology.[271,272] The device was then immediately placed on the microscope, incubated at

30°C and 5% CO₂ as in the other experiments. A brief acclimation period of approximately 20 minutes was given prior to data collection. Baseline (pre-electroporation) images were acquired for 10 minutes before applying the electroporation pulses. Cells were maintained in calcium-free DMEM for the duration of the experiment.

3.6.3 Sample Size and Inclusion Criteria

We analyzed U251 cells from three independent experiments for each voltage (500 V, 1000 V, 1500 V) and orientation ($\parallel$, $\perp$) condition. Table 3-2 presents the number of cells analyzed for the force analysis from each independent experiment. For each voltage and orientation tested, three independent experiments were conducted, with 20 or more total cells analyzed where possible. Ultra-low viability for the 1500 V $\perp$ condition limited sample number. Cells selected for analysis were well-centered on the fibers (for the duration of the experiment), had no interference from other cells, were adhered to nanofibers with fixed endpoints (orthogonal fibers well-fused), and showed an elongated and contractile phenotype. We performed three independent experiments of the 1000 V $\parallel$ condition in calcium-free DMEM and analyzed the response of 24 cells. Additionally, we performed two control (“sham”) experiments (Figure 3.8f). For our force analysis on additional cell types presented in Figure 3.6, we analyzed ≥ 8 cells from a single experiment for each cell type. C2C12 cells were electroporated at 2000 V due to their greater resistance to electroporation effects.

Table 3-1 Sample size for force analysis of U251 cells.

Condition	Number of cells analyzed per independent experiment			Total Cells Analyzed
	Exp1	Exp2	Exp3	

500 V	6	6	9	21
1000 V	9	6	11	26
1500 V	9	11	8	28
500 V ⊥	7	7	9	23
1000 V ⊥	8	6	8	22
1500 V ⊥	0*	0*	4	4
1000 V Ca ²⁺ -free	13	7	4	24
Control (Sham)	8	5	-	13

* no cells viable for analysis

Viability of U251 cells post-electroporation was analyzed from at least three independent experiments for each voltage (500 V, 1000 V, 1500 V) and orientation (||, ⊥, 2D Flat). Table 3-2 presents the number for cells analyzed for each independent experiment. Viability was assessed 180 minutes post-electroporation for 500 V experiments and 240 minutes post-electroporation for 1000 V and 1500 V experiments. (Note, 2D Flat data were collected from experiments investigating the ⊥ or || orientations.)

High-resolution brightfield data of individual cells enabled us to assess viability without the need for conventional live/dead staining. Viability was assessed at the single-cell level from brightfield data based on several criteria. Cells considered viable had (visually) intact membranes, had re-spread on the fibers, and were able to apply contractile forces to the fibers (i.e. deflected parallel nanofibers). Cells considered “dead” did not have (visually) intact membranes (cell lysis), did not re-spread on the fibers, and did not apply contractile forces (no fiber deflections). Using these criteria, cells considered “viable” were very distinct from cells considered “dead.” Figure 3.9b shows several examples of “viable” vs “dead” cells. As shown in

this figure, cell death generally occurred immediately following electroporation, suggesting necrosis (accidental cell death). For all experiments, all cells elongated between two parallel fibers with the characteristic elongated shape shown in Figure 3.1c were included in the viability analysis.

Table 3-2 Sample size for viability analysis.

Condition	Number of cells analyzed per independent experiment							Total Cells analyzed
	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	Exp 7	
500 V I	66	55	112	-	-	-	-	233
500 V $\perp$	68	25	37	26	25	-	-	181
500 V 2D Flat	34	26	32	103	-	-	-	195
1000 V I	60	62	60	33	27	48	126	416
1000 V $\perp$	39	45	93	21	-	-	-	198
1000 V 2D Flat	37	23	42	-	-	-	-	102
1500 V I	101	65	71	-	-	-	-	237
1500 V $\perp$	33	74	201	-	-	-	-	308
1500 V 2D Flat	15	24	20	93	48	126	-	326

3.6.4 Electroporation Parameters

Cells were electroporated with a high voltage pulse generator (BTX ECM 830, Harvard Apparatus). Ten, 100 μ s square-wave pulses were delivered at 500 V, 1000 V, or 1500 V to the device electrodes. Pulses were delivered at a frequency of 1 Hz. Measured current and voltage waveforms approximated ideal square waves (Figure 3.7a,b). Voltage waveforms were not affected by pulse number. Electrical current increased slightly with pulse number due to Joule heating. Finite Element modeling of the electric field within the device indicated that at applied

voltages of 500 V, 1000 V, and 1500 V, the cells within the scaffold region-of-interest (cut-out region of scaffold, 250 μm from edges) experienced electric field magnitudes of $441 \pm 12 \text{ V cm}^{-1}$, $882 \pm 23 \text{ V cm}^{-1}$, and $1323 \pm 35 \text{ V cm}^{-1}$ respectively for the parallel orientation, and $455 \pm 12 \text{ V cm}^{-1}$, $911 \pm 24 \text{ V cm}^{-1}$, and $1366 \pm 36 \text{ V cm}^{-1}$ respectively for the perpendicular orientation. See the Finite Element Modeling subsection of the Methods for more details.

Pulse application resulted in micron-diameter bubble formation on both electrodes, but did not result in electrical arcing. Undesired electrochemical effects were minimized by incorporating a large volume of fluid outside the electrode region (~ 2.5 times the volume of fluid between the electrodes), and locating the fiber network region several millimeters away from both electrodes. Media pH was minimally affected by electroporation (No electroporation (Sham): 7.11 ± 0.06 ; 1500 V: 7.16 ± 0.06 . pH measured 3 hours after electroporation).

3.6.5 Immunostaining and Confocal Microscopy

Cells were fixed and stained for actin, paxillin, and microtubules according to standard practices. Cells were fixed with 10% Formalin for 10 minutes, permeabilized with 0.1% Triton-X 100 in PBS for 15 minutes, and blocked with 5% goat serum in PBS for 30 minutes. Focal adhesion antibody (Paxillin, TYR31) ($5\mu\text{g/ml}$) and microtubule antibody (beta tubulin) ($1 \mu\text{g/ml}$) were prepared in an antibody dilution buffer (PBS with 10 mg/ml BSA and $1 \mu\text{l/ml}$ Triton X-100) and were added to the cells and incubated at room temperature for three hours. The device was washed with PBS and antibody dilution buffer supplemented with actin stain (rhodamine phalloidin, sc-301530; Santa Cruz Biotechnology, Dallas, TX, USA) diluted in a 1:80 ratio and secondary antibodies (Alexa Fluor 488 Goat Antibody (Green), Alexa Fluor 647 Goat Antibody (Cyan) both

diluted in a 1:400 ratio) were added for 45 minutes at room temperature while protected from light. The device was then washed with PBS and 300 nM DAPI diluted in PBS was added to the device for 5 minutes in the dark. The device was then washed with PBS and imaged using a 63x (1.15 NA) water-immersion objective on a confocal microscope (Zeiss LSM 880). The z-slice thickness was kept at either 0.36 μm or 0.5 μm .

Cell volume calculations were performed in MATLAB from z-stacks of fluorescent images (stained for actin, microtubules, and the nucleus) of fixed cells at various time points (pre-pulse, 0.5, 2, 8, 16, 32, and 128-minutes) after electroporation. (n = 6, 10, 8, 13, 7, 9 and 5 cells corresponding to pre-pulse, 0.5, 2, 8, 16, 32, and 128-minute timepoints respectively.) In MATLAB, images were converted into grayscale and subsequently binarized using a custom MATLAB routine to calculate the projected cell area as shown in Figure 3.9c. Cell volume was calculated as $\text{volume} = \Sigma(\text{projected cell area}) * \text{z-slice thickness}$.

Bleb analysis was performed in ImageJ. Bleb area was measured on the z-slice corresponding to the bleb's greatest diameter (n = 82, 126, 214, 131 and 23 blebs from 12, 12, 13, 11 and 4 cells for 0.5, 2, 8, 16 and 32-minute timepoints respectively). A membrane roughness ratio (Figure 3.4e) was used to quantify the increased length of the cell contour along the nanofiber due to the presence of blebs, and was measured as the ratio of the contour length to the cell end-to-end length (n = 6, 14, 16, 9, 10 and 6 for -2 (pre-pulse), 0.5, 2, 8, 16 and 32-minute timepoints respectively). Non-electroporated cells demonstrated negligible blebbing and thus had a roughness ratio very close to 1. Bleb analysis for calcium-free experiments (Figure 3.11a,b) followed the same procedure, however brightfield images were used to calculate the roughness ratio.

3.6.6 Membrane Permeability Imaging

To experimentally confirm membrane disruption by electroporation, we used YO-PRO-1 and Propidium Iodide (PI), both membrane-impermeant dyes. Membrane disruption by electroporation was visually demonstrated by the fluorescence of the membrane-impermeant dye YO-PRO-1 (1000 V || condition shown in Figure 3.2). For clarity in Figure 3.2a, we used a background subtraction technique (*via* a gaussian-blur) and a lower intensity threshold to remove background YO-PRO-1 signal and thus these images are for illustration purposes only. Experiments were performed with 1 μ l/ml (1 μ M) YO-PRO-1 (Thermo Fisher Scientific) in media.

We visualized the spatial distribution of PI uptake during the first 60 seconds after electroporation at 1000 V (||: 882 V/cm; $\perp$: 911 V/cm). A high concentration of PI (0.17 mg/ml) (Fisher Scientific) was used as in similar studies[273] to enable high frame-rate data collection (short exposure times) while maintaining strong fluorescence signal. We added media containing PI immediately prior to imaging and electroporating the cells. Ten, 1000 V pulses were delivered at 1 Hz. Images were captured at 1.2 s intervals at 63x magnification.

3.6.7 Force Calculation

Contractile force was calculated in MATLAB by comparing the deflection profile of the fiber with the best fit profile of a loaded fixed-fixed beam subjected to the cell forces at an angle, α_{Force} , measured as the angle between the resultant force vector and the undeflected nanofiber direction. Details on force analysis formulation and numerical scheme have been published

previously.[235] The cell's applied load to the fiber is assumed to be at each endpoint of the cell's protrusions where the f-actin stress fibers are anchored to the nanofibers *via* focal adhesions (Figure 3.1e).

The direction of the resultant force, α_{Force} , is estimated based on physiological structures from fixed cells. Elongated cells (cell before electroporation or long after electroporation) have well defined stress fibers which are $\sim 12.4^\circ$ from the horizontal (Figure 3.1e, bottom left). In instances where stress fibers were not present in recovering cells, we deferred to the angle of the dominant retraction fibers, (Figure 3.1e bottom center) known for their force bearing capabilities.[274,275] In cases where neither stress fibers nor retraction fibers are visible, and the cell is highly rounded, we took the resultant angle to be the bisection of the angle created by the membrane at the point of attachment to the fiber (Figure 3.1e, bottom right). For these situations, mechanical forces must be applied in-line with the membrane, thus, the resultant force would be the sum of the vectors radiating outward along the membrane, resulting in half the angle formed by the membrane. For a completely spherical cell, the half angle created by the membrane would point the force vector directly to the cell centroid. We fixed cells and imaged their cytoskeleton under no-electroporation conditions and 0.5, 2, 8, 16, 32, 128 minutes after electroporation at 1000 V ||. When α_{Force} was averaged for each cell, we arrived at the plot in Figure 3.1f (n=119). When cell length (L) is less than 63.4 μm in length, the angle of force application increases, and can be approximated as linear. Minimizing error for the data results in the following best fit:

$$\alpha_{Force}(L) = \begin{cases} 12.4 + .54(63.4 - L), & L < 63.4 \\ 12.4, & L \geq 63.4 \end{cases} \quad (1)$$

Where α_{Force} is in degrees and L is in μm . At each timepoint during cell recovery, the cell length was computed and the corresponding force angle was an input to the finite element model. The deflection profiles were measured by marking the fiber endpoints and eight points along the fiber length including the two endpoints of the cell. Fiber properties used in the computation of contractile force can be found in Table 3-5. Cell elongation was defined to be the length between a cell's endpoints on a fiber.

3.6.8 Finite Element Modeling

COMSOL 5.4 was used to model the electric field distribution in the device. We used the AC/DC module to perform a steady-state simulation. Computational models of the electric field within the device are shown in Figure 3.1d and Figure 3.7. Our models indicated that the scaffold region-of-interest (cut-out region of scaffold, 250 μm from edges) experienced electric field magnitudes of 0.88 V cm^{-1} for every 1 V delivered when the scaffold was in the parallel orientation, and 0.91 V cm^{-1} for every 1 V delivered when the scaffold was in the perpendicular orientation. Cells adherent to the glass slide (2D flat) experienced electric field magnitudes of 0.93 V cm^{-1} and 0.90 V cm^{-1} for every 1 V delivered when the scaffold was in the parallel orientation and perpendicular orientation respectively. Scaffold rotation is the cause of the slight increase in the electric field in the perpendicular orientation.

COMSOL was used to model the induced transmembrane potential (ITP) on cells. An accurate cell volume was reconstructed using 3D Slicer from a z-stack images of a fixed, actin-stained cell. Cell volume was meshed using 3-Matic. We performed a steady state model and

neglected the effect of electroporation-induced conductivity changes to the cell membrane.[276]

Over the volume, the Laplace equation (2) was solved:

$$\nabla^2 V = 0 \quad (2)$$

where V is voltage. A contact impedance boundary condition was used to model the boundary condition across the cell membrane:

$$n \cdot J = \frac{\sigma_m}{d_m} (V - V_{ref}) \quad (3)$$

where σ_m and d_m are the conductivity and thickness of the cell membrane respectively and V and V_{ref} are the voltages on either side of the cell membrane. Model parameters are presented in Table 3-6.

3.6.9 Mitigation of Thermal Effects

We performed experiments at 30 °C to mitigate thermal effects due to Joule heating. We analytically and experimentally determined Joule heating to be no greater than 8 °C (Figure 3.9). We calculated the worst-case scenario for Joule heating by assuming that all electrical energy was immediately converted to thermal energy to cause an instantaneous temperature rise.[273] Worst-case Joule heating was calculated for the delivery of 10 pulses at 1500 V using the equation:

$$\Delta T = IVt_{on}/(C_p m) \quad (4)$$

where ΔT is the change in temperature within the device, V is the voltage applied (measured), I is the current through the device (measured), C_p is the specific heat capacity of the media

(approximated as water: 4.184 kJ/(kg·K)), and m is the mass of the media being heated. Mass is calculated as $m = \rho lwh$, where ρ is the density (approximated as water: 1 kg/m³), l is distance between electrodes (1 cm), w is width of the channel (1 cm), and h is height of the channel (1.6 mm). We calculated that the worst-case scenario Joule heating would cause a temperature rise of less than 8 °C

Fiber optic temperature probe measurements within the device demonstrated that at the maximum electroporation condition of 10 pulses at 1500 V cm⁻¹, temperature rise was under 7 °C (Figure 3.9e-f). Our analytical temperature rise calculation is thus in good agreement with experiments. The rapid temperature rise was quickly dissipated by the surrounding fluid in the channel, and the temperature returned to within 2 °C of the pre-treatment temperature within a few minutes.

3.7 Supporting Information

Table 3-3 Sample size of cells analyzed for force response and percent of cells showing a biphasic stage of their response.

Condition	Number of cells analyzed per independent experiment			Number of cells with biphasic stage			Total cells with biphasic stage	Total Cells Analyzed	Total % of cells showing biphasic stage
	Exp1	Exp2	Exp3	Exp1	Exp2	Exp3			
500 V $\parallel$	6	6	9	5	2	4	11	21	52%
1000 V $\parallel$	9	6	11	6	6	8	20	26	77%
1500 V $\parallel$	9	11	8	7	8	4	19	28	68%
500 V $\perp$	7	7	9	0	0	0	0	23	0 %
1000 V $\perp$	8	6	8	5	2	1	8	22	36%
1500 V $\perp$	0*	0*	4	0	0	3	3	4	75%

Table 3-4 Sample size of cells analyzed for viability response and percent cell death for each experiment.

Condition	Ratio of dead cells to live cells (% cell death)							Total Cells analyzed	Percent cell death
	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	Exp 7		
500 V $\parallel$	0:66 (0 %)	0:55 (0 %)	0:112 (0 %)	-	-	-	-	233	0 ± 0 %
500 V $\perp$	0:68 (0 %)	0:25 (0 %)	0:37 (0 %)	0:26 (0 %)	0:25 (0 %)	-	-	181	0 ± 0 %
500 V 2D Flat	0:34 (0 %)	0:26 (0 %)	0:32 (0 %)	0:103 (0 %)	-	-	-	195	0 ± 0 %
1000 V $\parallel$	11:49 (18 %)	2:60 (3 %)	6:54 (10 %)	0:33 (0 %)	0:27 (0 %)	4:44 (8 %)	13:113 (10%)	416	7 ± 2 %
1000 V $\perp$	13:26 (33 %)	11:34 (24 %)	28:65 (30 %)	1:20 (5 %)	-	-	-	198	23 ± 6 %
1000 V 2D Flat	0:37 (0 %)	4:19 (17 %)	8:34 (19 %)	-	-	-	-	102	12 ± 5 %
1500 V $\parallel$	63:38 (62 %)	18:47 (28 %)	34:37 (48 %)	-	-	-	-	237	46 ± 10 %
1500 V $\perp$	33:0 (100 %)	72:2 (97 %)	194:7 (97 %)	-	-	-	-	308	98 ± 1 %
1500 V 2D Flat	13:2 (87 %)	14:10 (58 %)	15:5 (75 %)	66:27 (71 %)	46:2 (96 %)	102:24 (81 %)	-	326	78 ± 5 %

Table 3-5 Mechanical properties of polystyrene fibers for force calculation.

Property	Value
Length (μm)	275
Diameter (nm)	250
Young's Modulus (GPa) [234]	0.97
Pre-Tension (nN) [234]	201.2

Table 3-6 Parameters for induced transmembrane potential (ITP) COMSOL model.

Parameter	Symbol	Value	Reference
Extracellular media conductivity	σ_e ($\text{S} \cdot \text{m}^{-1}$)	1.28	Measured

Extracellular media relative permittivity	ϵ_e	80	[277,278]
Cytoplasm conductivity	$\sigma_c (\text{S} \cdot \text{m}^{-1})$	0.5	[278]
Cytoplasm relative permittivity	ϵ_c	60	[278,279]
Cell membrane conductivity	$\sigma_m (\text{S} \cdot \text{m}^{-1})$	3e-7	[278]
Cell membrane relative permittivity	ϵ_{cm}	8.57	[278,280]
Cell membrane thickness	$d_m (\text{nm})$	5	[278]

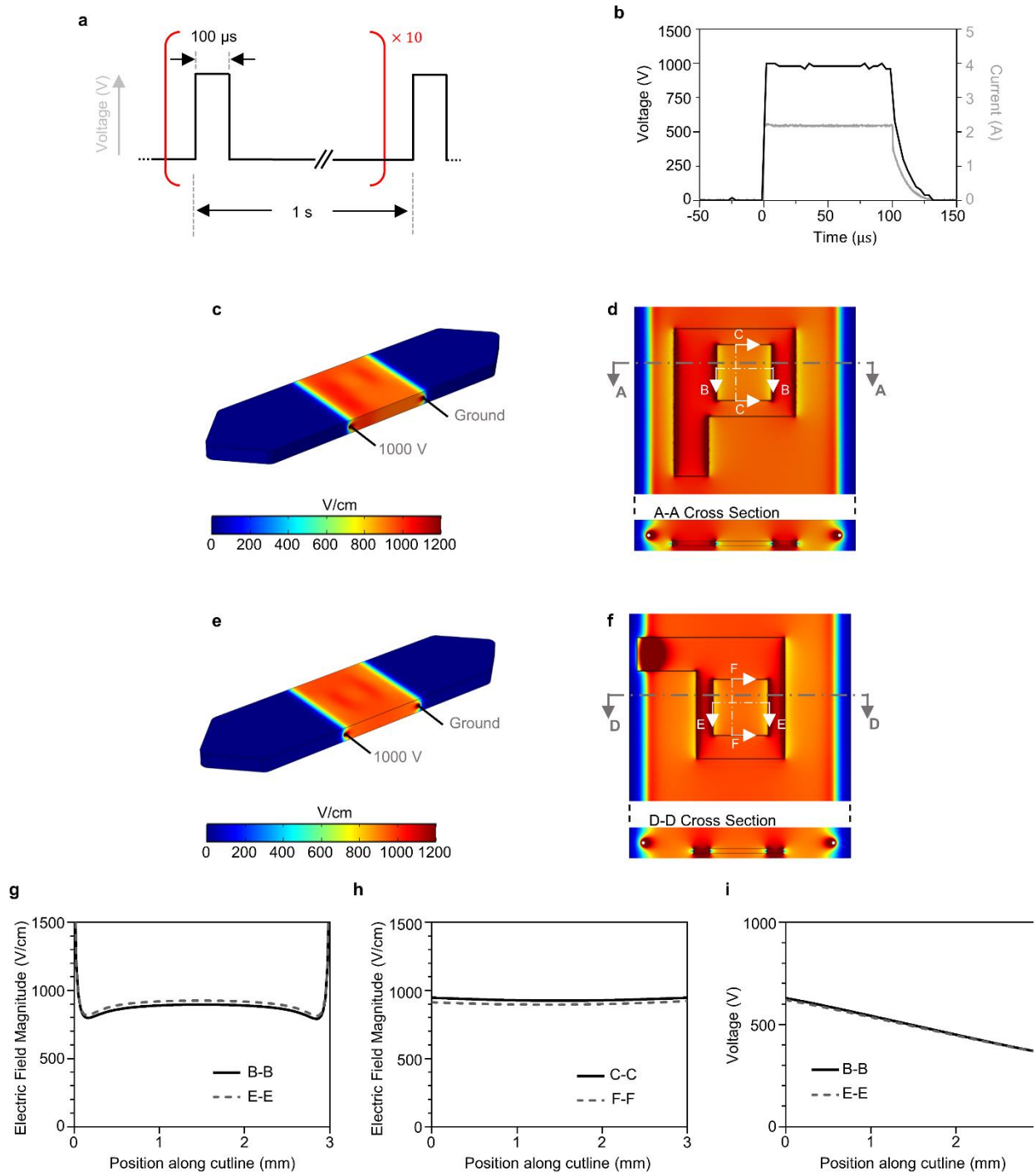


Figure 3.7 Electroporation waveforms the electric field within the device.

. a) Cells were treated with ten, 100 μ s square wave pulses at 1 Hz. b) Actual voltage and current waveforms measured during the first pulse. Pulses approximate idealized square waves. c) A finite element model shows the electric field distribution along the walls of the fluidic channel (c,e) and in the plane of the nanofiber scaffold (d,f). In c) and d) the scaffold is modelled in the parallel orientation while e) and f) are modelled with the scaffold in the perpendicular orientation. The nanofiber region within the scaffold has an approximately uniform electric across the center of the scaffold. g) Along the cutlines B-B and E-E the electric field magnitude is mostly constant, with asymptotic regions near the edges. h) Along cutlines C-C and F-F, electric field is quite uniform. i) The voltage drop is nearly linear along cutlines B-B and E-E. The generated electric fields over the region of interest were approximately 441 V cm⁻¹, 882 V cm⁻¹, and 1323 V cm⁻¹ respectively across the scaffold region in the parallel orientation and 455 V cm⁻¹, 911 V cm⁻¹, and 1366 V cm⁻¹ respectively across the scaffold region in the perpendicular orientation.

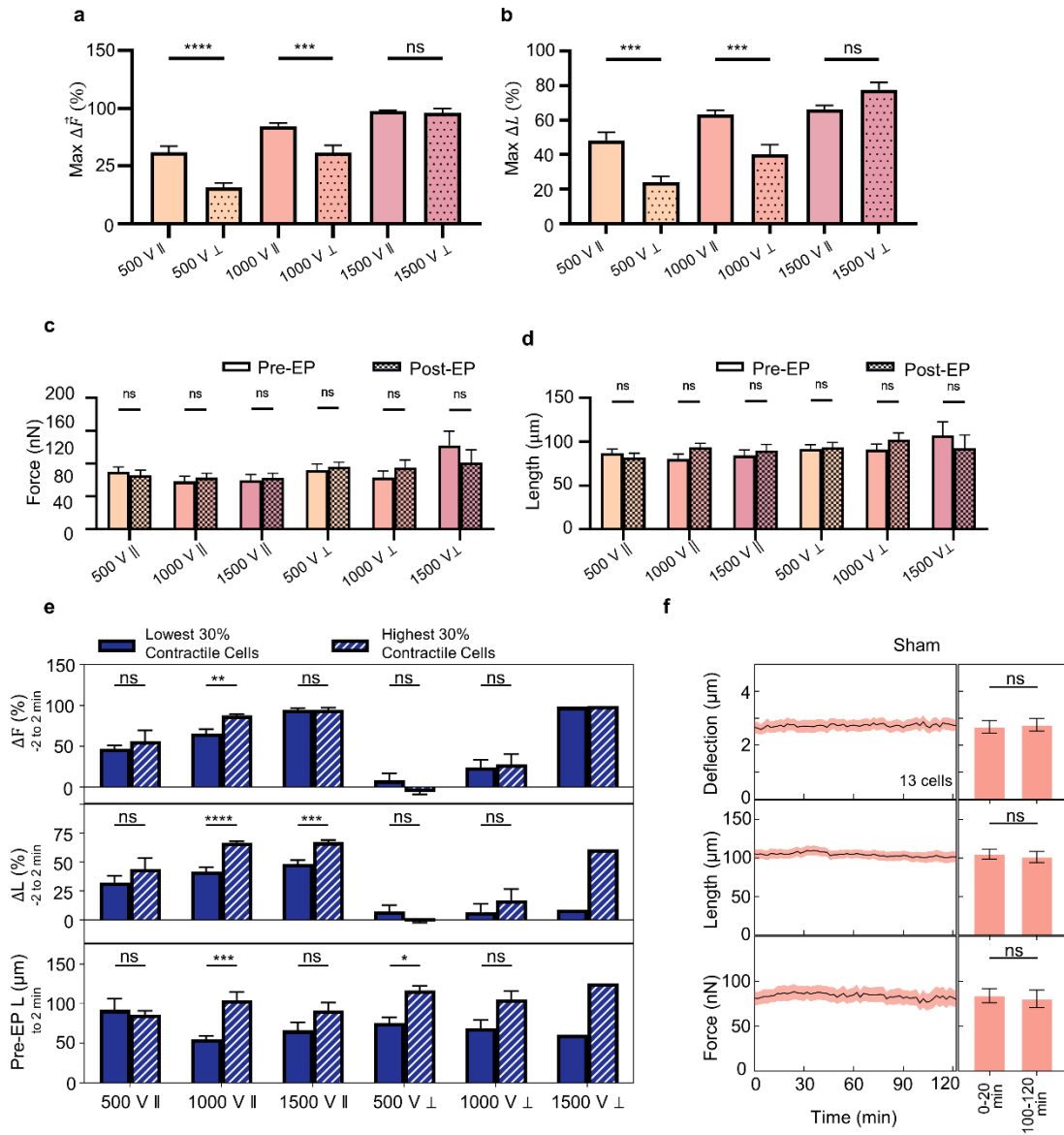


Figure 3.8 Force and length changes after electroporation

a-b) Data from Figure 3.2e-f, re-ordered to show significant differences due to electric field orientation. c-d) Cells post-electroporation do not show significant differences in contractile force or length compared to pre-electroporation values. Post-electroporation data collected at

180 min for 500 V and 240 min for 1000 V and 1500 V. e) For a given voltage and field orientation, high and low contractile cells show a similar force response in the first two minutes after EP. In the parallel orientation, the high contractile cells show a larger decrease in cell length immediately after electroporation when compared with low contractile cells, suggesting that contractility might increase the rate of rounding. However, high contractile cells also tend to also have longer initial lengths, so the rate of rounding may be dependent on both cell contractility and cell length. Stats not available for 1500 V $\perp$ (n=1 for both bars). f) Response of control cells ('sham exposure') kept at the same culture conditions as electroporation experiments. Over the course of 2 hours cells, demonstrated steady-state levels of contractility and maintained consistent cell shapes. EP, electroporation.

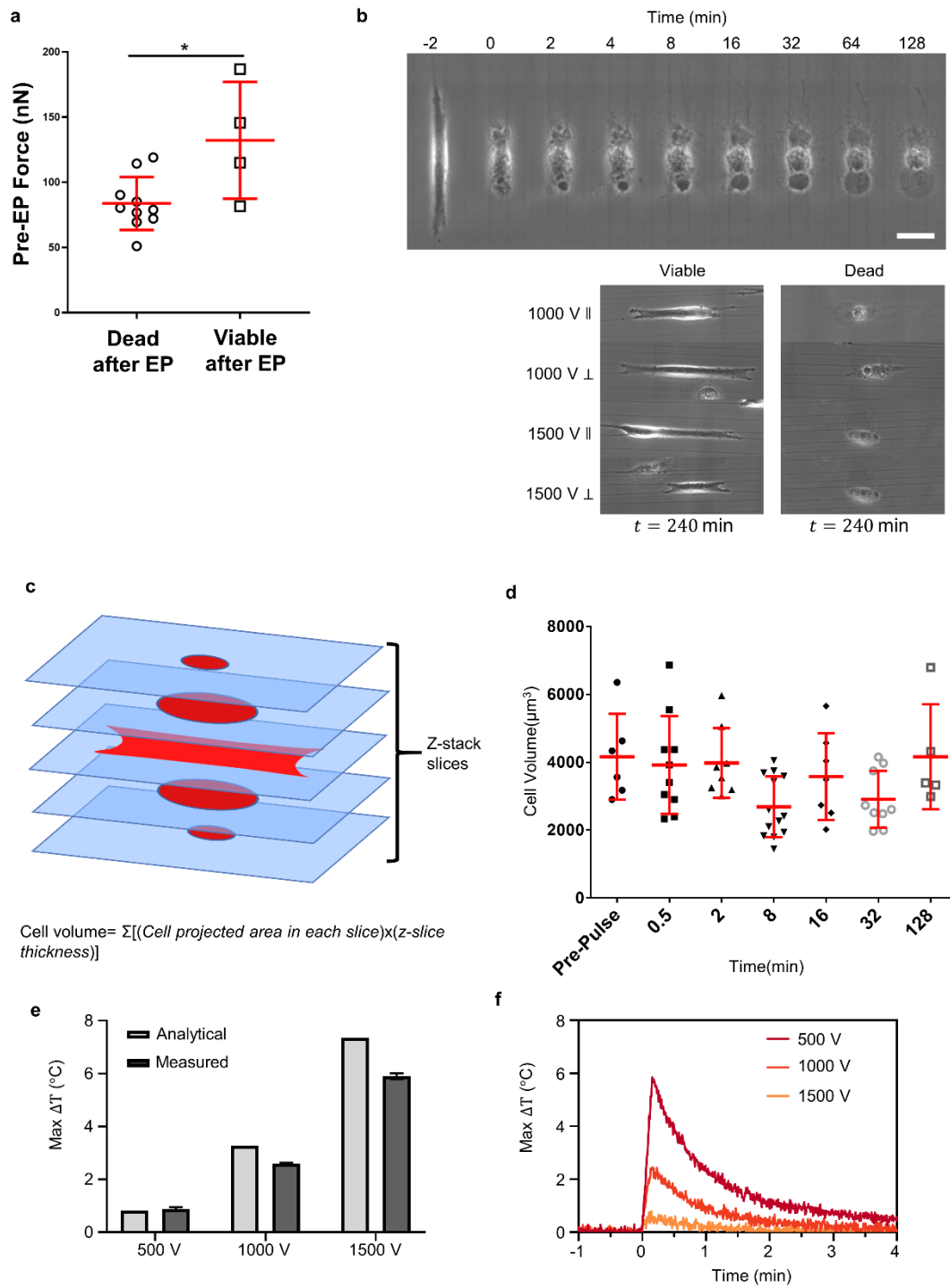


Figure 3.9 Additional analysis of cell death, cell volume and device Joule heating.

a) Comparison of pre-electroporation forces for cells treated at 1500 V in the perpendicular orientation. The few cells that survived electroporation at 1500 V in the perpendicular orientation showed higher pre-electroporation forces than cells that did not survive electroporation b) This image panel shows an example of cell death by accidental cell death. Very soon after electroporation, the cell takes on visual signs of cell death such as a loss of contrast and large membrane blebs. Additionally, the cell rapidly loses contractility as shown by the lack of fiber deflection after electroporation. Scale bar 25 μm . c-d) Cell volume change during cell recovery. Volume was calculated in MATLAB using z-stacks of fluorescent images (stained for actin, microtubules, and the nucleus) of fixed cells. The 2D area of the cell in each slice was used to estimate cell volume. d) Cell volume seems to decrease after electroporation (1000 V $\parallel$), with the greatest decrease between 8 and 32 minutes after electroporation. However, cell volume is not statistically significant between timepoints (ANOVA, $\alpha=0.05$). (n = 6, 10, 8, 13, 7, 9 and 5 corresponding to pre-pulse, 0.5, 2, 8, 16, and 32-minute timepoints respectively) e-f) Joule heating due to the electric pulses results in a rapid increase in temperature that returns to near pre-electroporation levels within minutes. The maximum temperature increase within the device due to Joule heating was experimentally and analytically verified to be under 8 $^{\circ}\text{C}$ for all conditions. Experimental measurements of Joule heating within the device was measured *via* a fiber optic temperature probe placed through the PDMS sidewall into the center of the electrode regions. f) The maximum temperature occurs immediately after pulsing and then returns to near pre-electroporation levels within 5 minutes. Fiber optic data collected at room temperature.

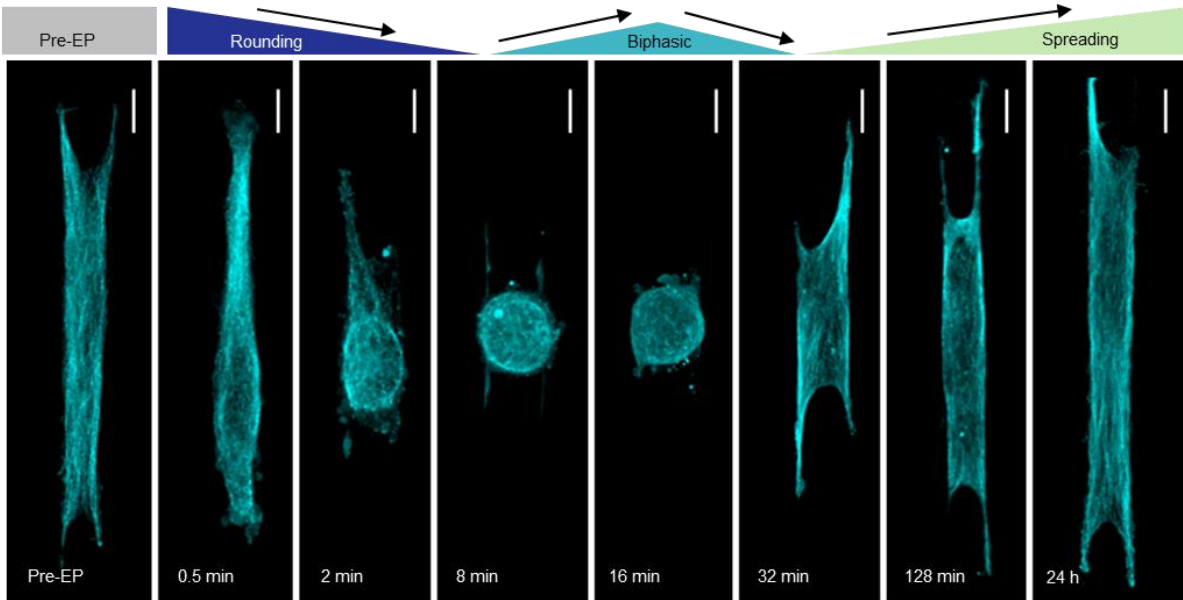


Figure 3.10 Microtubule dynamics after electroporation.

a) Maximum intensity projections of cells stained for microtubules before electroporation and during various stages of recovery post-electroporation (1000 V Electroporated cells (1000 V II) show a loss of microtubule alignment as the cells round, localization of tubulin at membrane blebs, and an eventual recovery of structure within 2 hours. Scales bars 10 μm .

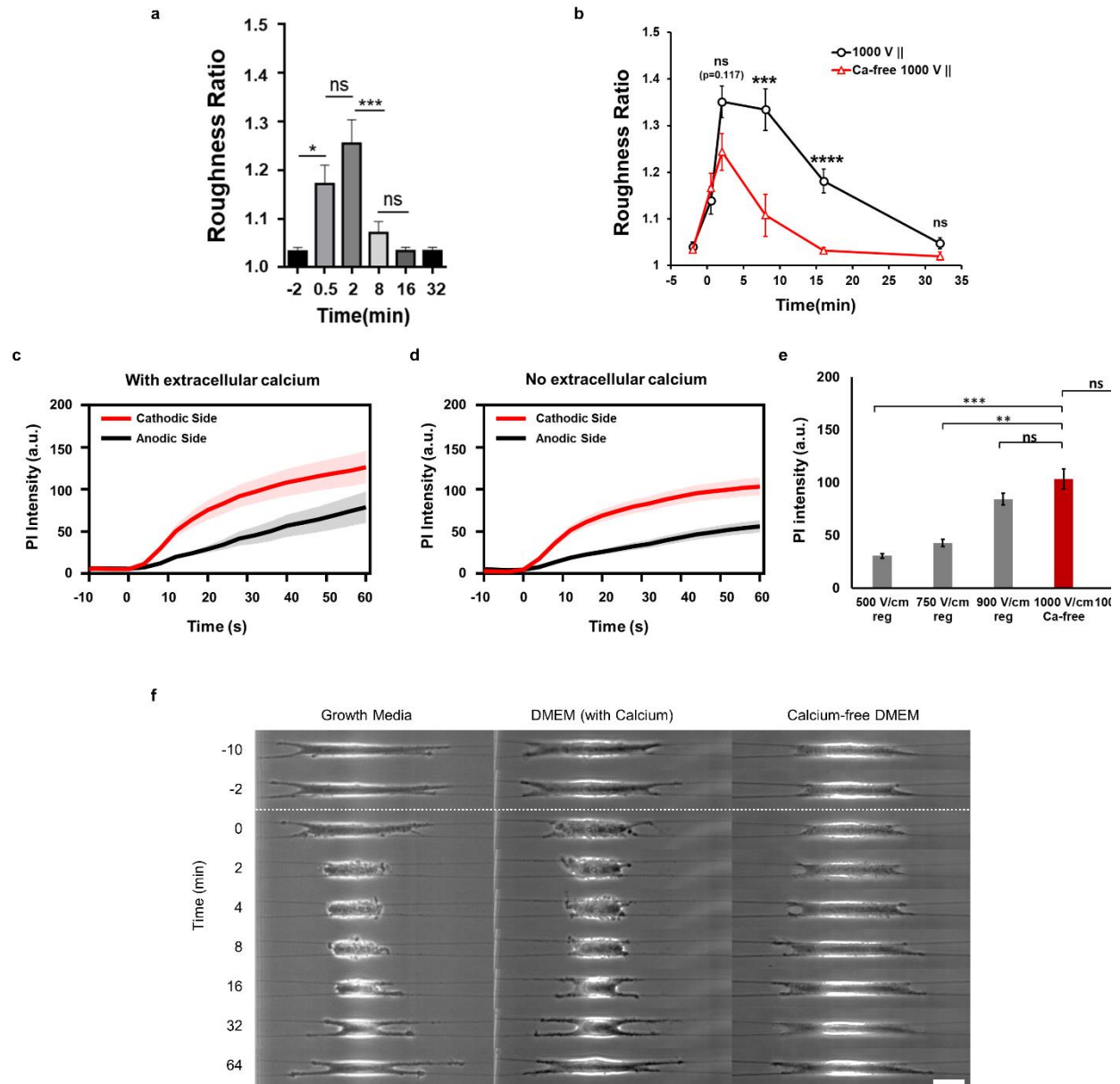


Figure 3.11 Analysis of blebbing, dye uptake, and cell rounding in calcium-free media.

a) Temporal dynamics of cell blebbing in Ca-free media (n=10 for each timepoint). The roughness ratio is defined in Figure 3.4f. b) Comparison of the blebbing dynamics after

electroporation in regular media *versus* Ca-free media. c,d) PI uptake after electroporation at 1000 V (parallel orientation) in c) growth media (1.95 mM calcium, n=18(N=4)) and d) Calcium-free DMEM (n=19(N=4)). PI uptake dynamics were recorded every 4 seconds with a 20x 0.8 NA objective. e) Comparison of PI uptake 60 s after electroporation at 500 V, 750 V, 900 V, and 1000 V in regular media (n= 14(N=2), 12(N=2), 18(N=2), 18(N=4) respectively) and at 1000 V in calcium-free DMEM (n=19 (N=4)). All experiments were conducted with cells in the parallel orientation. f) A representative cell electroporated at 1000V || in growth media (1.95 mM Ca; DMEM + 10% FBS + 1% PS), DMEM (1.8 mM Ca), and Calcium-free DMEM (~0 mM Ca). Scale bar 25 μ m.

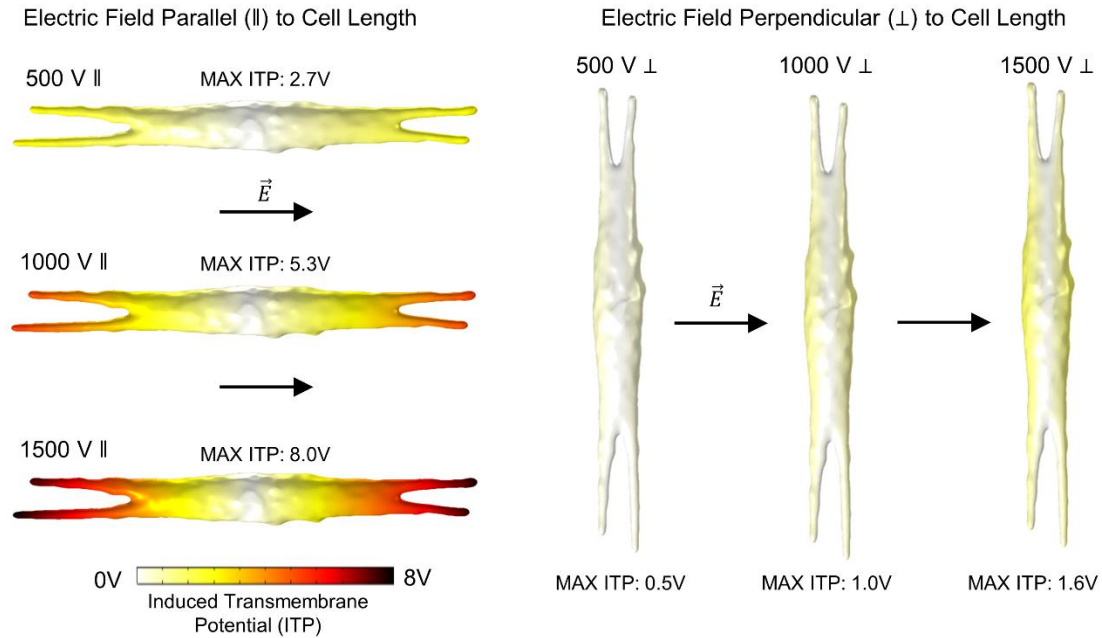


Figure 3.12 Induced transmembrane potential (ITP) model of a 3D cell in two orientations.

A steady-state computational model of the induced transmembrane potential (ITP) of reconstructed cells indicates differences in the spatial distribution and magnitude of ITP. Cells elongated parallel to the field show high ITP values distributed around the protrusions of the cell. ITP is defined as the voltage difference across the cell membrane at the moment prior to pore formation. Conversely, cells elongated perpendicularly to the field show lower ITP levels that are distributed around the sidewalls of the cell.

4 Chapter 4: A constriction channel analysis of astrocytoma stiffness and disease progression

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Attribution: Ravi Bollineni developed and analyzed the finite element model and neural network. Undergraduates John Moomaw and Evan Arents assisted with this project, such as device design and data processing.

4.1 Abstract

Studies have demonstrated that cancer cells tend to have reduced stiffness (Young's modulus) compared to their healthy counterparts. The mechanical properties of primary brain cancer cells, however, have remained largely unstudied. To investigate whether the stiffness of primary brain cancer cells decreases as malignancy increases, we used a microfluidic constriction channel device to deform healthy astrocytes and astrocytoma cells of grade II, III, IV and measured entry time, transit time, and elongation. Calculating cell stiffness directly from the experimental measurements is not possible. To overcome this challenge, finite element simulations of the cell entry into the constriction channel were used to train a neural network to calculate the stiffness of the analyzed cells based on their experimentally measured diameter, entry time, and elongation in the channel. Our study provides the first calculation of stiffness for grades II and III astrocytoma and is the first to apply a neural network analysis to determine cell mechanical properties from a constriction channel device. Our results suggest that the stiffness of astrocytoma cells is not well-correlated with cell grade. Furthermore, while other non-central-nervous-system cell types typically show reduced stiffness of malignant cells, we found that most astrocytoma cell lines had increased stiffness compared to healthy astrocytes, with lower-grade astrocytoma having higher stiffness values than grade IV glioblastoma. Differences in nucleus-to-cytoplasm ratio only partly explain differences in stiffness values. Although our study does have limitations, our results do not show a strong correlation of stiffness with cell grade, suggesting that other factors may play important roles in determining the invasive capability of astrocytoma. Future studies are warranted to further elucidate the mechanical properties of astrocytoma across various pathological grades.

4.2 Introduction

Tumors derived from glial cells, known as gliomas, account for more than 70% of primary brain tumors.^[48] Of the many types of gliomas, the most common form is astrocytoma—a tumor originating from astrocytes and their progenitor cells. Astrocytes are the most abundant cell type in the brain and support neural function.^[49] The World Health Organization classifies astrocytoma into four grades based on histological and molecular characteristics of the tumor: Grade I (pilocytic astrocytoma), Grade II (diffuse astrocytoma), Grade III (anaplastic astrocytoma), and Grade IV (glioblastoma), with increasing grade corresponding to decreased expected outcomes.^[281] Grade I astrocytoma is slow growing and benign, occurring mostly in children and young adults. Treatment of grade I astrocytoma by complete surgical resection is typically curative, and grade I tumors only rarely progress to higher-grade tumors.^[282,283] Grade II astrocytoma tumors do not have well-defined edges, and thus complete surgical resection is generally not possible. Grade II astrocytoma is generally slow-growing and considered benign. Grade III astrocytoma is fast-growing and malignant. Glioblastoma (GBM), or grade IV astrocytoma, accounts for 82% of malignant gliomas.^[52] Glioblastoma is highly aggressive and tumors show significant mitotic activity, vascular proliferation, and necrosis and overall survival is only 1-2 years post diagnosis.^[52] Primary (*de novo*) GBMs account for the majority of astrocytomas, while a minority first begin as grade II or III tumors and typically progress over years.^[284] It is unclear what drives the progress of low-grade astrocytomas.^[285] Primary and secondary GBM are morphologically indistinguishable, but arise through different pathways of genetic mutation.^[284]

All grades of astrocytoma rarely metastasize (< 2% of cases^[286]), but typically remain within the central nervous system and are generally unifocal.^[52] Instead, astrocytoma of grades II

through IV are characterized by significant infiltration of tumor cells into the brain at the tumor margin. Extensive infiltration of tumor cells makes complete resection impossible and limits radiation therapy efficacy.^[284] For example, 70-90% of GBM tumors will reoccur within a 2-cm margin of the primary tumor.^[287,288] Unlike many tumors that rely on intravascular and lymphatic migration, astrocytoma migration exploits the brain's natural structures and extracellular spaces.^[289] Studies reveal that glioma cells actively seek out blood vessels and travel along the basement membrane, displacing astrocyte endfeet and disrupting the blood-brain barrier.^[290] Glioma cells also actively hijack white matter fiber tracks for migration through interstitial spaces of the parenchyma.^[290] To transit the brain's submicron spaces, astrocytoma cells employ several migratory behaviors including degradation of the extracellular matrix^[289] and volume reduction^[291] to squeeze through constrictions. Because glioma cells infiltrate this mechanically-challenging environment, it is reasonable to suspect that the intrinsic mechanical properties of the glioma cells change to enhance motility. The mechanical properties of astrocytoma, therefore, may provide insight into migration capability, migration rates, and deviation from the normal mechanical phenotype of healthy astrocytes.

The mechanical properties of primary brain cancers have not yet been well-characterized. Only a few studies have investigated the mechanical properties of primary brain cells and primary brain cancer cells. Most existing studies focused only on healthy astrocytes^[292] and/or GBM.^[293,294] One study showed that a healthy astrocytes from prenatal rat embryos were stiffer than a human GBM (LN229, ATCC) cell line, and that showed a strong correlation between substrate stiffness and cellular stiffness.^[294] Another study measured astrocyte mechanical

properties and showed that astrocytes are less stiff than neurons, suggesting that astrocytes may not act as a structural support but rather as a compliant embedding for neurons.[292] One recent study used a microfluidic manometer device to compare the stiffness of healthy human astrocytes to GBM cell lines (A172, 1321N1).[295] This study, although evaluating a small number of cells, suggested that GBM may have increased stiffness compared to healthy normal astrocytes. Studies of non-central nervous system cancers such as breast,[296-300] prostate,[301] liver,[302] bladder,[303-305] lung,[225] oral,[306] thyroid,[307] and ovarian[308] cancer cells have demonstrated reduced stiffness of cancer cells compared to their healthy counterpart cells. Furthermore, studies of breast cancer,[309-311] ovarian cancer,[224,308,312] and colon cancer[313] demonstrate that high-grade cancer cells are more compliant than low-grade cancer cells, indicating a link between stiffness and disease progression. Widespread confirmation of the aforementioned trends has led to the paradigm that a reduction in cell stiffness correlates to metastatic potential in cancer.[314] Researchers have hypothesized that the decreased cell stiffness of cancer cells allow the cells to squeeze through the endothelium of blood vessels and enter circulation. However, some studies challenge this paradigm and indicate that increased malignancy and invasion correlate with stiffer cells in some cases.[301,315-320] The unique microenvironmental niche of glioma cells and the low rates of metastasis of glioma suggest these cells may have mechanical properties and stiffness trends differing from previously studied non-brain cell types. The brain is composed mainly of proteoglycans (lectican family) and associated binders hyaluronan and tenascins, creating a soft gel-like environment that is quite different from the fibrous collagen environments of other tissues.^[290]

Conventional methods for testing the mechanical properties of cells include micropipette aspiration[321] and atomic force microscopy.[322,323] These techniques are commonly used but require expensive equipment and are low-throughput. Microfluidic techniques, alternatively, can be used to investigate the mechanical properties of cells at low cost and high throughput. Microfluidic techniques generally force cells to deform by imposing narrow constrictions,[296,324-328] high fluid shear stress,[329-332] optical stress,[333-339] or electric fields.[340-344] Constrictions channel devices[295,296,324,325,345-361] are perhaps the simplest microfluidic technique, and force cells to deform as they enter and transit through a narrow channel. Constriction channels mimic pores/capillaries *in vitro* and can be designed to include multiple constrictions[362,363], deformable channel walls, or tapered constrictions. In this study, we use a simple constriction channel microfluidic device to deform cells under an applied hydrostatic pressure and measure key parameters of cell stiffness: entry time, transit time, and shape-change parameters.

Stiffness, or Young's modulus (E), cannot be directly measured from constriction channel experiments, and thus additional analysis, computations, or experiments must be performed to convert experimental results (entry time, transit time, elongation, etc.) to cell stiffness (Young's modulus). For example, if two cells with equal diameters are considered, the cell with a slower entry into the channel (longer entry time) will have a higher stiffness; however, if the two cells have differing diameters, it is not possible to determine which is stiffer by comparing their entry times. There is not a simple relationship between the experimentally measured parameters (entry time, transit time, elongation, cell diameter) and cell stiffness. To obtain stiffness values from experimental parameters, previous studies have used several methods including histogram

matching of strain and pressure,[364] calibration using gel beads[350], analytical relationships[348], and 2D finite element modelling[365-367]. In this paper we establish a novel framework to complement existing methods. We construct a computational framework by combining 3D finite element modeling (FEM) with an artificial neural network (ANN) that together are capable of calculating the cell's stiffness based on experimental data such as cell diameter, entry time, and shape-change parameters. Over 150 finite element simulations of cell entry into a constriction channel were performed across a range of cell diameters and stiffness values. In each simulation, the entry time and cell elongation within the constriction were calculated. These FEM results were used to train a neural network to determine cell stiffness from input parameters. We then used the trained ANN to calculate the stiffness of each cell in the experiments by inputting cell diameter, entry time, and cell elongation.

Several studies have used finite element modeling of cell deformation during micropipette aspiration[368-370] or entry into a constriction channel[365-367] for mechanical characterization of cells. In almost all the studies, cells were assumed to be an incompressible visco-hyperelastic solids. Elastic solid behavior was modeled using the incompressible neo-Hookean model and viscous relaxation was modeled using the Prony series.⁵¹ In almost all prior studies, cell deformation in three dimensions was approximated using two-dimensional or even two-dimensional axisymmetric plain strain models to reduce the computational cost. Using these computational results, the authors derived numerical or analytical relationships to evaluate mechanical properties of cells. Experimental data was then applied to the derived numerical or analytical relationships to qualify cell mechanical properties. These numerical/analytical models,

however, are inconsistent in literature because there is no exact analytical equation for finding the stiffness (Young's modulus) of a cell because of the assumptions considered while deriving the relations. In this paper, instead of deriving the analytical equations using the results of finite element simulations, we use artificial neural networks for quantifying the mechanical properties of any cell type using the trained network.

An artificial neural network is a nonlinear statistical analysis technique which is suitable for systems hard to describe using the existing physical models. Neural networks provide a way for linking input data to the output data using a set of nonlinear functions. Generally choosing the optimum number of layers (i.e., hidden layers) in the network is one of the challenging steps in the neural network modeling. Usually a trial-and-error procedure is applied to get the most accurate neural network. There are some existing studies in which artificial neural networks are used for predicting the mechanical properties of metallic alloys that are tough to be evaluated with the existing models. In these works, computational data are used for training a network, and experimental results are then given as input into the trained network, and used to evaluate the mechanical properties of complex systems.[371,372] Additionally, machine learning and artificial neural networks have been used to classify and sort cells populations.[373-376] According to the authors' knowledge, this is for the first time that artificial neural networks are being used in this field of cellular mechanical property characterization by constriction channels. Prior studies have employed numerical relationships to relate experimental data from constriction experiments with FEM simulations.[325] However, numerical relationships become complex when many parameters are considered. Neural networks provide an alternative approach and excel at pattern recognition

data interpretation. Relative to numerical relationships, neural networks become comparatively more beneficial as the number of input parameters increases. For this study we used three input parameters (cell diameter, cell elongation, and entry time), for which numerical relationships for Young's modulus could have been contrived. However, we anticipate that future studies may include additional parameters, making neural networks a preferred method for extracting cell properties. Therefore, our use of neural networks in this paper lays the groundwork for more complex studies in the future.

In this study, we calculate the stiffness of four established astrocyte/astrocytoma cell lines (Healthy: NHA; Grade III: SW1783; Grade IV: U251, U87) and two patient-derived astrocytoma cell lines (Grade II: VTC-018 Grade III: VTC-055, VTC-085). Grade II astrocytoma cell lines are not commercially available, so we investigated a patient-derived astrocytoma grade II cell line. We investigated if a decrease in astrocytoma cell stiffness would correlate with increased pathological grade. We found that, contrary to the prevailing trends with other cancer types, astrocytoma stiffness was not well-correlated with cell grade and in general showed increased stiffness compared to healthy astrocytes.

4.3 Methods

4.3.1 Microfluidic Device and Experimental Setup

The constriction channel device was fabricated from a single layer of polydimethylsiloxane (PDMS) using standard fabrication methods. Briefly, a master mold was negatively patterned by deep reactive ion etching of a silicon wafer and then silanized. PDMS was cast molded on the

wafer (10:1 base to cross-linker), cured at 100 °C for 1.5 hours, and peeled off. Inlet and outlet holes were punched with a 0.75 mm biopsy punch. The PDMS layers were plasma bonded (Harrick Plasma) to clean glass cover slips and kept under vacuum until experimentation. The assembled devices created a narrow constriction region 8 μm wide, 8.3 μm tall, and 130 μm in length (Figure 1a).

Two, 1-ml syringes without plungers were used as reservoirs for the hydrostatic pressure system and connected to the constriction device with 30 AWG tubing (Cole Palmer). Because of the extremely low fluid flow rate through the narrow constriction, the reservoir levels remained constant for the duration of experiments. The fluid interfaces were at atmospheric pressure and were precisely aligned using a custom laser-cut acrylic stand (Figure 1a, Figure S1) to ensure a consistent pressure drop across experiments. The reservoir liquid levels were separated by exactly 30 cm, producing a pressure drop across the system of 3.0 kPa. Nearly all of the pressure drop occurs across the narrow constriction due the very high hydraulic resistance of this channel. A pressure of 3.0 kPa is within the upper pressure range for intracranial hypertension[377], but is a bit high for physiological relevance. The high pressure was required for cell deformation and entry into the narrow constriction.

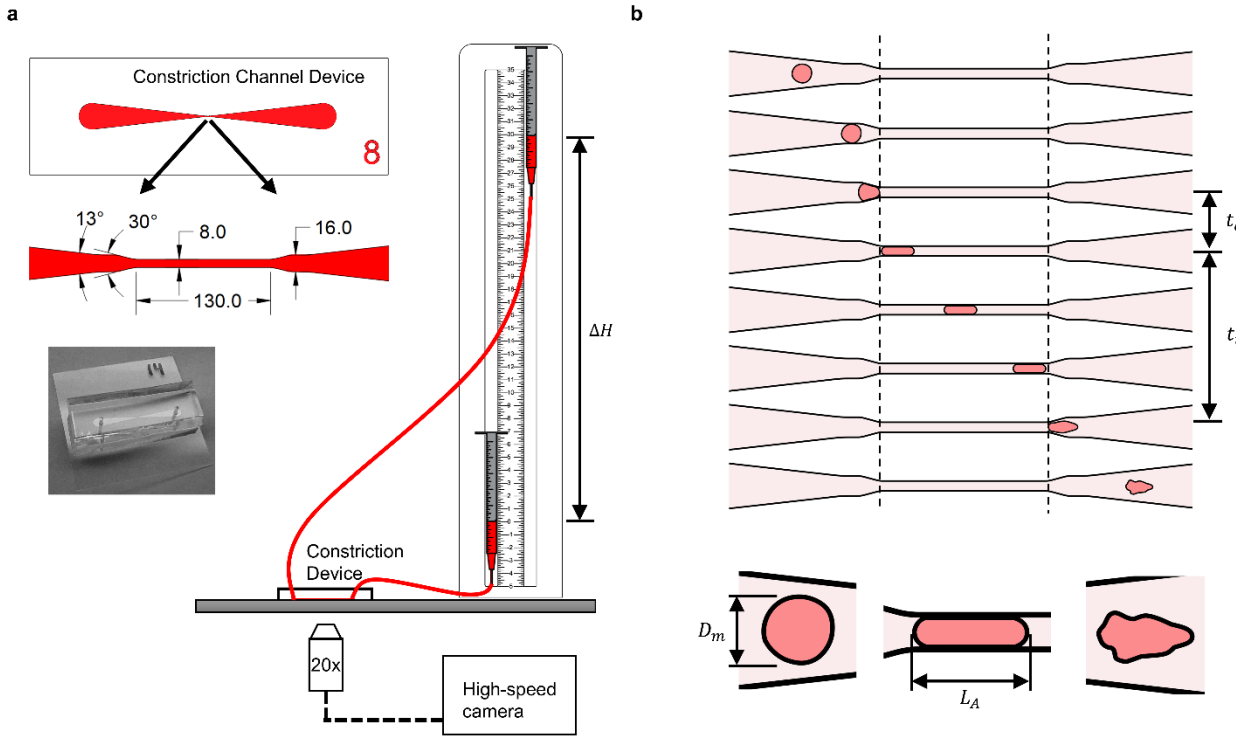


Figure 4.1 The constriction channel device and experimental setup.

(a) The constriction channel device and experimental setup. (a) The microfluidic constriction channel device is composed of a single channel that tapers to a very narrow constriction. The constriction is 8 μm wide, 8.3 μm tall, and 130 μm long. The device is composed of a PDMS layer bonded to a glass slide. Pressure-driven flow through the device is achieved by the vertical offset of two syringe reservoirs aligned on a custom stand to create constant, uniform hydrostatic pressure. A highspeed camera captures the cells passage through the constriction. b) A schematic of a cell traversing the constriction channel. Entry time (t_e) is defined to be the time taken for the cell to completely squeeze into the entrance of the constriction - when the leading edge of the cell reaches the constriction to the time when the trailing edge of the cell is fully within the

constriction. Transit time (t_t) is the amount of time taken by the cell to travel the whole length of the channel (130 μ m). Cell size is measured before the cell enters the constriction by approximating the cell as an ellipse with a major and minor diameter (D_m). Cells elongate within the constriction cells, and elongation is measured as the ratio of the elongated length (L_A) to the cell minor diameter (D_m). Cells exit the channel significantly deformed.

Prior to an experiment, a constriction channel device was placed on the stage of an inverted microscope (Leica DMI6000). A highspeed EM-CCD camera (Hamamatsu 9100-02) and MicroManager software were used to capture high-speed videos of cells passing through the constriction. A 20x objective (NA 0.4) was used to capture a field of view of approximately 400 μ m x 40 μ m. Data was captured at 200 fps.

4.3.2 Cell Culture

Six cell lines (healthy astrocytes and astrocytoma grades II – IV) were cultured according to standard culture procedures. Flasks were routinely passaged at 70-90% confluency, and only low passage number cells were included in the experiments. Normal human astrocytes (NHA, Lonza) were cultured with AGM astrocyte growth media bullet kit (Lonza) consisting of 500 mL ABM Basal Medium (CC-3187) supplemented with 15.0 ml Fetal bovine serum (FBS), 5.00ml L-Glutamine, 0.50 ml GA-1000, 0.50 ml Ascorbic Acid, 0.50 ml HEGF, 1.25 ml Insulin. Grade II astrocytoma cell lines are not commercially available, so we established a grade II astrocytoma cell line (VTC-018) from excised patient tissue. Likewise, we also established a grade III (VTC-

085) astrocytoma cell line from excised patient tissue. Both patient-derived cells lines were cultured in Dulbecco's Modified Eagle's Media (DMEM) with supplemented with 15% FBS and 1% Penicillin Streptomycin (PS). The SW1783 cell line (ATCC) is a commercially available grade III astrocytoma, and was cultured in Leibovitz's L-15 media supplemented with 10% FBS and 1% PS. We cultured two glioblastoma (grade IV astrocytoma) cell lines, U251 and U87 cells. U251 cells (Sigma Aldrich) were cultured in DMEM supplemented with 10% FBS and 1% PS. U87 cells (ATCC) were cultured in EMEM media supplemented with 10% FBS and 1% PS. All cell lines except SW1783 were cultured at 37°C under 5% CO₂. SW1783 cells were cultured at 37°C under 100% atmosphere.

To prepare a suspension of cells for experimentation, the following protocol was used. Cells cultured in T75 flasks were washed twice with 5 ml phosphate buffered saline (PBS), trypsinized with 1.5 ml of trypsin, and incubated for 1.5 – 2 minutes at 37°C. A 2 ml pipette was used to break apart cell clumps before 4 ml of cell culture media was added to deactivate the trypsin in the flask. The 4 ml of the cell suspension was transferred to a centrifuge tube, and 1 ml was transferred to an automatic cell counter (ViaCELL, info) to determine the cell density and viability. The cell suspension was centrifuged at 150 x *g* for 5 minutes. The supernant was decanted and 5 ml of our experimental buffer solution was added to resuspend the cells. For our experiments, we used a buffer solution of DPBS (Ca/Mg⁺²-free) supplemented with 1% bovine serum albumin (BSA), 5 mM EDTA, DNase I (25ug/ml). This buffer was formulated to minimize cell-cell and cell-surface adhesion, both of which adversely affect constriction channel experiments. After resuspension in the buffer, the cells

were recentrifuged at $150 \times g$ for 5 minutes. The supernant was decanted and the pellet was resuspended at 1×10^6 to 3×10^6 cells/ml. A cell strainer (Fisher, $40 \mu\text{m}$ filter) was used to filter out cell clumps. A 1 ml syringe was connected to a 15-inch #30 AWG tube with a plastic dispensing tip (Jensen Global) and the syringe was filled with $500 \mu\text{l}$ of the cell solution. The syringe and tubing were then transferred to the hydrostatic pressure stand. During the experiment, the plunger of the syringe was removed and the contents of the syringe were at atmospheric pressure.

4.3.3 Measured Parameters

This study measured several parameters as cells traversed the constriction channel including cell size (D_m), entry time (t_e), transit time (t_t), cell elongation (L_e), and cell circularity. Image data was processed semi-manually in ImageJ according to the following procedures.

Cell size: Before cells were deformed by the constriction, cells generally had a circular or slightly elliptical shape when viewed from the microscope. To quantify cell size, we used ImageJ software to trace a cell's perimeter using a spline, and then fit an ellipse to the spline using ImageJ's Fit-Ellipse function. This analysis yielded the major-diameter (D_M) and the minor-diameter (D_m) for each cell. Most cells were highly circular, and yielded very similar values for D_M and D_m . During experiments, we found that cells typically rotated such that their minor-diameter was oriented parallel with the constriction width as they approached the entrance to the constriction channel (data not shown). For this reason, we used the cell's minor diameter as the metric for cell size (Figure 4.1b). For brevity, we refer to the minor diameter of cells as simply cell diameter.

Entry Time (t_e): Entry time was defined to be the time taken by the cell to enter the constriction. Entry time was calculated from the time the leading edge of the cell crossed into the constriction (8- μm wide region) to the time when the cell's trailing edge had completely entered the constriction. Entry time is illustrated in Figure 4.1b.

Transit Time (t_t): Transit time was defined as the time taken by the cell to travel the entire length of the constriction (130 μm). Transit time was calculated from the time the trailing edge of the cell entered the constriction to the time when the trailing edge of the cell exited the constriction channel as show in Figure 4.1b.

Elongation (L_E): Cell elongation (L_E) was defined as the ratio of the length of a deformed cell (fully within the constriction channel) to the diameter of the cell prior to entering the constriction region.

$$L_E = \frac{L_A}{D_m} \quad 1$$

Where L_E is cell elongation, L_A is the deformed cell length of the cell within the constriction, and D_m is the minor-diameter of the cell prior to entering the constriction (Figure 4.1b).

Cell shape change: We measured cell circularity before and after the constriction channel to measure cell shape change. Circularity was calculated using the formula:

$$\text{Circularity} = 4\pi \left(\frac{A}{P} \right)^2 \quad 2$$

Where A is the z-projected area of the cell, and P is the z-projected perimeter of the cell. Circularity values range from 0.0 - 1.0, with a value of 1.0 representing a perfect circle. ImageJ was used to trace a cell's edges with a spline in ImageJ and circularity was automatically calculated using ImageJ's Analyze Menu. When measuring circularity after exiting the constriction channel, contents of ruptured cells were not included in this calculation as they were difficult to resolve due to low contrast.

4.3.4 Numerical Simulations

Finite element simulations of the cell entry in to the constriction channel were performed using the finite element package ABAQUS53.[378]

4.3.4.1 Geometrical and Material Modeling.

Three-dimensional cell deformation was conducted in ABAQUS/Explicit solver. Cells were modeled as a continuum sphere across a range of cell diameters to collect as broad a data set of numerical results as possible. The constriction channel was modeled as a rigid body, since the stiffness of PDMS (~ 1 MPa) is much higher than that of the cell stiffness. The model of the constriction channel had the same dimensions as in experiments, but incorporated an initial entrance region with increased channel height (20 μm), such that cells could be initially modeled as spheres before being flattened by the low channel height (8.3 μm) just prior to the constriction channel entrance. The material model for cells was taken to be incompressible neo-Hookean with viscoelastic behavior.[379] The constitutive equation for the neo-Hookean material is:

$$\sigma_s = C_{10} \left(\bar{B} - \frac{1}{3} \text{tr}(\bar{B})I \right) + \frac{2}{D_1} (J - 1)I \quad 3$$

Where σ_s is the Cauchy stress tensor, $\bar{B}$ is defined as $\bar{F} \cdot \bar{F}^T$ with $\bar{F}$ being the distortion gradient which is equal to $J^{1/3}F$, where J is elastic volume ratio, D_1 is temperature dependent material parameter, and I is identity tensor. C_{10} and D_1 are given as:

$$C_{10} = \frac{G_0}{2} \quad 4$$

$$D_1 = \frac{3(1 - 2\nu)}{G_0(1 + \nu)} \quad 5$$

where G_0 is the initial shear modulus and ν is the Poisson's ratio.

Viscoelastic behavior is defined using the Prony series expansion of time dependent shear and volumetric behavior formulated[369]:

$$g_R(t) = 1 - \sum_{i=1}^N \bar{g}_i^p (1 - e^{-t/\tau_i^G}) \quad 6$$

$g_R(t)$ is a dimensionless shear relaxation modulus, $\bar{g}_i^p$ is the dimensionless Prony series parameter for shear modulus, and τ_i^G is the relaxation characteristic time.

$$k_R(t) = 1 - \sum_{i=1}^N \bar{k}_i^p (1 - e^{-t/\tau_i^K}) \quad 7$$

where $k_R(t)$ is a dimensionless bulk relaxation modulus, $\bar{k}_i^p$ is the dimensionless Prony series parameter for bulk modulus, and τ_i^K is the relaxation characteristic time. The bulk relaxation term will be equal to zero because the material is considered to be incompressible. The density of the cell was considered as 1000 kg/m^3 and the elastic behavior was modeled using the term C_{10} .^[365] For incompressible material D_1 is equal to zero. The viscoelastic behavior was modeled using the Prony series where specified the dimensionless shear modulus and relaxation characteristic time.

4.3.4.2 Boundary conditions and Meshing.

As the spherical cell entered the constriction channel, the cell is significantly deformed due to smaller cross-sectional area of the channel compared to that of the cell. To model these large deformations, geometrical nonlinearity was considered in the analysis. Friction between the channel and cell was modeled using a simple coulomb friction model, in which critical shear stress on the sliding surfaces was directly proportional to contact pressure between the surfaces with a constant frictional coefficient f_c . The rigid channel was kept fixed throughout the simulation while the spherical cell was acted up by a uniform pressure loading of 2.45 kPa, equal to the pressure that was used in the experiments. As a cell deforms and enters into the constriction channel, the surface area which is exposed to the pressure keeps changings. Due to this change, a uniform pressure could not be used on a pre-selected region of the cell surface. To overcome this issue, a user subroutine VDLOAD was used for applying the pressure loading on the outer boundary of the cell that was outside of the constriction channel. This area was updated in each time step during the simulation. Cells were discretized with C3D10M quadratic elements, which are very efficient

in modeling large deformations and complex contacts situations. The constriction channel was meshed with a default discrete rigid element mesh.

4.3.4.3 Artificial neural network model.

We used an artificial neural network designed by the available MATLAB neural network toolbox. Generally, the number of neurons in the input and output layers of a neural network are determined by the number of input and output variables. Here we used three input nodes, entry time, cell diameter, and elongation, and only one output node for Young's modulus. For training and validating the network, we used the results collected from the finite element simulations. A total data set of 155 simulations were used for the training and validating the network. Among that data set, 150 data points were used for training, which was performed using a batch training process. The remaining 5 data points were used for validating the network, which was useful in quantifying the number of hidden layer neurons to be used in the network. In order to get the optimal neural network, different numbers of hidden layer neurons were considered and the error of the predicted values for each network were calculated. We choose to use a network with a hidden layer consisting of eight neurons, which our validation process demonstrated was capable of predicting stiffness with less than seven percent error.

4.3.5 Nucleus-to-Cytoplasm (NC) ratio calculation

The nucleus-to-cytoplasm (NC) ratio is the ratio of nuclear volume to cytoplasmic volume. To calculate the NC ratio, we stained cell nuclei with NucBlue (2 drop per ml) and the cytoplasm with Calcium AM (4 μ l/ml). Cells were stained in suspension, and then

transferred to a well-plate and imaged at 10x before cell adhesion occurred. Thus, cells were assumed to be spherical. To calculate the NC ratio, nuclear and cytoplasmic channels were individually thresholded and binarized to black and white. ImageJ's Analyze Particle function was used to calculate the z-projected area, the major-diameter (of a fitted ellipse), and the minor-diameter (of a fitted ellipse) of the nuclei and cells. Only cells (and nuclei) that were relatively circular (circularity > 0.75) were analyzed. The average of the major and minor diameters was assumed for the out-of-plane direction, and volumes were calculated with these diameters. To accurately compare NC data to the constriction channel data, we adjusted cell diameters (originally measured on spherical cells) to be equivalent with the diameters calculated for cells just prior to entering the constriction channel (flattened due to low channel height). See Supplemental Information for derivation.

4.3.6 Statistics

Statistics were performed in JMP Pro 15. Statistical differences were determined by ANOVA tests at $\alpha=0.05$. Multiple comparisons Student-t tests were used to compare statistical significance between multiple groups ($\alpha=0.05$).

4.4 Results

Healthy astrocytes and astrocytoma grade II, III, and IV cells were analyzed in high numbers ($81 \leq n \leq 270$) as presented in **Table 4-1**. The average minor-diameter of cells analyzed ranged from 18 to 25 μm depending on cell type (**Table 4-1**). Cells with diameters larger than 25 μm often did not reach the constriction channel because of the relatively low channel height of the

device. Furthermore, many large cells (>25 μm) that reached the constriction channel could not enter the constriction at the experimental pressure (2.45 kPa) due to the large deformation required, and thus became stuck prior to entering the channel. For these reasons, we focused our analysis on cells with diameters between 10 μm and 25 μm .

Figure 4.2b-c shows two representative cells traversing the constriction. Prior to entering the constriction channel, cells displayed a high degree of circularity and high velocity. Upon encountering the constriction, cell velocity was dramatically reduced as significant deformation was required for a cell to enter the constriction channel. Deformation usually occurred as an elongation of the leading (aspirated) edge of a cell and the lateral compression of the minor-diameter of a cell to the width of the channel. In many cases, especially larger cells, the leading (aspirated) surface of the membrane of the cell and the trailing edges (at both contact point with channel sidewalls) showed blebbing, which indicates membrane rupture and leakage of cytoplasmic contents (Figure 4.2c). While confined to the channel, cells elongated in the direction of the channel. Upon exiting the constriction, cells had highly deformed shapes with significantly decreased circularity (circularity decrease = 0.34 ± 0.13 , average for all cell types).

Table 4-1 Cell grades, origin, and number analyzed.

Grade	Cell Line	Cell Type	Number Analyzed	Cell diameter in μm (Mean $\pm$ STD)
Healthy	NHA	Primary	n = 270	16.9 ± 3.4
Grade II	VTC-018	Primary*	n = 110	19.4 ± 3.3
Grade III	VTC-085 SW1783	Primary*	n = 81	18.9 ± 3.5
		Immortalized	n = 130	21.4 ± 4.3

Grade IV	U251	Immortalized	n = 129	20.9 ± 3.9
	U87	Immortalized	n = 113	16.0 ± 2.1

* Patient-derived cell line

4.4.1 Entry Time, Transit Time, and Elongation

The entry times (t_e) for all cells analyzed are plotted in Figure 4.2d against cell diameter. The relationship between entry time and diameter is nonlinear, but can be roughly approximated as a log relationship as shown by the best-fit lines on the semi-log plots in Figure 4.2d. Interestingly, while the slopes of the best fit semi-log relationships for healthy cells through grade III cells are quite similar, the slopes for grade IV cells appear quite different – with U251 cells showing a low slope and U87 cells showing a high slope. We used cell entry time as an input to the neural network for predicting the stiffness of cells.

The transit times (t_t) for all analyzed cells are show shown Figure 4.2d. As with entry time, transit time is roughly linearized when plotted on a log scale against diameter. Best fit log relationships show similar trends in their slopes as with entry time. Because our finite element simulations could not recapitulate cell transit through the constriction (See next section), transit time was not included in the analysis of cell stiffness.

Cells elongated in the direction of the channel when confined within the constriction (Figure 4.2b-c). We measured cell elongation (L_E , see Methods), and found that the elongation was approximately linearly-correlated with cell diameter, as shown in Figure 4.2d. We use elongation as an input to the neural network for predicting the stiffness of cells.

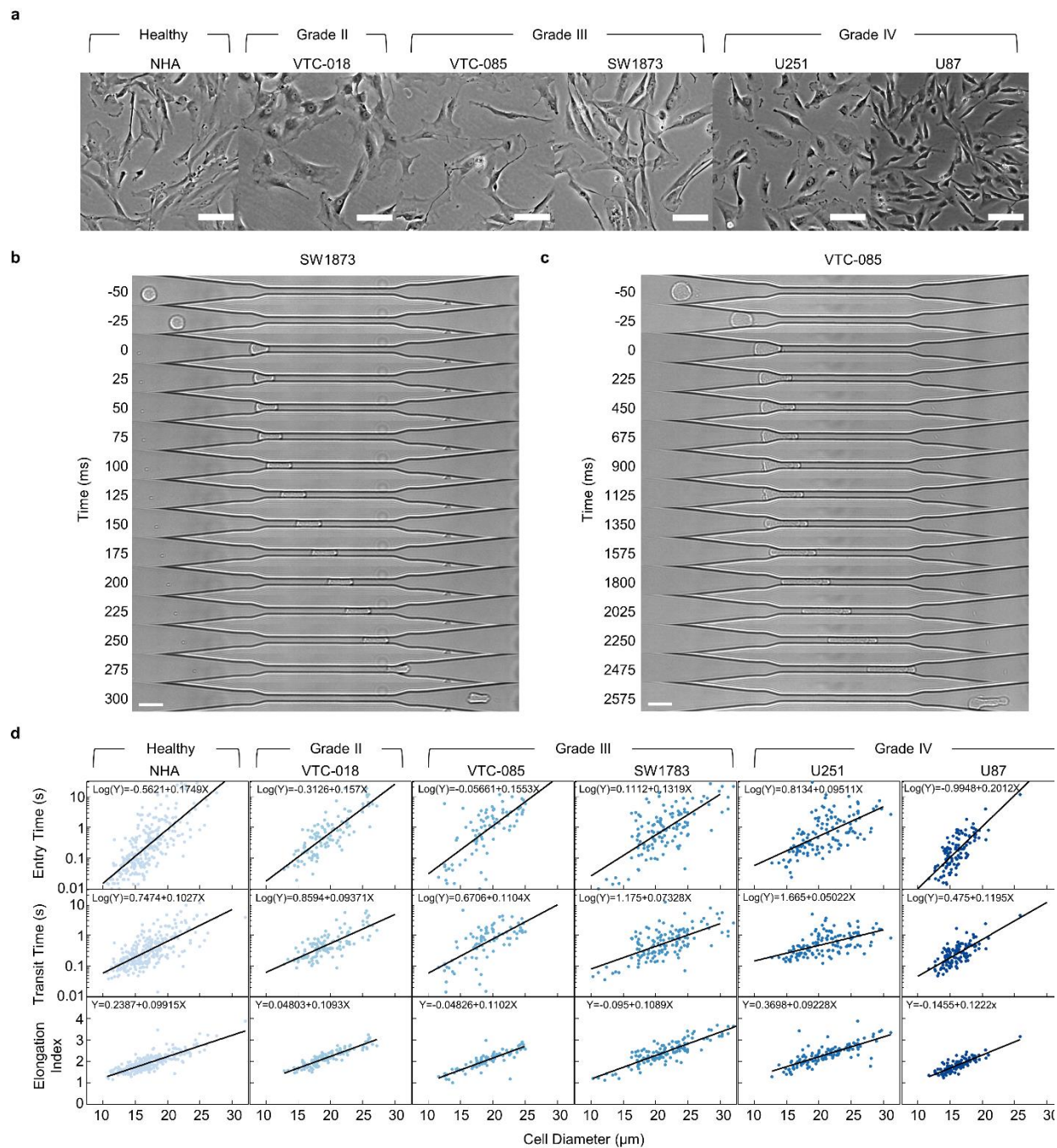


Figure 4.2 Constriction channel results.

a) Brightfield images of each cell line showing cell morphology of adherent cells. Scale bars 100 μm . b-c) Timeseries of two representative cells traversing the constriction channel. c) A small diameter cell (SW1873, diameter 15.8 μm) entering and transiting the constriction. d) A larger-diameter cell (VTC-085, diameter 20.3 μm) entering and transiting the constriction channel. Scales bars 25 μm . d) Entry time, transit time, and elongation index for all cell types plotted against cell diameter. Entry time and transit time are roughly linearized on a log scale when plotted against cell diameter.

4.4.2 Analysis of cellular entry process and numerical simulations.

Using finite element modeling, we simulated cell entry into the constriction channel. Figure 4.3 shows a sequence of images illustrating the characteristics of cell entry. Cell deformation from our numerical simulations agreed well with the sequential cell entry process into the channel measured in the experiments that is shown in Figure 4.2. Aspiration length, definite as the distance traveled by the leading edge of the cell past the entrance of the constriction, plotted against time revealed that the process of cell entry is divided into three stages as in Figure 4.3f. In the first stage, aspiration length shows a rapid, nonlinear increase as the cell's leading edge enters the constriction. However, the forward progress of the leading edge is quickly impeded in the second stage as the cell becomes deformed by the constriction (see Figure 4.3b). This deformation causes the second stage to have a linear relationship between time and the aspiration length, with a small slope. The slow movement of the cell into the channel at this stage is illustrated in Figure 4.3c-d.

In third stage of entry, the trailing edge of the cell completely enters the constriction, leading to an increased velocity as the cell begins transiting the constriction (Figure 4.3e).

In this study we performed multiple simulations by varying the stiffness between 3.6 to 21.6 kPa (3.6, 4.2, 4.8, 5.4, 6, 6.6, 7.2, 7.8, 8.4, 9.0, 9.6, 10.2, 10.8, 11.4, 12.0, 13.2, 14.4, 15.6, 16.8, 18, 20, and 21.6 kPa) for a set of cell diameters varying from 11 μm to 19 μm in an interval of approximately 1 μm , while keeping all the remaining parameters constant in all the finite element simulations. From these simulations, we calculated entry time (t_e) and elongation (L_E) for each simulation of cell diameter (D_m) and Young's modulus. We optimized our simulations to capture the largest range of cell diameters and stiffness values as possible. However, simulations with cell diameters greater than 19 μm were not possible due to the very large deformations, and entry times of greater than 2s were excluded from analysis to the limited accuracy of model predictions.

4.4.3 Artificial neural network training and validation.

We trained a neural network to predict the stiffness of a cell using our collected data from the finite element simulations. A schematic of the network is shown in the Figure 4.4. We found that a neural network with one hidden layer composed of eight neurons was able to predict the

stiffness for the finite element data with an error less than seven percent. The network, once trained, could estimate the stiffness for any cell, when time entry (t_e), entry diameter (D_m) and

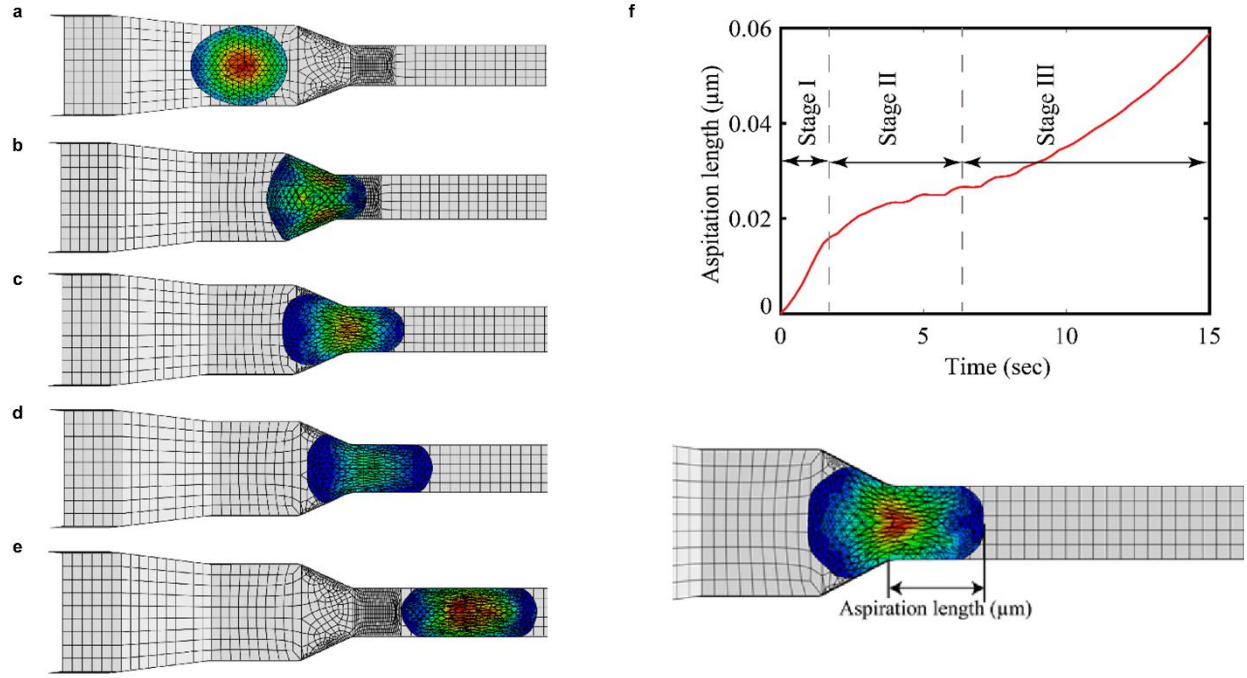


Figure 4.3 Numerical simulations of cell entry into the constriction channel.

a) Numerical simulations of cell entry into the constriction channel. (a-e) Snapshots showing cell deformation during cell entry into the constriction channel. (a) Cells approach the constriction with some deformation present due to the low channel height. (b-d) The leading-edge of the cell enters the channel (stage I), and the cell undergoing slow deformation (c, d) as the entire cell enters the constriction (stage II). (e) After entry, the cell transients through the channel (stage III). (f) Aspiration length versus time illustrates the three stages of cell entry process

elongation index (L_E) values for that cell were given as input into the network. For example, Figure 4.4b-d shows contour plots for stiffness for multiple combinations of the input parameters (t_e , D_m

and L_E).

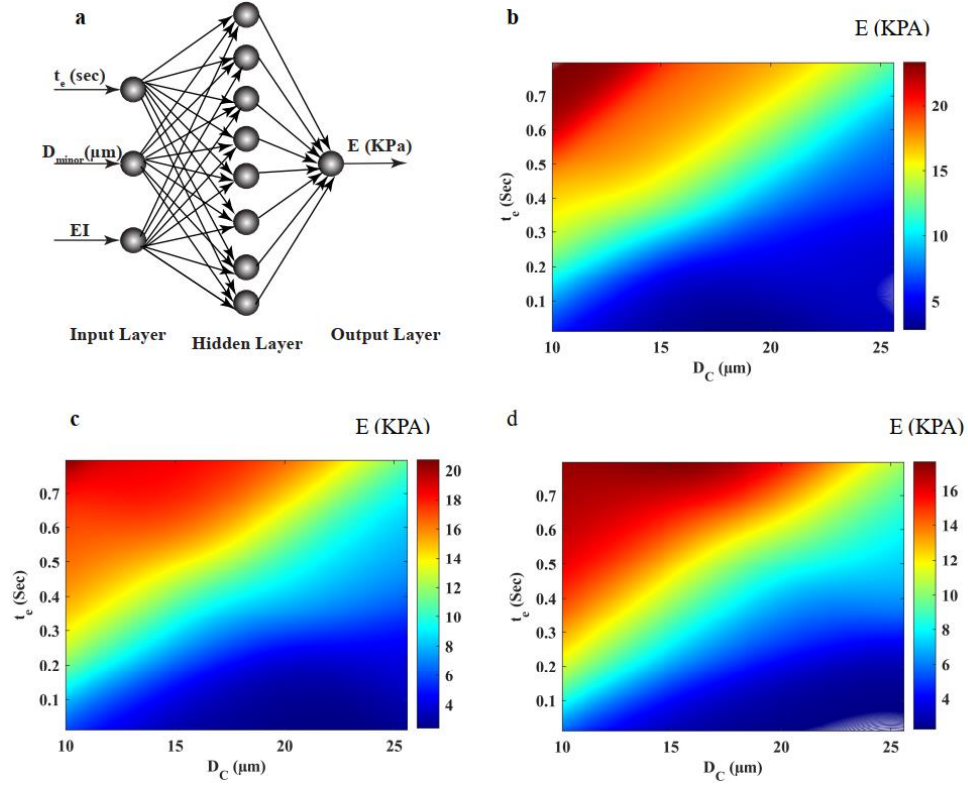


Figure 4.4 Neural Network architecture and results.

(a) Schematic representation of the neural network architecture. The network predicts stiffness (E) based on three inputs: entry time (t_e), diameter (D_m), and elongation (L_E). (b-d) Contour plots show stiffness values for different combinations of cell diameter and entry time (t_e) when elongation values are (b) 1.6, (c) 1.9, and (d) 2.

4.4.4 Stiffness values of astrocytes and astrocytoma cell lines

Using the trained neural network, we calculated the stiffness values for our experimental data, as shown in Figure 4.5. **Table 4-2** presents the average and median values of the stiffness for each cell type. We found that average stiffness values varied between 7 and 13 kPa depending on cell type, and an ANOVA test revealed significant differences between cell types. **Table 4-2** also includes the groupings from multiple comparisons Student t-tests; each cell type not connected by the same letter had significantly different stiffness. We found that cell grade did not show a strong correlation with average (or median) cell stiffness. Interestingly, NHA cells (healthy astrocytes) and U87 cells (grade IV) had significantly lower average (and median) stiffness values compared to the other cell types. Contrarily, the patient-derived grade III astrocytoma cell line (VTC-085) showed the highest average (and median) stiffness, with significantly higher stiffness than all other cell types. To better understand the origin of the lower stiffness, we calculated the nucleus-to-cytoplasm (NC) ratio for all cell types.

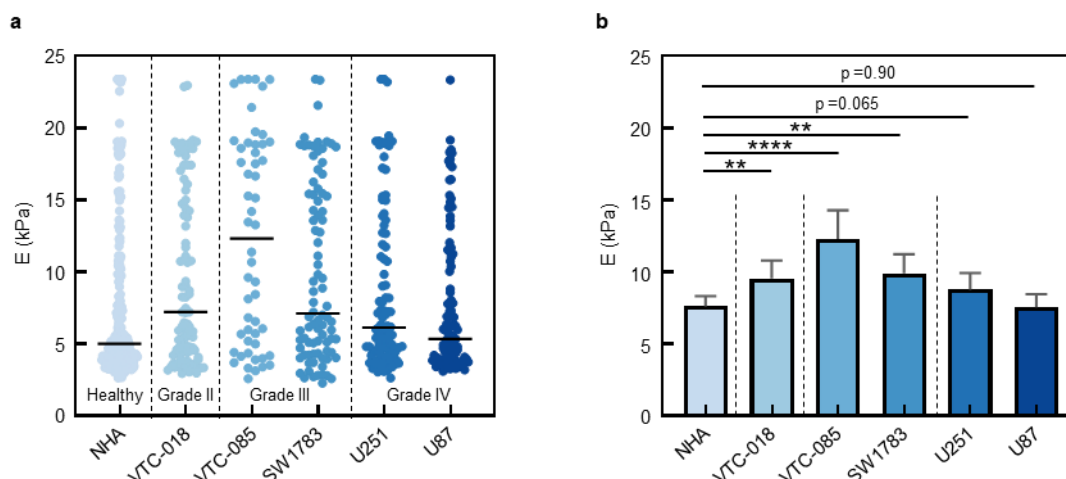


Figure 4.5 Neural Network calculation of stiffness.

a) Stiffness for each cell analyzed. Horizontal black lines show media values. b) Average Stiffness values with 95% confidence intervals for all cell types. Dashed black lines separate cell grade (healthy through grade IV left to right. Bars show significant differences between astrocytoma cell lines and healthy astrocytes (student-t tests).

Table 4-2 Stiffness (E) of all cell types.

Grade	Cell	Number Analyzed	Stiffness (E)			Student t-test groupings		
			Mean	STD	Median			
Healthy	NHA	234	7.6	5.4	5.0	A		
Grade II	VTC-018	91	9.5	6.0	7.2		B	
Grade III	VTC-085	52	12.3	7.2	12.3			C
Grade III	SW1783	86	9.9	6.1	7.1		B	
Grade IV	U251	98	8.8	5.8	6.1	A	B	

Grade IV	U87	111	7.5	4.9	5.3	A		

4.4.5 Nucleus-to-Cytoplasm (NC) ratio

To better understand the results of our stiffness measurements, we calculated the nucleus-to-cytoplasm (NC) ratio for all cell types. Figure 4.6 shows the NC ratio plotted against cell diameter for cells with 10-25 μm diameter. Cell diameters were adjusted for equivalence with the measurement method for the constriction channel experiments (See Methods). As can be seen from our results, the NC ratio differs significantly between cell types. Most notably, SW1783 (grade III) and U87 (grade IV) cell show different NC characteristics. Both SW1783 and U87 cells have significantly lower NC ratios compared to the healthy astrocytes (NHA). Furthermore, we found that U251 cells had a significantly higher NC ratio than the healthy astrocytes (HNA). Supplemental Figure 4.8 shows the density plots of NC ratio versus cell diameter for all cell sizes. From the distribution, it is clear that SW1783 and U87 cells show different NC ratio characteristics compared to other cell lines.

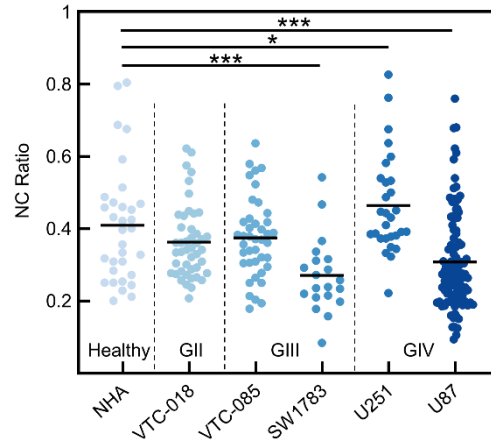


Figure 4.6 Nucleus to cytoplasm (NC) ratio for all cell types.

Compared to healthy astrocytes, SW1783 cells (Grade III) and U87 cells (Grade IV) have significantly lower NC ratios, while U251 cells (Grade IV) have a significantly higher NC ratio. NC ratios calculated for cells with diameters between 10 μm – 25 μm , for consistency with constriction channel analyses.

4.4.6 Discussion

The prevailing paradigm in cancer progression and metastasis is that increased metastatic potential of cancer cells correlates with decreased cell stiffness. Due to the significant deformations that must occur for a cell to migrate through confined spaces, decreased stiffness is thought to increase a cells ability to traverse these mechanically challenging spaces. In this study, we investigate whether the malignancy of astrocytoma correlates with cell stiffness. We used a microfluidic constriction channel device to deform healthy astrocytes and astrocytoma cells of

grades II-IV to investigate the stiffnesses of these cells, and were able to analyze the passage of over 75 cells for each cell type through a microfluidic device consisting of a single constriction channel ($8 \times 8.3 \times 130 \mu\text{m}$). Using finite element simulations of cell entry into the constriction, we trained a neural network to predict stiffness (E) values based on cell diameter, cell entry time, and cell elongation within the channel.

While neural networks have been used to sort cell populations based on their mechanical properties,[373,374] this study is the first to use an artificial neural network to compute the stiffness of cells. Furthermore, our study represents the first investigation of cell stiffness for grade II and grade III astrocytoma. Unfortunately, research on low-grade glioma's (grades II and III) has been limited. Glioma research is generally directed toward grade IV astrocytoma (glioblastoma), as these grade IV gliomas presents the most severe pathology and many grade IV cell lines are readily available and easy to work with. Unfortunately, at this time there are no grade II astrocytoma cells commercially, further limiting research efforts. Here, we investigated a patient-derived grade II and grade III astrocytoma cell line and measured cell stiffness. Additionally, we investigated another grade III astrocytoma cell line that is commercially.

Several prior studies using constriction channels have used cell entry time directly to infer cell stiffness, rather than calculating stiffness.[295,355] However, entry time values alone have limited usefulness since they cannot be compared across studies due to the dependence on channel geometry and pressure drop across the channel. Our calculation of stiffness via finite element simulations enables our results to be easily compared with other studies. In good agreement with previously calculated values of stiffness for astrocytes and GBM cells,[292,294] we found that

average stiffness values varied between 1.5 and 3.5 kPa depending on cell type. Interestingly, we found that the average stiffness of the cell lines tested did not have a strong correlation with cell grade. Compared to the healthy astrocytes (NHA cells), most astrocytoma cell types were found to have higher stiffness than their healthy counterpart. Only U87 cells (grade IV) did not show a statistically significant increase in stiffness. The patient-derived grade III cell line showed the highest average stiffness. To calculate these stiffness values, we included three input values (cell diameter, entry time, and elongation) into our neural network.

The nucleus is the stiffest organelle of the cells, and thus we calculated the nucleus-to-cytoplasm (NC) ratio for each cell type to understand the impact of nuclear size on our results. NC ratios revealed that U87 and SW1783 cells had significantly smaller nuclei than other cell types. Furthermore, U251 cells showed higher NC ratios compared to other cell types. Low stiffness values of U87 seem to be explainable by their smaller-than-normal nuclei. Since the nucleus is generally 5-10 times stiffer than the surrounding cytoskeleton,[380] smaller nuclei require less deformation to enter the constriction channel, leading to decreased entry times and decreased stiffness values for these cells. Likewise, the large nuclei of U251 cells may explain their higher-than-normal stiffness values. NC ratios do not explain all trends in cell stiffness, however, as both grade II and III patient-derived cells had high stiffness values, but showed nearly equivalent or lower average NC ratios than healthy astrocytes (NHA).

Several additional factors may contribute to the trends in cell stiffness we observed. Patient-derived primary cells, used at low passage number, may be mechanically different than established or immortalized cell lines that have been adapted for cell culture. Studies have shown

that astrocytes change their stiffness and behavior depending on the stiffness of their culture platform,[294,381] thus patient-derived cells may differ from established cell lines. Low-grade cells may be innately stiffer than healthy astrocytes due to their pathology. Astrocytes are generally quiescent and non-migratory, but can become highly migratory when activated by brain injury.[382] Healthy astrocytes, therefore, may be less stiff than low grade gliomas due to this migratory capability. Our culture conditions for the astrocytes may have promoted a migratory phenotype due to the inclusion of insulin in the culture media, which has been shown to induce migration in other cell types.[383-385] Given the migration potential of healthy astrocytes, it may prove insightful for future studies to include grade I astrocytoma, which typically does not show cell migration *in vivo*. Finally, patient-derived cells were more difficult to work with (due to cell clumping and clogging in the channels), and therefore their stiffness values may have more error. Interestingly, low grade astrocytoma cells showed equal or higher stiffness values compared to glioblastoma. Future studies are warranted to better understand the nature of high stiffness values we found in low-grade astrocytoma.

The increased invasion and malignancy of higher-grade astrocytoma is well documented[52,284]. However, while grade IV gliomas showed lower stiffness than low-grade gliomas, healthy astrocytes had approximately equal stiffness with grade IV gliomas. Our finding that astrocyte and astrocytoma stiffness is not well-correlated with malignancy goes against the prevailing paradigm in cancer mechanics, and warrants future studies to investigate this further. A few studies with other cell types, have also reported higher stiffness values for malignant and late-stage cancer cells compared to healthy or less malignant cells. Bastatas *et al.* found that highly-

metastatic prostate cells were stiffer than lowly-metastatic cells.[315] Similarly, Faria *et al.* showed that a highly-invasive prostate line had higher stiffness than a non-invasive prostate line.[301] Zang *et al.* found that hepatocellular carcinoma cells were more stiff than healthy hepatocytes.[302] Finally, Darling *et al.* found no correlation between malignancy and stiffness in chondrosarcoma cell lines, with the cells of greatest malignancy showing the lowest stiffness.[386] Our results add to the findings of these studies that suggest that malignancy is not always correlated with stiffness. Limited or no correlation between astrocytoma grade and cell stiffness may suggest that stiffness is not the driving (or rate-limiting) factor in astrocytoma migration. Several active cellular processes are known to contribute to glioma invasion. Glioma cells are able to degrade the extracellular matrix by secreting proteases and other pro-migratory molecules.[289] During migration, cells reach forward to attach to the ECM, degrade their ECM attachments at their posterior, and contract via myosin II and their actin cytoskeleton to squeeze themselves (especially their nucleus, the stiffest intracellular organelle) through constrictions.[387,388] Additionally, ligand-mediated cascades allow glioma cells to exchange ions and water and decrease their volume up to 30% to squeeze through narrow passages.^[291] Other studies, however, have shown evidence that suggests nuclear stiffness may be the rate-limiting step in glioma invasion.[387-390] For example, knockdown of lamin A, an important component of nuclear lamina, decreases nuclear stiffness in U251 cells and increased transmigration across a transwell membrane.[391] Lamin A/C linked with nuclear stiffness and rate-limiting migration.[389] Future studies are warranted to investigate if there are differences of nuclear stiffness between grades. Since we forced cell deformation in our experiment, we cannot capture active cell processes, such as volume regulation,

that are involved in migration. Therefore, future studies may provide more information by measuring the rate of active cell migration through constrictions for various grades of astrocytoma.

In our experiments, pressure was held constant by a hydrostatic pressure difference maintained by vertically-displaced syringe pumps. Due to the low fluid flow, the fluid level in the syringe pumps remained constant. However, our device did not enable real-time monitoring of pressure levels, as more complex microfluidics devices[293] or pressure-based flow controllers[348,354] can enable. During experiments, large cells (or clumps of cells) progressively became lodged upstream of the constriction channel, potentially decreasing the pressure drop across the constriction channel. To minimize unwanted pressure drops upstream of the channel, we performed our experiments on multiple constriction channel devices and only performed experiments on constriction channel devices for short durations (around 5 minutes) before replacing the device. Additionally, we created a buffer solution to minimize cell-cell and cell-wall interactions.

The geometry of our microfluidic channel (particularly the low channel height, 8.3 μm) limited the size of cells that reached and transited through the constriction at the hydrostatic pressures used in these experiments. Constriction channel devices are known to be sensitive to cell size, since constrictions too narrow do not allow cell passage but channels too wide do not cause significant deformation. Unfortunately, the astrocytoma cells lines in this study showed a very wide range of sizes, making it difficult or impossible to collect data for all cell diameters. With the exception of U87 cells, most cell types had a significant percentage of cells too large for analysis with our device. Consequently, future work is warranted to confirm that stiffness trends from this

experiment apply to cells with larger diameters. Smaller cells, however, tend to have less cytoplasm and thus larger NC ratios. Therefore, contributions from the nuclear stiffness to the overall cell stiffness may be more pronounced in our experiments. This hypothesis seems reasonable, since NC ratios show some correlation with cell stiffnesses. A higher sensitivity to nuclear stiffness may be desirable, since previous studies have demonstrated the nucleus, the stiffest part of the cell, may play an invasion-limiting role by slowing the rate that cells can migrate.[387-390] To improve the throughput and reduce clogging events in future studies, a multi-constriction channel device with a higher channel height could be implemented. Increasing the device channel height and constriction cross-sectional area would have enabled the entire distribution of cell diameters to enter the channel and may have improved our results. Improvements of our device could also include a method for real-time pressure measuring to ensure any small pressure changes during and between experiments are known.

Due to the high deformity required by cells to enter the constriction channel, finite element modeling of cell entry into the constriction was very difficult due to large mesh deformation leading to model convergence errors. We maximized the parameter space possible for modeling cells of various diameter. However, at cell diameters greater than 19 μm , cell deformation was quite large and convergence of simulations was not possible. Consequently, our neural network estimated stiffness for cell diameters greater than 19 μm by extrapolating data from smaller diameters. Therefore, our calculation of stiffness for cells 19 μm may have some error. Limiting our experimental data set to cells with diameters below 19 μm was not practical due to the low number cells within this range for some cell types. Additionally, the large deformations required

for larger cells to enter the constriction channel lead to cell blebbing/rupturing. Blebbing/rupturing is a result of the lipid bilayer detaching from cortical actin that supports the membrane and provides mechanical strength. This phenomenon cannot be replicated in simulations, as it involves discrete cell components with different mechanical properties. Our simulations treat cells as homogenous visco-elastic bodies with uniform properties. Thus, significant cell blebbing and rupturing may lead to inaccuracies of our simulations. In future work, more experimental parameters (transit time, nuclear size, etc) could be used to train a neural network for even more reliable prediction of stiffness from constriction channel data.

4.5 Conclusions

In this study we used a constriction channel microdevice to deform cells for the purpose of calculating cell stiffness of healthy astrocytes, and grade II, III, and IV astrocytoma cells. Using measured values of cell diameter, entry time, and elongation, we calculated the stiffness of cells using neural network trained with finite element simulations. Our study provides the first calculation of stiffness for grade II and grade III astrocytoma, and is the first to apply a neural network analysis to a constriction channel device. Our findings suggest that low-grade gliomas have elevated stiffnesses compared to healthy and high-grade gliomas, and warrants future studies to investigate this further. Differences in NC ratios between the cell types partly explain the stiffness values, suggesting that nuclear deformation plays a significant role in modulating the rate of entry into constrictions. While most prior studies show reduced stiffness of cancer cells compared to their healthy counterparts, we found that most astrocytoma cell lines had higher stiffness values than the healthy astrocytes. Increased migratory potential of higher-grade

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astrocytoma cells may be driven by factors other than cell stiffness, such as active cell processes like volume regulation and ECM degradation.

4.6 Conflicts of interest

There are no conflicts to declare.

4.7 Acknowledgements

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4.8 Supporting Information

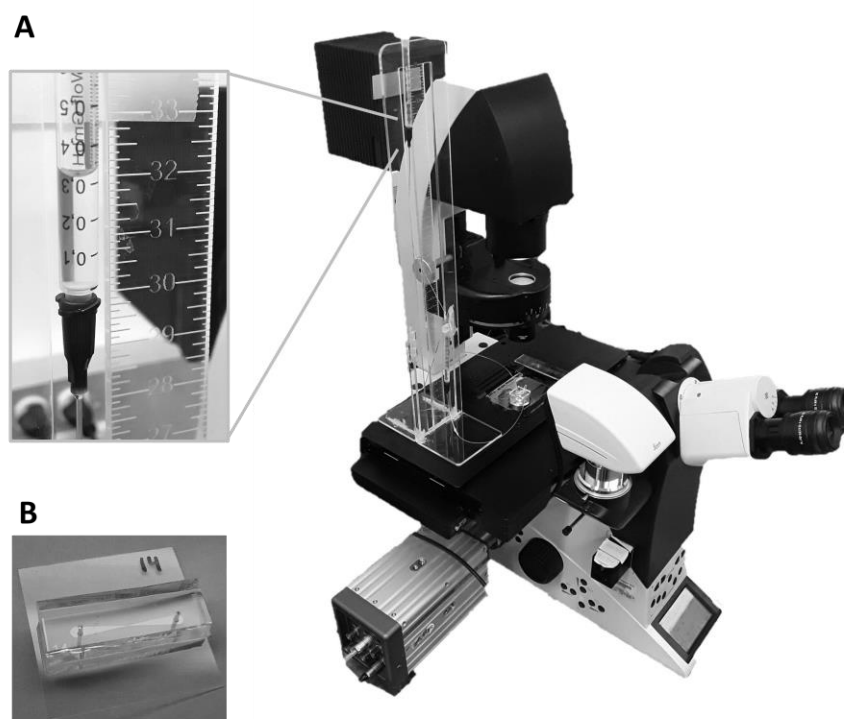


Figure 4.7 Photographs of the experimental setup and device.

(a) Photograph of the experimental setup with enlarged view of a syringe reservoir. (b) Photograph of the PDMS device.

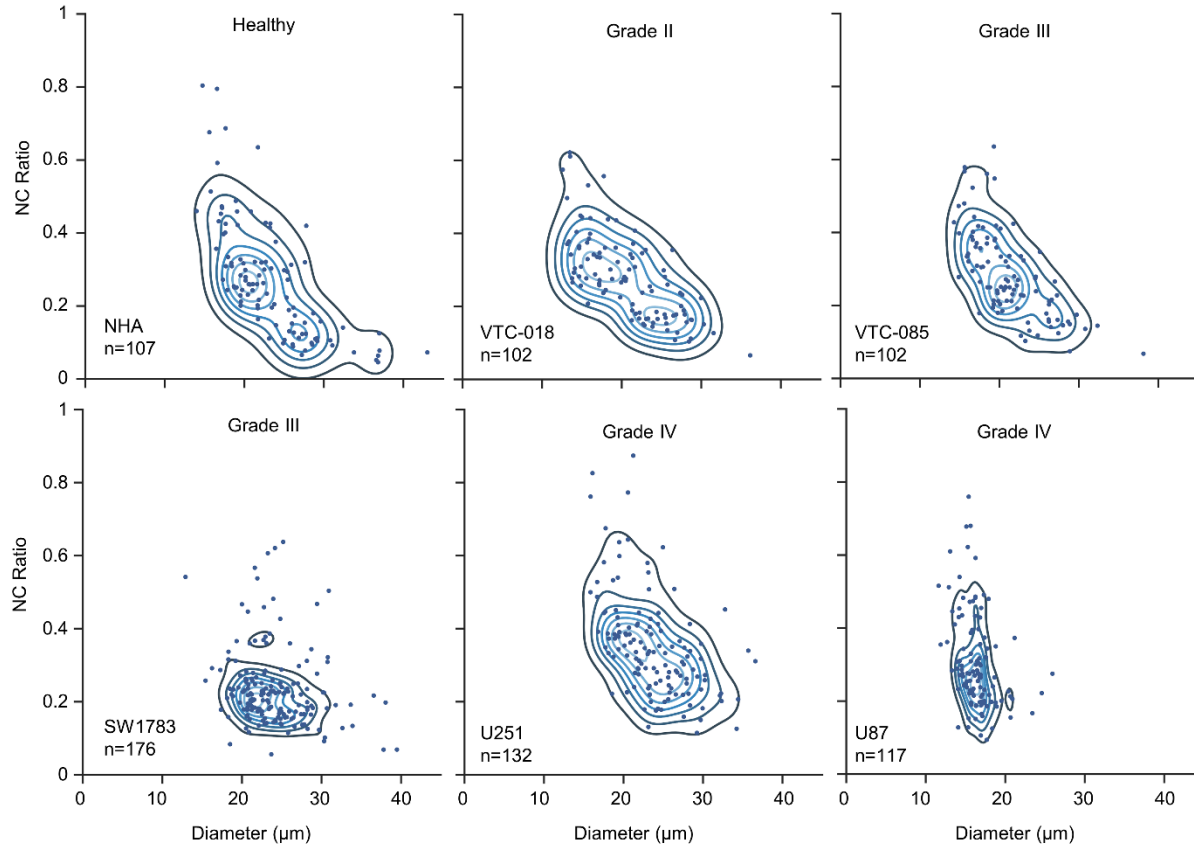


Figure 4.8 Nucleus to cytoplasm (NC) ratio for all cell types.

Diameters measured from cells approximated as spheres.

4.8.1 Calculation of nucleus-to-cytoplasm (NC) ratio.

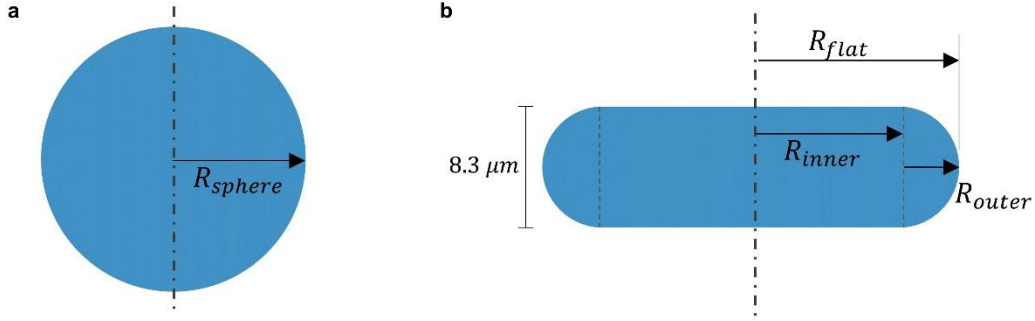


Figure 4.9 Cells shapes in suspension and within the PDMS device.

a) Spherical cell with average radius R_{sphere} . b) Flattened cell, such as when cells are approaching the constriction channel. Flattened height is given by the channel height at $8.3 \mu m$.

NC ratios were measured on newly-adherent cells that were approximated as spherical, with an average radius R_{sphere} . Taking the cells to be incompressible, we calculated the theoretical values of R_{flat} from measured values of R_{sphere} . Flattened cells, due to a channel height of $8.3 \mu m$, were assumed to have rounded edges with a radius, R_{outer} , equal to half the channel height ($4.15 \mu m$). Cell size measurements during constriction channel experiments were taken by tracing the perimeter of cell (top view) along the edge of the cell measured as R_{flat} . Volume of a spherical cell (V_{sphere}) was calculated as:

$$V_{sphere} = \frac{4}{3} \pi R_{sphere}^3$$

The volume of a flattened cell can be calculated by integration, and is given by:

$$V_{flat} = 2\pi \left(R_{flat}^2 R_{outer} - \frac{R_{outer}^3}{3} \right)$$

Assuming incompressibility and equating $V_{sphere} = V_{flat}$, we find that:

$$R_{flat} = \left(\frac{2R_{sphere}^3 + R_{outer}^3}{3R_{outer}} \right)^{\frac{1}{2}}$$

5 Chapter 5: A multiplexed microfluidic device to measure blood-brain barrier disruption by pulsed electric fields

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5.1 Abstract

Local modulation of the blood-brain barrier (BBB) by electroporation shows significant potential for treating neurological conditions. Optimal electroporation parameters, however, have not yet been identified. Due the large number of parameters such as pulse duration, magnitude, number, and frequency, high-throughput electroporation experiments are highly desirable to explore a large

a large parameter space. Microfluidic BBB models can provide low-cost, controlled experiments with human cells, but are generally limited by low-throughput per device. In this study, we developed a multiplexed BBB device that enables a single device to test a three-fold range of electric field magnitudes against an unexposed control group in a single experiment. A custom-designed tapered-channel creates in a linear gradient of electric field within the device, and an asymmetric branching channel enables placement of a control group within the same device. We validate our device by optically monitoring BBB permeability in real-time using the diffusion of a fluorescent marker across a monolayer of human cerebral microvascular endothelial cells (hCMECs) to determine the time-dependent permeability changes after applying high-frequency electrical pulses. We show that HFIRE pulses can transiently open the BBB. Unexpectedly, electrofusion of cells resulted in decreased permeability for some HFIRE conditions. Our device can enable multiple electroporation parameters to be investigated in a single experiment, which may help identify optimal electroporation parameters for opening the blood-brain barrier.

5.2 Introduction

Despite decades of research, many neurological diseases and disorders remain challenging to treat, in part because of inadequate drug penetration to the brain. For example, glioblastoma, an aggressive brain cancer, is almost universally fatal within 1-2 years.[392] Parkinson's disease and Alzheimer's disease, two common degenerative diseases, are rapidly increasing in prevalence worldwide with no cure.[393,394] Treatment of these conditions and other neurological diseases and disorders is hampered by the protective nature of the blood-brain barrier (BBB). The blood-brain barrier regulates transport between the blood and the brain tissue, a critical function

maintaining a neuro-protective microenvironment.[395] The protective nature of the BBB, however, becomes a severe blockade to effective therapy of neurological disease. Therapeutic drugs delivered intravenously often cannot penetrate the BBB and consequently fail to reach target areas in sufficient concentrations for satisfactory therapeutic effect. Alarming, nearly 100% of all large-molecule drugs and ~98% of small-molecule drugs cannot penetrate the BBB.[53] There is no question that timely development of new drug delivery mechanisms to overcome the BBB is needed.

The BBB is composed of specialized endothelial cells surrounded by supporting cells such as astrocytes and pericytes.[396] Cerebral endothelial cells express high levels of tight-junctions (TJ) proteins that seal-off transport between adjacent cells. Tight junctions are complex multi-protein structures that connect the actin cytoskeleton of adjacent endothelial cells and cause a profound restriction of the paracellular transport (transport between adjacent cells). Tight junctions restrict paracellular transport to small hydrophilic molecules. Transport across the endothelial cells, or transcellular transport, is restricted by specialized influx/efflux transport on their membranes and cytoplasmic enzymes that actively or passively regulate the transport of molecules. Passive transport across the cell membrane by diffusion is limited to small (< 400 Da) lipophilic molecules.[53] Unfortunately, nearly all large molecules, such as most chemotherapies, are prevented from entering the brain.

Recent *in vivo* and *in vitro* research has demonstrated that high-voltage pulsed electric field (PEFs) are able to transiently disrupt the BBB. . [42-46,63] PEFs disrupt cell membranes and enable unregulated transmembrane molecular diffusion through the creation of membrane pores, and

effect known as electroporation. In addition, PEFs can cause significant cytoskeletal disruption.[231] Electroporation-induced disruption is reversible if the disruption does not exceed the adaptive ability of a cell to recover. However, high voltages and high pulse numbers can lead to cell death, an outcome exploited for non-thermal cancer ablation techniques known as Irreversible electroporation (IRE) and High-frequency Irreversible Electroporation (HFIRE). Both IRE and HFIRE shows promise in animal studies for ablating brain tumors such as glioblastoma. In canine studies, H-FIRE treatment has achieved complete remission of glioblastoma multiforme.[397] IRE uses 100 μ s monopolar pulses, while H-FIRE replaces these 100 μ s pulses with a series of 1-10 μ s bi-polar pulses. Both IRE and HFIRE are safe for ablation near critical vasculature. HFIRE pulses offer several advantages over IRE such as minimization of muscle contractions, more uniform and predictable ablation sizes, and possibly greater selectivity toward cancer cells.[398] HFIRE treatment is a minimally invasive treatment typically administered using two parallel electrodes probes or a single bi-polar probe.

Electroporation techniques show significant potential for BBB disruption, however determination of optimal parameters for maximizing BBB disruption have not yet been identified. The large number of parameters such as pulse duration (ns-ms), magnitude (V-kV), number (1-thousands), and frequency (1-90 Hz) making determining optimal parameters difficult. HFIRE pulse waveforms add additional variables such as intra-pulse delay. Therefore high-throughput experiments are highly desirable to explore this large a large parameter space.

Microfluidic *in vitro* BBB models provide an excellent method for exploration of the vast parameter space of PEFs therapies. Although *in vivo* analysis of the BBB provides a

physiologically-relevant system, *in vivo* studies are limited by high costs (limiting animal numbers), labor-intensive experiments, confounding variables, and species-specific responses. Microfluidic *in vitro* models, however, can overcome these *in vivo* limitations to provide low-cost, high-throughput, well-controlled experiments using human cell types.

In a recent paper, we have demonstrated that electrical energy can increase the permeability of an endothelial monolayer.[399] Using a double-layer microfluidic device similar to the device of this study, we found that IRE pulses can increase the permeability of the BBB in a reversible or irreversible manner based on the electric field magnitude and pulse number. High magnitude 100 μ s pulses of 833V/cm caused irreversible electroporation for 90 pulses but primarily reversible electroporation for 10 pulses at the same magnitude. Electroporation at 833V/cm for 10 pulses caused the permeability of the BBB to reach a maximum of 1.5x baseline 15-20 minutes after pulsing, followed by return to near baseline permeability 50 minutes after treatment. Lower magnitude pulses of 333V/cm and 200V/cm also showed transitory BBB disruption, but of lower magnitude and for shorter durations. The observed increase in permeability due to these pulses was attributed to electroporation of the cell membrane, consistent with a previous study that quantified the uptake of fluorescent dyes by hCMECs after IRE pulses to determine cell death percentages and total FITC-dextran uptake by endothelial cells.[400] Furthermore, we found some evidence that short (10 μ s), low-magnitude (25 V/cm) pulses, typical of DBS waveforms, may also disrupt the BBB to increase permeability. It has been hypothesized that low-level electric fields may increase the permeability of the BBB by disrupting tight junctions. The results of several

studies, including the aforementioned *in vitro* study, hint at this low-level BBB disruption although conclusive results remain elusive, as does the mechanism of action.

Current microfluidic devices to model the BBB suffer from low-throughput. In nearly all cases, microfluidic BBB devices offer only one experimental condition per device. Since microfluidic device can require significant time and (possibly) costs to fabricate, improving the data collection for a single device is very desirable. Additionally, BBB *in vitro* experiments typically run several hours to many days, thus increasing the time and cost of studies if only a single experimental condition can be tested per device. Another limitation of most microfluidic BBB devices is the lack of a on-chip control. Although control device can be prepared separately, BBB experiments can be very sensitive to device-specific characteristics such as BBB integrity, which is difficult to control precisely between experiments. On-chip controls enable nearly all experimental parameters except those of study to be eliminated from experimental results. A few microfluidic multiplexed BBB devices have been proposed,[401] however no multiplexed devices have been designed for investigation with pulsed electric fields. This this study we seek to overcome these challenges with low-throughput of BBB devices by validating a multiplexed BBB device for investigating BBB disruption by pulsed electric fields across multiple electric field magnitudes simultaneously.

Our results demonstrate that our multiplexed device is able to measure the differing responses of the BBB to varying electric field magnitudes simultaneously, while tightly controlling for undesired experimental variability via an on-chip control. Using our device, we show that bipolar HFIRE pulses transiently increase BBB permeability in the first 30-60 minutes, and that BBB disruption is both field- and pulse-number- dependent. Unexpectedly we found that some pulsing

conditions actually reduced BBB permeability below pre-electroporation values following the transient increase in permeability.

5.3 Materials and Methods

5.3.1 Device Design and Fabrication

A simple geometry, illustrated in Figure 5.1Figure 1.1a, enables multiple electric field magnitudes to be tested in a single experiment. The microfluidic device is fabricated from two polydimethylsiloxane (PDMS) channel layers: an upper, tapered channel (red in Figure 5.1a) and a bottom, branching channel. The two channel layers are separated at their intersections by a polyester track-etched membrane having pores of 0.4 μ m in diameter (Figure 5.1b). Human cerebral microcapillary endothelial cells (hCMECs) are cultured within the tapered channel on the upper side of the permeable membrane to form a confluent monolayer. During an experiment, fluorescent sodium salt is introduced to the upper channel, and the diffusion of this tracer across the monolayer is measured in the bottom branching channels downstream of the intersection's points (Figure 5.1c). The double-layer BBB device is bonded to a glass slide, and located adjacent to a single-layer PDMS adaptor chip that functions as a bubble trap and adaptor. H-FIRE pulses are applied to the device via acupuncture electrodes, one located at the wide end of the tapered channel and another located between the branched channels of the bottom layer. Both the tapered channel and branching channel have specialized geometry to maximize the effectiveness of data collection.

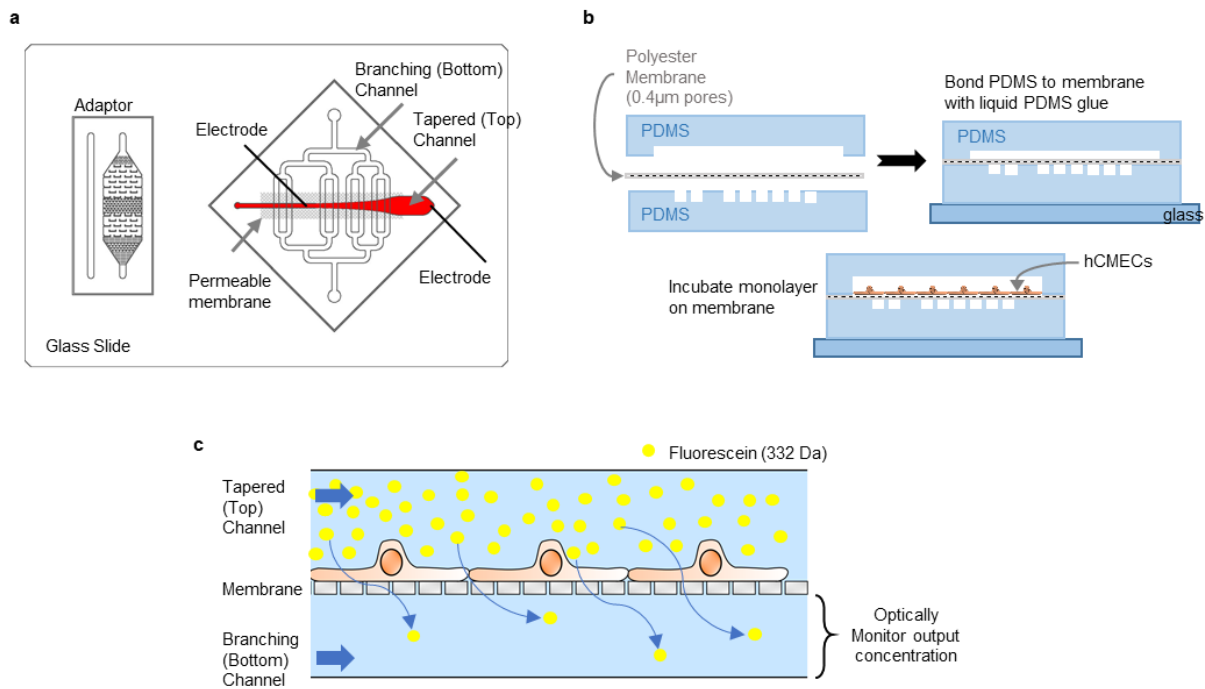


Figure 5.1 Schematic of the multiplexed double-layer BBB device.

The multiplexed BBB device. a) Schematic of the double layer device with a tapered channel (red) of varying cross-section to produce a linear gradient of electric field along the channel length. A branching channel enables six electric field magnitudes to be tested in a single experiment. The tapered channel is separated from a lower, branching channels by a permeable polyester membrane (gray). b) A transparent, permeable polyester membrane with 0.4µm pores is bonded between two PDMS layers using uncured PDMS. Human cerebral microvascular endothelial cells are cultured on the upper side of the membrane. c) Permeability across the monolayer is determined by diffusion of a fluorescein sodium salt from the upper channel to the lower channel.

Fabrication: This device was fabricated using established microfabrication techniques, which are briefly described here. Deep reactive ion etching (DRIE) was used to pattern silicon master molds, which were then salinized. Polydimethylsiloxane (PDMS) was cast molded onto the master molds, cured, and peeled off. The PDMS layer were cut out, and inlet and outlet holes were punched using a 0.75mm biopsy punch. After cleaning particulates off the surface by scotch tape and sonication in isopropanol alcohol, dry PDMS layers were placed under vacuum for at least 15 minutes prior to bonding. Bonding of the layers was achieved with a liquid PDMS glue. PDMS and toluene, mixed in a 1:1 w/w ratio, were spun onto a glass slide at 1600 RPM for 1 minute. Immediately, the PDMS layers were removed from vacuum, and placed face down on the glue. After a few minutes, or when the PDMS layers had absorbed the glue, the layers were carefully peeled off the glass slide and placed face up on a flat surface. Before bonding, a pre-cut polyester membrane was aligned on the bottom layer, and then layers were then carefully aligned and bonded. The devices were cured at room temperature for 24 hours. The cured devices and an adaptor/bubble trap PDMS layer were plasma bond side by side on the glass slide. Acupuncture needle electrodes were inserted into the channels, and fixed in place with epoxy.

Branching Channel: The bottom channel branches from a single inlet into eight channels, as shown in Figure 5.2a. The branched channels are 100 μm in depth, 0.8 mm wide, and 8 mm long. The branching design creates eight intersection points with the top channel, enabling eight independent measurements of BBB permeability. Two channels (rightmost channels in Figure 5.2a), are offset from the other channels such that a needle electrode can be inserted in the tapered channel between channels 2 and 3 of the bottom channel. When electrical pulses are administered

to the device, the endothelial monolayer above channels 1 and 2 remains unaffected because these channels are positioned outside electrodes, while channels 3-8 capture the effects of the electric field on the monolayer, since the monolayer is exposed to the electric fields above these channels. The control channels allow the effects of electrical pulses to be distinguished from other miscellaneous effects such as flow rate inconsistencies, temperature changes, or changes in endothelial behavior due to experimental conditions (fluid flow, media, fluorescent dye, etc). Furthermore, the control channels serve as a baseline for analysis of viability following H-FIRE treatments.

The offset of the two “control” channels from the six “exposed” channels creates an asymmetric branching pattern that causes flow rates between the eight channels to differ due to differing hydraulic resistance between the channels. To correct for this difference, the hydraulic resistance of the branching network was equalized by adjusting the position of the inlet branching point. (see SI for hydraulic resistance network).

Tapered Channel: The top channel has a constant-width of 0.7 mm at its intersections with channels 1 and 2, and a custom tapered region (expanding from 0.7 mm to 3.0 mm) at its intersections with channels 3-8. The tapered section of the channel has a specific profile to produce a linear gradient of electric field, thereby enabling six electric field magnitudes to be tested over a three-fold range in a single experiment. To determine the taper shape to produce a linear gradient of electric field, we used Ohm’s Law to relate the electric field to the current density and thus the cross-sectional area in the following way,

$$E(x) = \frac{1}{\sigma} J(x) \sim \frac{1}{\sigma A(x)} \quad (1)$$

where $E(x)$ is the electric field, σ is the conductivity of the medium, $J(x)$ is the current density, and $A(x)$ is the cross-sectional area of the channel.[400] Since PDMS is an excellent insulator, current can be assumed to travel only through the channel. Furthermore, low-conductivity media is flowed through the branching channels during experiments, thus maintaining the electrical current primarily within the tapered channel. Therefore, by controlling the cross-sectional area of the tapered channel (via variable width), we altered the electric field magnitude. The electric field inversely varies with channel width; thus, a three-fold reduction in channel width will produce a three-fold increase in the electric field magnitude. To find the profile for a 10 mm long tapered region of width of 0.75 mm to 2.25 mm, we solved the following equations for a and C :

$$w(x) = \frac{C}{x} \quad (2)$$

$$\begin{cases} w(a) = 0.375 \\ w(a + 10) = 1.5 \end{cases}$$

Where a and C are unknown constants, and $w(x)$ is the width of the channel. We used the calculated values of where a and C to determine the device taper. The tapered channel height is 250 μm .

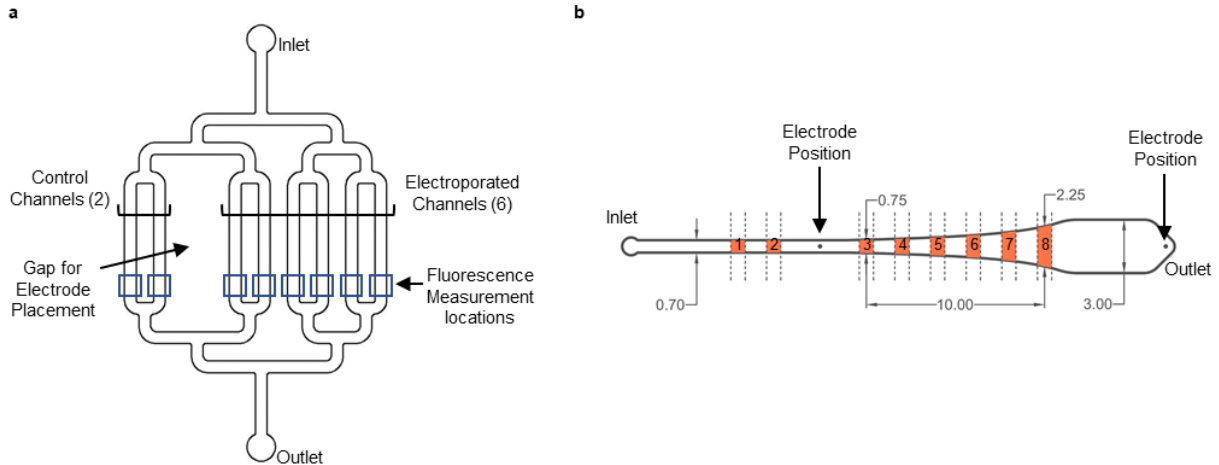


Figure 5.2 The branching channel and tapered channel geometry.

a) The branching channel includes two control channels separated from six test channels by a gap that enables electrode placement. A slight offset of inlet and outlet channels equalizes the hydraulic resistances between the channels. Fluorescent measurements are taken in each channel downstream of the intersections points with the tapered channel. b) The tapered channel has a width of 0.7 mm at the the control channels, but increases in width up to 3.0 mm due to a custom taper that creates a linear gradient of electric field. The three-fold increase in width between channels 3 and 8 enables a three-fold change in electric field magnitudes to be tested.

5.3.2 Permeability measurement in the device

Permeability is measured in real-time via the diffusion of fluorescent sodium salt (367 Da) across the endothelial monolayer from the top channel to the bottom channels (Figure 5.1c). During experiments, the fluorescent intensity of the eight lower channels were measured every 30 seconds by imaging at four locations downstream of the intersection's points. Due to laminar flow in the

device, the fluorescence intensity of all channels to be monitored simultaneously at the confluence of the branching channels. Permeability of the system (membrane and monolayer) can be described:

$$P = \frac{1}{AC_0} \frac{\partial Q}{\partial t} \quad (3)$$

where P is the permeability [cm/s], A is the intersection point area [cm²], C_0 is the concentration of sodium salt in the top channel [μg/cm³], and $\partial Q/\partial t$ is the rate of flux across the intersection [μg/s]. Because the bottom channel continually discharges the diffused sodium salt away from the intersection point due to the constant flow, the concentration of sodium salt in the bottom layer can be assumed to constant. Thus,

$$\frac{\partial Q}{\partial t} = CU \quad (4)$$

Where C is the concentration of sodium salt in the bottom channels, and U is the flow rate. This enables the simplification:

$$P = \frac{U}{A} \left(\frac{C}{C_0} \right) \quad (5)$$

Since fluorescence intensity is linearly correlated with channel height, exposure time, and concentration [400], it is possible to relate concentration to fluorescence intensity:

$$P = \frac{U}{A} \left(\frac{I_F}{I_{F0}} \right) \quad (6)$$

Where I_F is the fluorescence intensity measured at the outlet, and I_{F_0} is the baseline intensity as measured in the adaptor channel. Finally, when calculating the permeability of the monolayer, the permeability of the polyester membrane must be accounted for by the following expression:

$$\frac{1}{P_c} = \frac{1}{P_t} - \frac{1}{P_m} \quad (7)$$

where P_c is the overall permeability, and P_t and P_m are the membrane and monolayer permeabilities respectively.

5.3.3 H-FIRE parameters

To validate the usefulness of this device, we treated the monolayer with HFIRE pulses and measured the permeability of the monolayer following treatment. Table 5-1a,b present the HFIRE parameters tested. HFIRE pulses were delivered by a custom pulse generator as bursts of bipolar pulses: 2 μ s positive polarity pulses were followed by a 5 μ delay and a 2 μ s negative polarity pulse (2-5-2 μ s waveform) (Figure 5.3a). Actual waveforms closely approximated ideal square waves (Figure 5.3b). The 2-5-2 μ s bipolar pulses were applied in series 25 times to achieve 100 μ s total pulse time to make complete ‘pulse’. Pulses were delivered at 1 Hz, and pulse number was varied from 10 to 900.

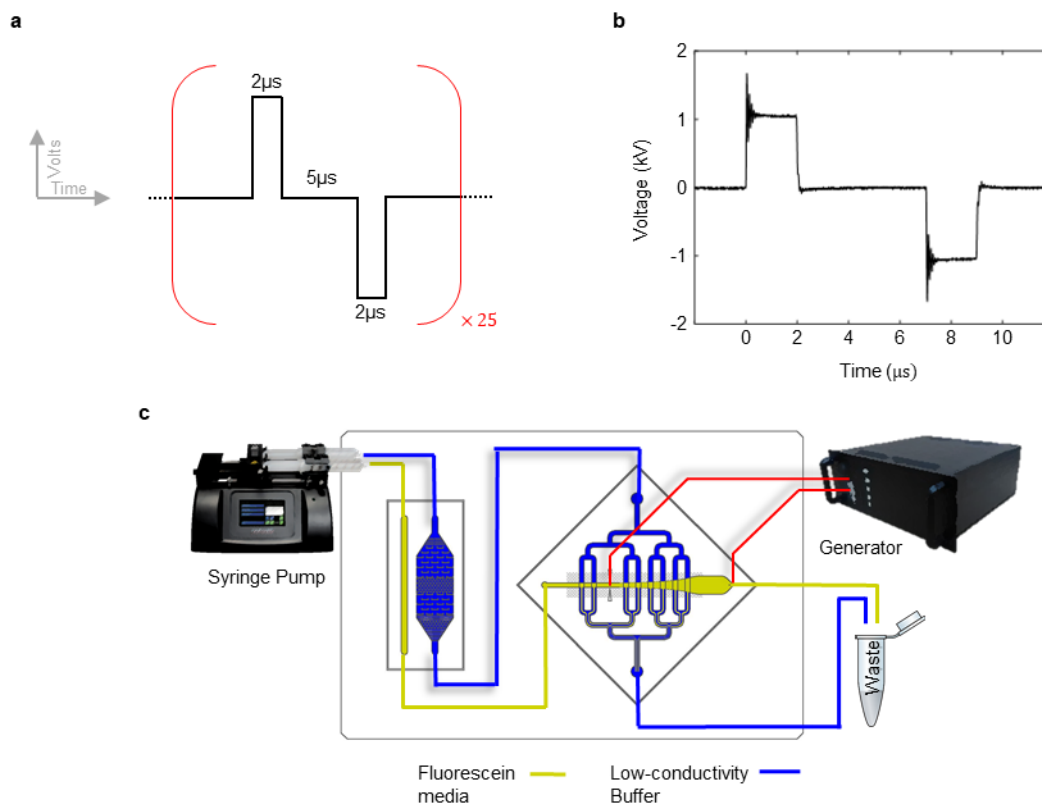


Figure 5.3 H-FIRE pulses and experimental setup.

a) H-FIRE waveforms consist of bipolar pulses of 2 μs pulses of opposite polarity separated by a short intrapulse delay of 5 μs . b) Actual voltage waveforms approximate ideal square waves. c) Constant fluid flow is delivered via a dual syringe pump. Fluid flows into a bubble trap before entering the microfluidic device. A high-voltage pulse generator supplies the HFIRE pulses via needle electrodes within the device. The microfluidic device is enclosed in an on-stage incubation chamber for testing.

Prior to administering the H-FIRE pulses, permeability was monitored for 30 minutes before pulsing to collect a baseline measurement. Experimental results were analyzed using Image-J. After the pulse administration, the permeability was monitored for 1.5 hours to capture permeabilization and recovery of the BBB.

Table 5-1 H-FIRE Test Conditions

Voltage Applied (V)	Pulse Number	Electric Field at each channel (V/cm)							
		Ch1	Ch2	Ch3	Ch4	Ch5	Ch6	Ch7	Ch8
1034	10, 100, 900	0	0	900	780	660	540	420	300
3102	20, 200	0	0	2700	2340	1980	1620	1260	900

5.3.4 Experimental set-up

Experiments were performed on an inverted microscope (Leica). Figure 5.4c shows the experimental setup. Fluid flow in the upper and lower channels was established by two 5ml syringes, both supplying a flow rates of 10 ul/min. The syringe feeding the tapered channel contained EndoGRO-MV complete media with 167 ug/ml of Fluorescein sodium salt. The syringe feeding the bottom channel was filled with low-conductivity buffer composed of 8.5% sucrose [w/v], 0.3% glucose [w/v], and 0.725% RPMI [v/v]) modified with 0.1% of BSA [w/v] and 0.1% of Kolliphor P188 [w/v], and 0.1 mM EDTA. Syringes were connected to the PDMS devices with leur-lock tips and #30 gauge plastic tubing. Prior to connecting to the BBB device, both syringes connected to an adapter channel. The syringe feeding the tapered channel was connected to a straight adaptor channel that was used to determine the fluorescence intensity of the media for the

given microscope parameters. The syringe feeding the branching channel was connected to a bubble trap to prevent any bubbles from entering the BBB device.

5.3.5 Cell culture

Human cerebral microcapillary endothelial cells (hCMECs, EDM Millipore, Billerica, MA) were cultured using standard cell culture procedures. hCMECs were cultured at 37 °C in flasks and routinely passaged at 90% confluence. Cell flasks were incubated with rat-tail collagen 1 coating solution for 1 hour and washed with PBS prior to cell seeding. Cells were cultured in hCMEC EndoGRO-MV media (Sigma Millipore) comprised of EndoGRO basal medium supplemented with EndoGRO-LS supplement (0.2%), rh EGF (5 ng ml⁻¹), L-glutamine (10 mM), hydrocortisone hemisuccinate (1 µg ml⁻¹), heparin sulfate (0.75 U ml⁻¹), ascorbic acid (50 µg ml⁻¹) and FBS (5%) all from Simga Millipore.

We seeded hCMEC cells in the double layer chips in a procedure similar to that described in previous studies.[399,400] First, the double layer chips were sterilized with 70% ethanol, washed with PBS, and filled with 50 µg ml⁻¹ human fibronectin (Life Technologies) in PBS and incubated for 1 hour at 37 °C. After 1 hour of incubation, the PBS was replaced with complete media and incubated for an additional hour. hCMECs were the seeded at a density of 10 million cells per ml. Following 1-2 hours of incubation, pipette tips loaded with complete media were inserted into the inlets and outlets to supply nutrients during incubation in static condition.

Confluent monolayers typically formed after 2-4 days. To sustain cells during long experiments, a custom-made stage-top incubator was used to incubate cells during experiments. The incubator

was connected to a 5% CO₂ tank (Airgas, Radnor, PA) and heated with a built-in ITO electrical heater.

5.3.6 Live-dead analysis

We performed live-dead staining using Calcein-AM and Propidium Iodide (PI). Calcein-AM (4ug/ml) and PI (50ug/ml) were added to culture media and incubated within the device for 15 minutes. Devices were then washed with cell culture media and imaged.

5.4 Results and Discussion

5.4.1 Fluid Flow Modelling

To verify that the asymmetric branching channel had equal flow rates, as predicted by the hydraulic resistance calculations, we modeled the fluid flow in branching channel. Our simulation results showed that the slight offset of the inlet established uniform flow rates between all channels (< 1 % flow rate differences).

Finite element simulations of the fluid flow demonstrate that despite the asymmetric branching, the hydraulic resistance of all channels was equalized to establish a uniform flow rate between all eight channels, illustrated in Figure 5.5a. Simulation results indicate that the flow rate differs 0.1% or less between the parallel channels. This small degree of difference is trivial, and may arise from the fillets, or be an artifact of limited computational resolution. The equalized flow rates of this study is a significant improvement to a previously used design.[399]

5.4.2 Electric Field Modelling

To verify that the channel design generated a linear profile, we performed finite element simulations with COMSOL Multiphysics. Figure 5.4c shows the electric field magnitude for each of the channel intersection points. Finite element modelling of the electric field in the tapered channel indicated that a linear gradient of electric field magnitude was established within the tapered region. Furthermore, Figure 5.4c demonstrates that the electric field magnitude is fairly consistent across the width of the tapered channel. Over the 6 intersection points, the electric field magnitude was verified to differ by a factor of three.

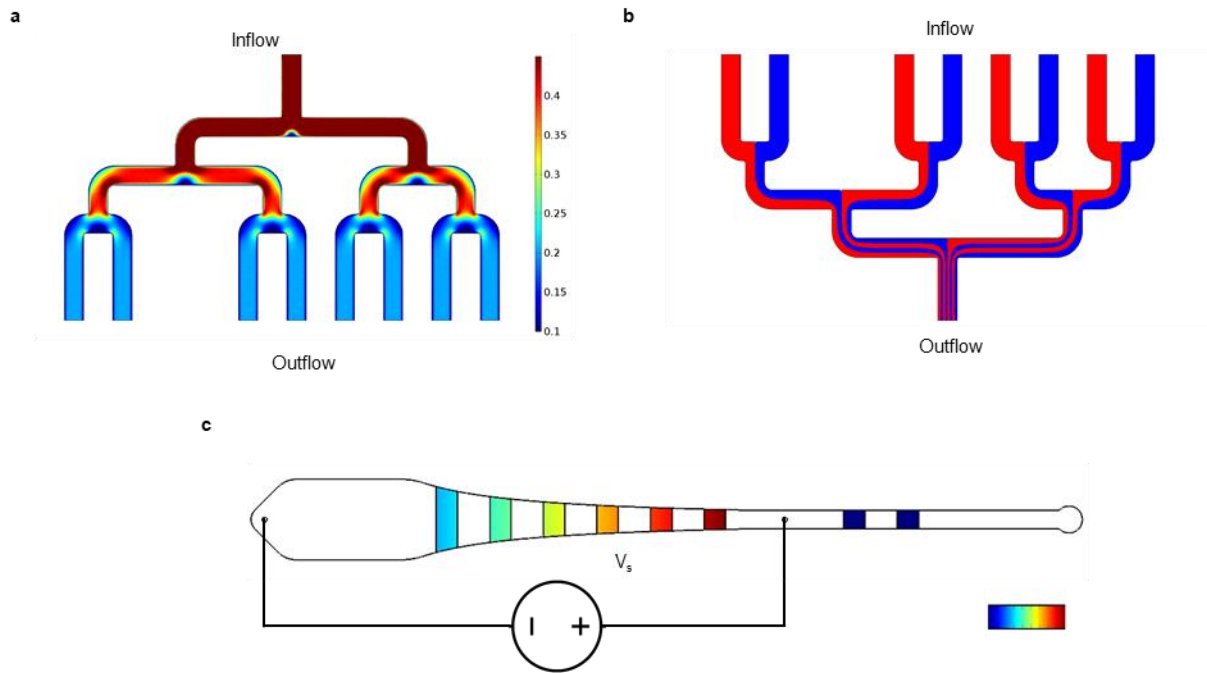


Figure 5.4 Finite element simulations of fluid flow and electric field magnitude.

a) Fluid flow within the asymmetric branching channel is constant between all channels. Flow rates differ less than 0.1%. b) Fluid flow streamlines show flow paths of the channels. c) Electric field magnitude within the tapered channel. The tapered channel creates a linear gradient of electric field, with magnitudes 3-fold higher in the narrowest tapered-channel compared to the widest channel. control channels lie outside the electrodes and do not experience electric fields.

5.4.3 Reduction of experimental variability by on-chip control channels

Permeability measurements in our experiments are very sensitive, and thus small variation in

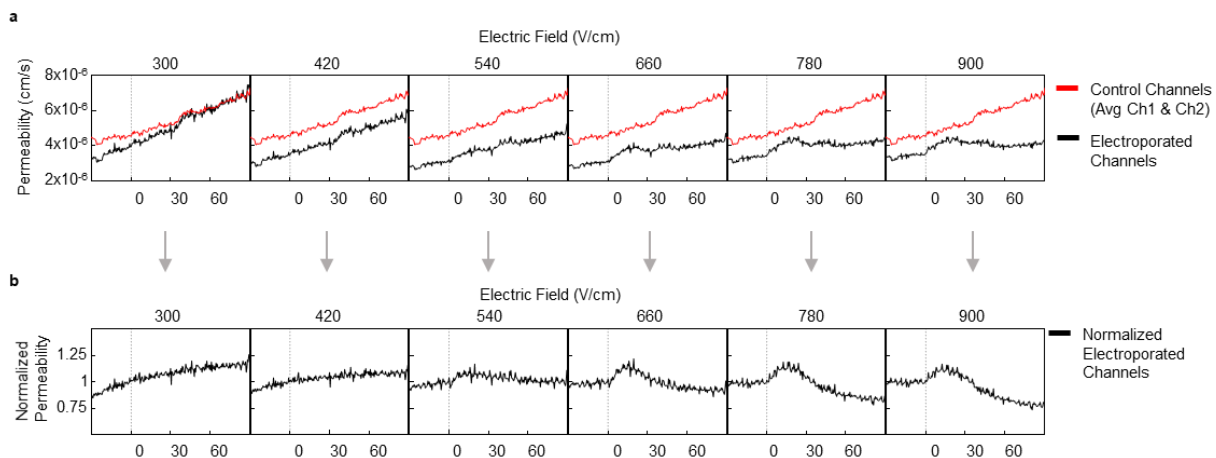


Figure 5.5 On-chip control channels reduce miscellaneous effects

a) Representative data for an electroporation experiment (300-900 V/cm, 100 pulses) Raw permeability values of the control and test channels show value show increasing permeability during the experiment with some random variations. b) Normalizing the electroporated channels by the control channels significant reduce the miscellaneous effects so the electroporation effects become more clear.

experimental conditions can introduce unwanted noise in the data. For example, our measurements are extremely sensitive to flow rate changes. Even a small change in the flow rate can distort permeability measurements. We minimized flow rate changes using 5 ml gas-tight glass syringes (Hamilton Company) and a precise syringe pump (Harvard Apparatus PHD Ultra). Small changes

in flow rate still occurred, and thus monitoring the control channel permeability enabled us to eliminate this unwanted noise. Furthermore, we noticed that endothelial permeability generally increased during our experiments. We believe this is primarily due to the use of low-conductivity buffer in the branching channels, as endothelial cells are quite sensitive to their media. The increasing permeability during experiments made resolving just the changes in permeability due to electroporation difficult.

Using the permeabilities measured in the control channels, we normalized the permeability data from the six test channels, as shown in Figure 5.5. As this figure demonstrates, the normalized data significantly reduces noise. Furthermore, at the 900 V/cm condition, the raw permeability data appears to show that the permeability of the monolayer remains elevated for the duration of the experiment after electroporation, however when normalized by the control channels, we actually find that after a brief period of increased permeability, monolayer permeability unexpectedly decreases below baseline. As shown by the 300 V/cm and 420 V/cm data, normalization does not completely correct the upward-trending permeability pre-electroporation for the lowest-electric field channels. We believe that this is due to differences between the width of the control channels (0.7 mm wide) and the lowest-electric field channels (2.25 mm wide), which may cause cells in these differing widths to behave slightly differently (for instance, due to the differences in fluid shear stress).

5.4.4 BBB disruption after HFIRE

First, we applied 1034 V to the devices to investigate cell response at 300, 420, 540, 660, 780, and 900 V/cm (Figure 5.5a). We investigated these electric field magnitudes for 10, 100, 900 180

HFIRE pulses. Our results showed that BBB disruption was electric field magnitude and pulse number

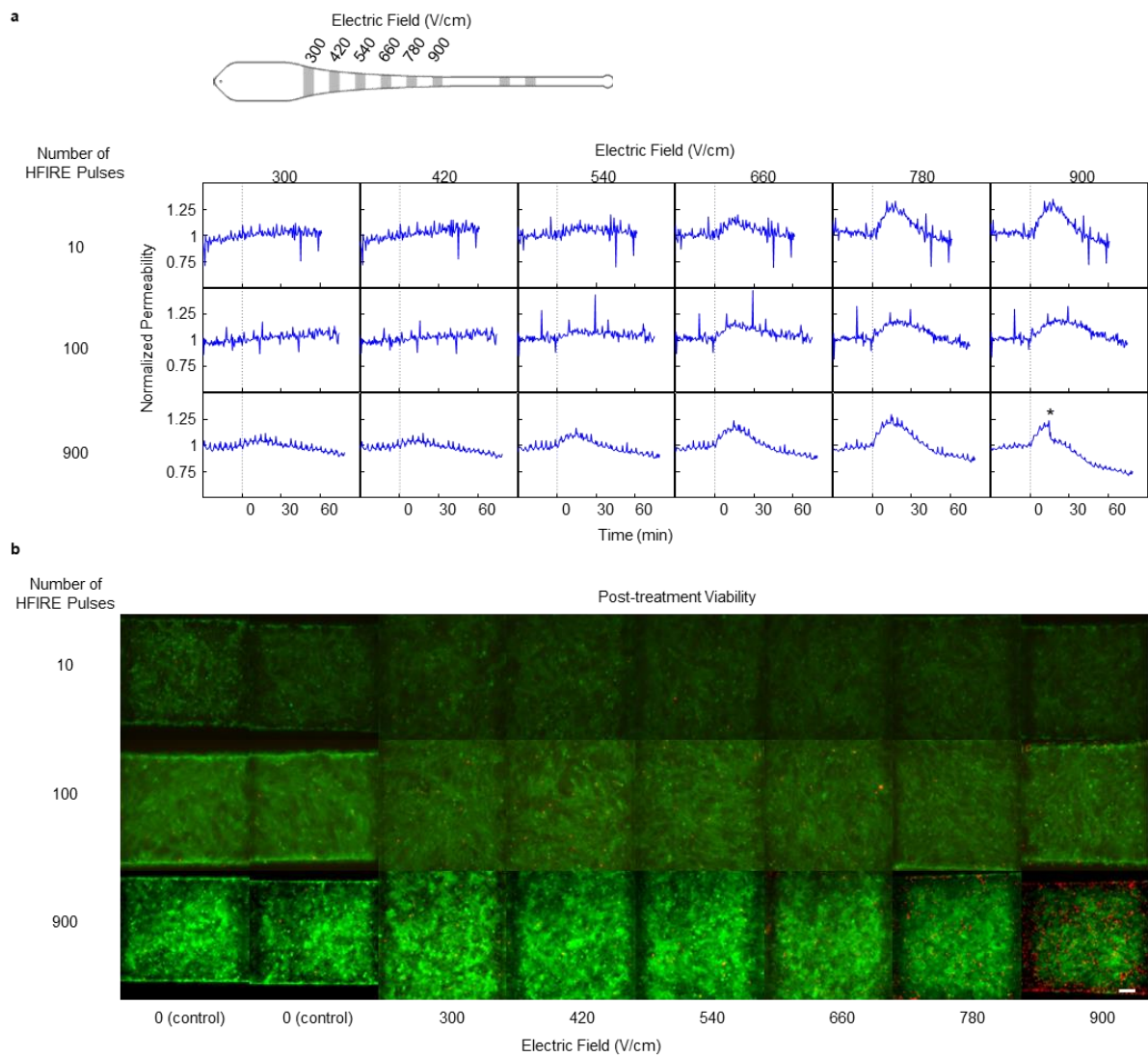


Figure 5.6 Permeability after HFIRE at 300-900 V/cm

a) Normalized permeability for HFIRE treatments with 10, 100, and 900 pulses from 300-900 V/cm. Higher voltages (660-900 V/cm) show transiently increased permeability after HFIRE. Each row of plots represents a single experiment. b) For all pulse numbers, HFIRE between 300-900 V/cm resulted in very high cell viability post-treatment.

dependent. For 10 pulses, BBB disruption was minimal at 540 V/cm, moderate at 660 V/cm, and significant at 780 and 900 V/cm. Maximal disruption was reached approximately 15 minutes after electroporation, with permeabilities returning to near baseline by about 40 minutes. For 900 pulses, all tested voltages show some BBB disruption. Electric fields of 300 and 420 V/cm produced minimal BBB disruption. Higher voltages, however, produced significant BBB disruption and unexpectedly had approximately the same magnitude and duration as for 10 pulses. The asterix in the 900 V and 900 pulse plot indicates where a bubble within the channel rapidly altered the flow rate, and thus introduced an undesired downward shift in the data.

We performed live-dead imaging to determine if cell viability after treatment. For treatments of 10 and 100 HFIRE pulses at 300-900 V/cm high viability was found for all conditions. This finding agrees with our permeability finds, which demonstrated that permeability returned to baseline values after treatment. Likewise, cells showed high viability after 900 HFIRE pulses between 300-600 V/cm, but showed noticeable cell death for 780 and 900 V/cm treatments. Interestingly, our permeability results for this condition indicate that the monolayer still regains near baseline permeability values despite the cell death that occurs at these electric fields.

To increase cell death, we then applied 3102 V to the device to create electric fields of 900, 1260, 1620, 1980, 2340, and 2700 V/cm within the device, and investigate treatments of 20 and 200

HFIRE pulses. For 200 Pulses, we found significant cell death for electric fields greater than 900 V/cm (Figure 5.6b). At 1620 V/cm and above, 200 HFIRE pulses resulted in essentially 100% cell death. Corresponding to this complete loss of cell viability, for these conditions our permeability data show that after electroporation, cells never regain their pre-electroporation values, but instead show a two-to-three-fold increase permanent increase in permeability. For 200 pulses at 900 and 1260 V/cm, however, we find a significant increase in membrane permeability during the first 60

minutes after electroporation. Despite significant cell death at 1260 V/cm membrane permeability returns to near baseline values.

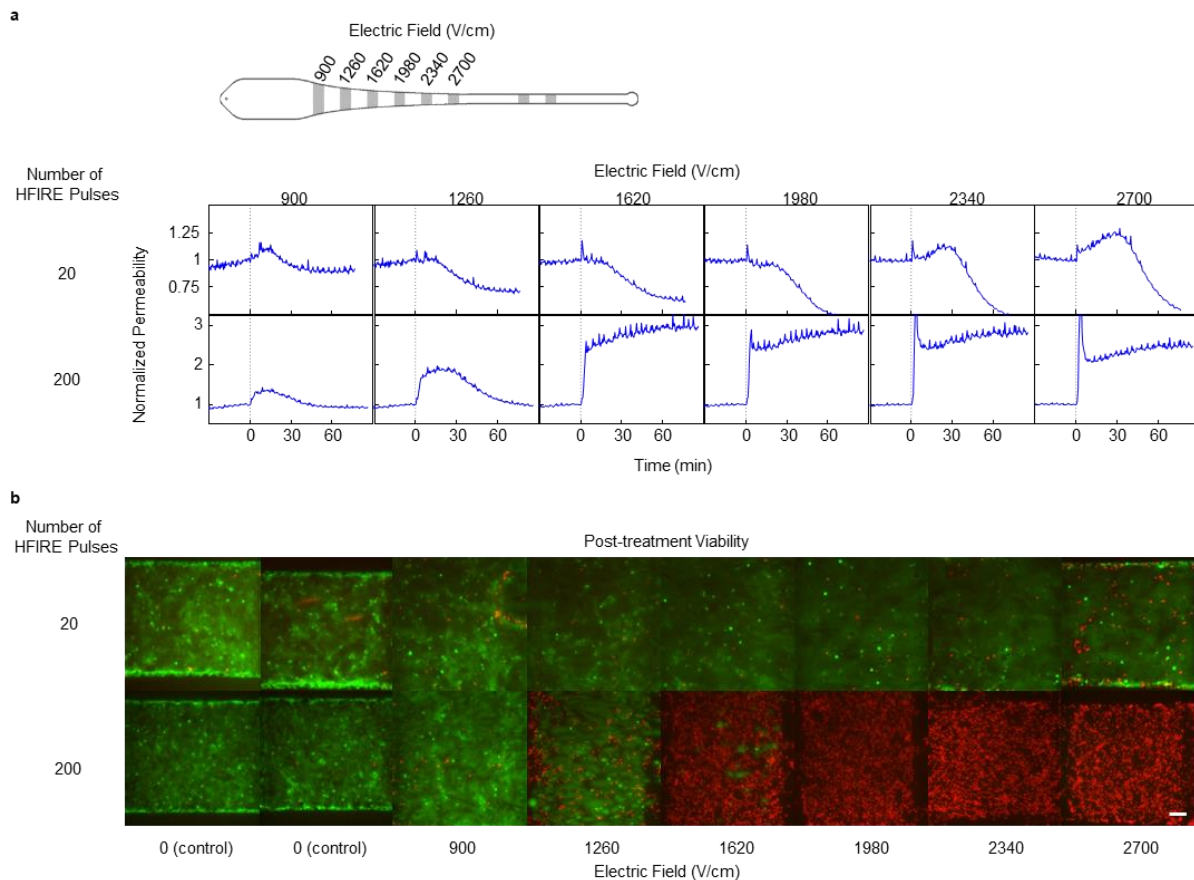


Figure 5.7 Permeability after HFIRE at 900-2700 V/cm

a) Normalized permeability for HFIRE treatments with 20 and 200 pulses from 900-2700 V/cm. Twenty HFIRE pulses from 900-2700 V/cm increase BBB permeability, but subsequently cause permeability to decrease substantially. Two hundred HFIRE pulses results in an irreversible increase in permeability for 1620 V/cm and greater, however reversible permeabilization for 900-1260 V/cm. b) Twenty HFIRE pulses do not induce significant cell death after 2 hours, however cells show significant electrofusion. Two hundred HFIRE pulses cause almost 100% decrease in cell viability for 1620 V/cm and greater.

Treatment with 20 pulses at these electric fields resulted in unexpected permeability trends. Electroporation with 20 pulses at 900 V/cm yielded a transitory increase in monolayer permeability followed by a return to baseline values. Higher voltages from 1260 to 1980 V/cm, however, resulted in a minimal increase in permeability followed by a significant decrease in permeability. This is unexpected as electroporation is known to disrupt cell membranes, with disruption increasing with increasing voltage. Likewise, at 2340 and 2700 V/cm a period of increased membrane permeability is followed by a period of significantly decreasing permeability. For the 20 pulse conditions, our live-dead stain indicates that most cells had recovered cell membranes, as indicated by the presence of the green stain in Figure 5.6a. Likewise, only a few scattered cells

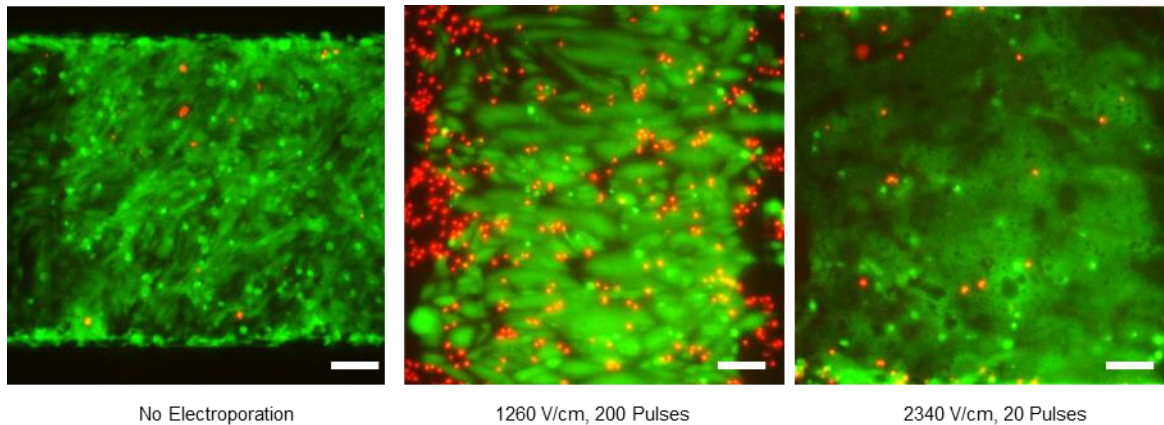


Figure 5.8 Electrofusion due to HFIRE.

While unexposed cells show distinct cell boundaries, HFIRE pulses delivered at higher voltage causes significant electrofusion, as indicated by large multi-cell bodies.

showed uptake of PI, which would indicate compromised cell membranes.

A closer look at the morphology of electroporation cells revealed that high voltages resulted in significant amounts of electrofusion (Figure 5.7). Adjacent cells, while appearing distinct from each other in the control channels, appears to be fused together to create large, multicellular bodies after electroporation with high voltages. Figure 5.7 compares the morphology of untreated endothelial cells with cells treated at 1260 V/cm with 200 pulses and 2700 V/cm with 20 pulses. For both conditions, we find that significant changes in morphology have occurred, indicated of cell electrofusion. Remarkably, for 20 pulses at 2700 V/cm, it appears that nearly all cell membranes have been fused together across the entire monolayer.

5.4.5 Discussion

Our results demonstrate that our multiplexed microfluidic BBB device is able to simultaneously deliver electric pulses over a three-fold range of magnitudes, and measure membrane permeability of the treated endothelial monolayer in real-time. A custom branching design with electrode placement between the branches established enables unexposed control channels while maintain uniform flow rates for ease of analysis. On-chip control channels enabled a significant reduction of undesired effects from electroporation effects. Our study uses bi-polar HFIRE pulses to electroporation the cell membrane, and is the first study BBB disruption with these fields.

Our permeability data, acquired for electric fields between 300 and 2700 V/ cm and for 10 to 900 HFIRE pulses, reveals electric field and pulse number dependent trends. Reversible electroporation due to lower fields (300-900 V/cm) leads to a transitory increase in monolayer permeability in the first 30 to 60 minutes after treatment. Followed by a recover of pre-pulse permeability. At very low electric fields (300 V/cm) increase in permeability are only apparent for

high pulse numbers (900 pulses). Fields less than 900 V/cm resulted in very higher cell viability post-electroporation. Interestingly, reversible electroporation achieved with higher electric fields resulted in a transitory increase in permeability that was followed a decrease in permeability significantly below pre-electroporation levels. The decrease in permeability is likely due to significant levels of electrofusion between adjacent cells, thus limiting paracellular transport of the fluorescent marker. HFIRE pulses that induced cell death showed irreversible increases in membrane permeability after treatment.

A significant amount of electrofusion was observed at high electric fields, and future studies should be conducted to determine if electrofusion occurs in vivo for these pulsing conditions. Electrofusion is well-known to occur between adjacent cells, and thus electrofusion events may also occur in vivo. Endothelial cells in vivo, however, form three-dimensional vessels with complex geometries that are not replicated by our simple 2D endothelial monolayer. Furthermore, our device applies the electric field parallel to the endothelial monolayer, which may increase electrofusion events beyond what would be physiologically relevant in a 3D geometry where the field is not always parallel with the endothelial cells. If electrofusion does not occur in vivo, simple 2D monolayer devices may be insufficient for investigating physiologically-relevant BBB disruption by pulsed electric fields. Alternatively, future devices could be constructed to apply electric fields across the cell monolayer, which may yield different results.

The normalization of permeability data by the control channels was quite successful at eliminating unwanted noise. A significant source of error in these experiments was subtle changes in flow rate of the bottom channel. Even the nucleation of bubbles inside the bubble trap could cause small

changes in flow rate. Endothelial permeability changes due to experimental conditions also were well eliminated by controls, normalized by the control channels. Normalization of the two widest channels (lowest field channels) resulted in a slight upward-trending normalized permeability that was most pronounced at the beginning of the experiments. It is unclear what caused the normalized permeability to increase in widest channels (ch7 & 8). The impact of BBB adaptation to the application of shear stress may contribute to this trend. Many studies have found that under physiologically relevant shear stress ($\sim 5\text{-}10$ dynes/cm²) that BBB permeability decreases. Thus, when normalized to the narrow control channels (highest shear stress), the widest channels (lowest shear stress) may show an increasing trend due to BBB tightening in the narrow channels. However, it seems unlikely that this is the only factor, because at the experimental flow rates, the maximum shear stress in the channel is ~ 0.25 dynes/cm², at least an order of magnitude lower than physiological values. Furthermore, experiments using only one-tenth the flow rate had showed the same trends. One possibility is that the monolayer behaves slightly differently along the edges of the channel in the center of the channel. Because all of the channels have different intersection areas-to-edges values, this may contribute to some of the normalization challenges in this experiment.

Our device measured the actual permeability of an hCMEC monolayer to fluorescein sodium salt (376 Da). With the current device and experimental setup, our experiments are limited to 2-3 hours. It is therefore not possible, to investigate how electroporation impacts monolayer permeability beyond this timeframe. The maximum duration of experiments is determined by syringe volume and the endothelial cells tolerance of the low conductivity buffer solution. For longer experiments,

electrodes could be integrated below and above the monolayer for electrical impedance spectroscopy (EIS). EIS uses electrical resistance measures to determine the monolayer integrity, and would be preferable for longer-duration experiments.

5.4.6 Conclusions

Our microfluidic device can increase the efficiency of traditional microfluidic BBB experiments, by providing six measurements of BBB permeability simultaneously. Furthermore, an on-chip control enables comparison with treated monolayers, and can be used to remove unwanted noise by normalizing the data. We measured the permeability of an endothelial monolayer in real-time before, during, and after treatment by H-FIRE electric field magnitudes 300-2700 V/cm and pulse numbers 10-900. We show that BBB permeability can be increased by HFIRE pulses reversibly or irreversibly. Some pulsing conditions lead to significant electrofusion and a subsequent decrease in permeability after treatment. Our design could be used to better understand BBB response to a variety of electroporation parameters (pulse lengths, pulse numbers, pulse frequency, etc). Understanding how pulse parameters effect BBB disruption will be important for actuate treatment planning for electroporation procedures of the brain.

5.5 Supplemental Tables and Figures

Table 5-2 Table of intersection point area and electric field magnitudes relative to a 900V/cm field in channel 3.

<i>Channel</i>	<i>Intersection area (mm²)</i>	<i>Normalized Intersection Area</i>	<i>Average EF</i>	<i>STD EF</i>	<i>Max EF</i>	<i>Min</i>
1	0.56	1	--	--	--	--
2	0.56	1	--	--	--	--
3	0.6001	1.0716	900	13.8526	924.4394	876.1165
4	0.6925	1.2366	780.0016	13.85035	804.4553	756.0338
5	0.8185	1.4616	660.0346	13.84447	684.8285	636.0836
6	1.0007	1.7870	540.1518	13.82943	565.566	516.068
7	1.2871	2.2984	420.5272	13.7903	446.359	396.0633
8	1.8039	3.2213	301.7835	13.65235	330.3015	277.4709

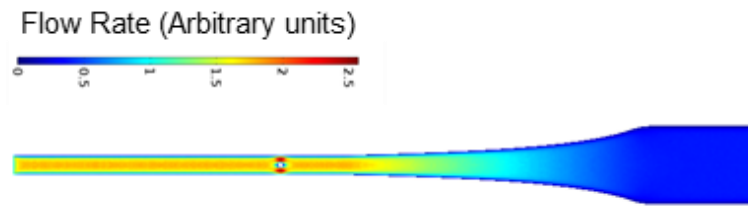


Figure 5.9 Fluid flow within the tapered channel.

Due to the variable width, the fluid flow rate varies along the channel length. COMSOL simulations of the flow profile in the tapered channel reveal that the flow is only disrupted locally at the mid-channel electrode insertion point, and does not impact the flow conditions over the intersection points.

5.5.1 Hydraulic resistance network Calculations

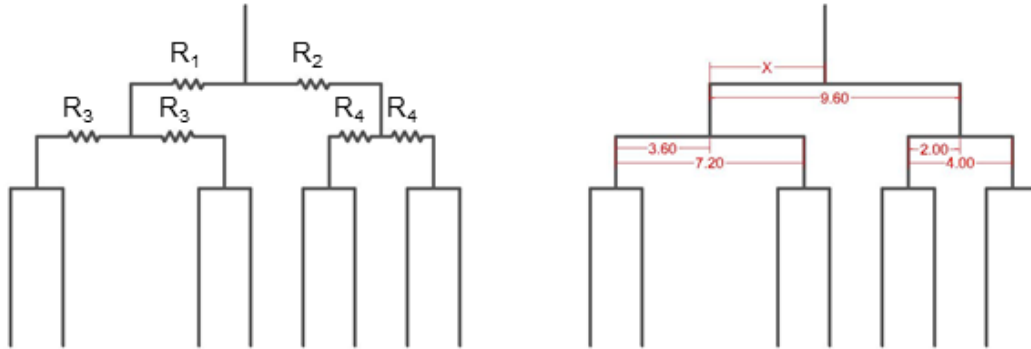


Figure 5.10 A simplified schematic of the branching channel using a hydraulic resistance analogy.

(a) Image of the branching geometry as an electrical resistance network. (b) Dimension of the branching channel (in mm) with the unknown length, X .

To equalize the flow rate of the eight branch channels, we first construct an equivalent hydraulic resistance circuit, shown in Figure 5.9a. Due to symmetry, we simplify the analysis by considering only one half of the symmetric branch geometry, and ignoring fillets.

For laminar flow, the pressure drop ΔP across a channel can be related to the flow rate Q and the hydraulic resistance R_{Hyd} by $\Delta P = QR_{Hyd}$. This relationship is equivalent to Ohm's Law for electricity: $V = IR$ where V is the voltage drop, I is the current, and R is the electrical resistance. We can quickly see that when the hydraulic resistance is equalized between channels, we will achieve equal flow rates for a given pressure drop.

For a rectangular channel, the hydraulic resistance is described by:

$$R_{Hyd} = \frac{12\eta L}{h^3 w (1 - 0.63(h/w))}$$

where η is the dynamic viscosity, h is the channel height, w is the channel width, and L is the channel length.[402]

Because our channels are of constant height and width, we find that

$$R_{Hyd} \sim L$$

Thus, we can easily solve for the unknown length L_X by equalizing the hydraulic resistance of the left-hand-side (LHS) branches and the right-hand-side (RHS) branches.

$$R_{LHS} = R_{RHS}$$

$$L_X + \left(\frac{1}{L_{R_3}} + \frac{1}{L_{R_3}} \right)^{-1} = L_{R_2} + \left(\frac{1}{L_{R_4}} + \frac{1}{L_{R_4}} \right)^{-1}$$

$$L_X + \frac{L_{R_3}}{2} = L_{R_2} + \frac{L_{R_4}}{2}$$

$$L_X = L_{R_2} + \frac{L_{R_4} - L_{R_3}}{2}$$

Substituting numerical values for our system, we find that:

$$L_X = (9.2 - L_X) + \frac{2.0 - 3.6}{2}$$

$$L_X = 4.4 \text{ mm}$$

5.5.2 Alternative Straight-Channel Design

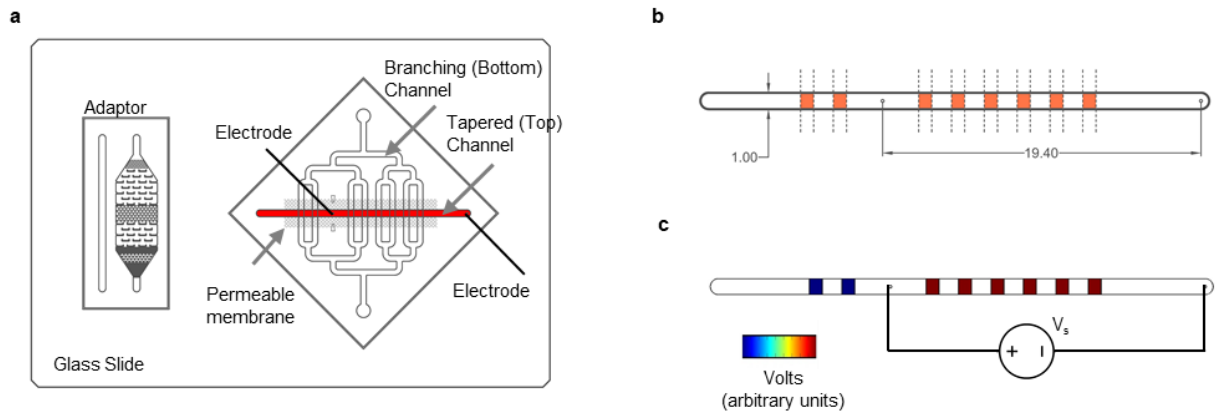


Figure 5.11 Straight-channel BBB design.

a) As an alternative to the tapered channel, a straight channel can be used as the upper channel, enabling six measurements of permeability for the same electric field treatment. b) geometry of the straight channel. c) COMSOL modeling of the electric field confirms that the electric field is equivalent between the six test channels.

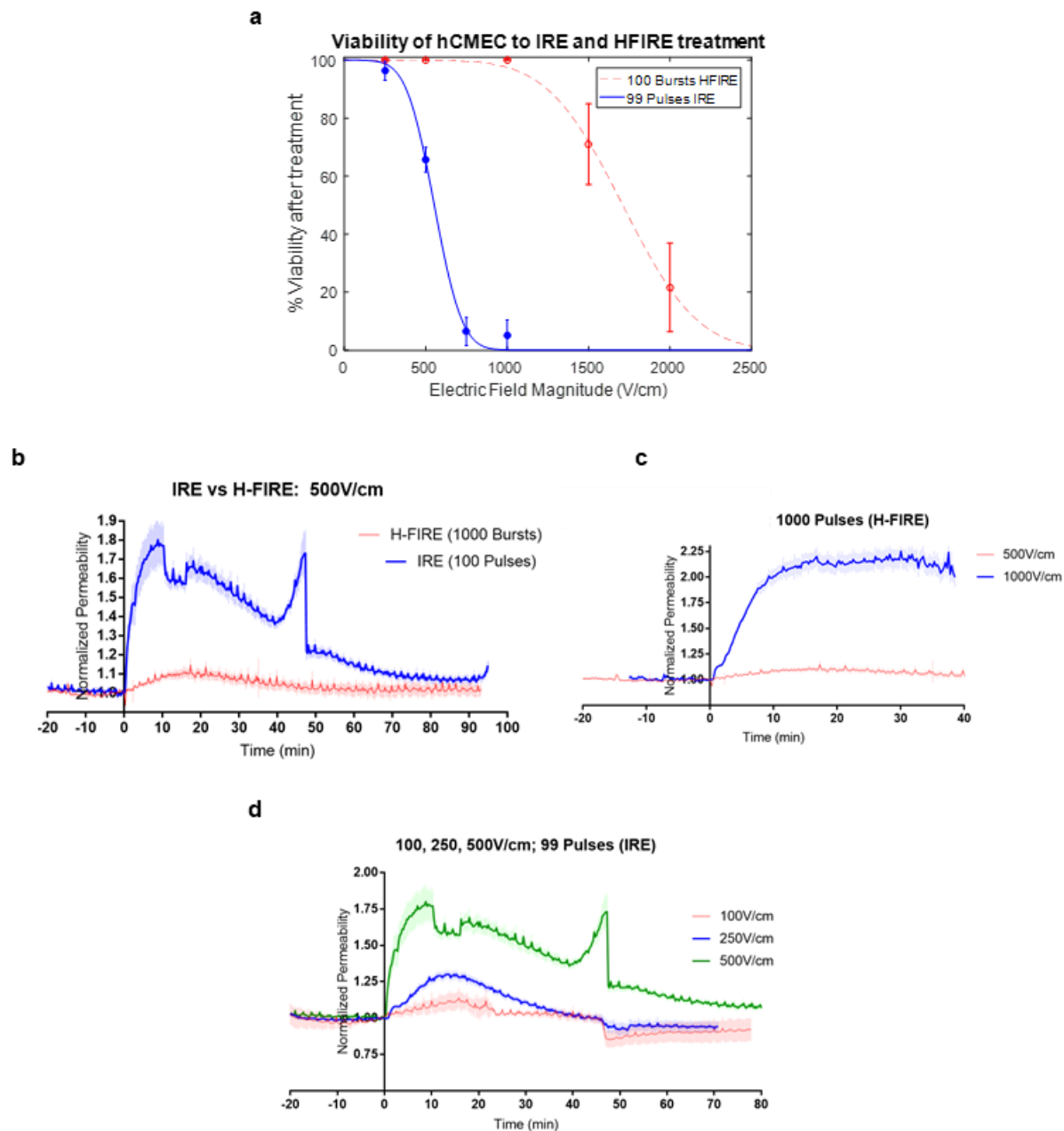


Figure 5.12 Additional results from IRE and HFIRE using the straight-channel device.

a) Cell viability is significantly lower for IRE compared to HFIRE for a given electric field when pulse number is constant. (or nearly constant). b) IRE induces significantly more BBB disruption at 500 V/cm compared to HFIRE, even when 10x more HFIRE pulses are applied compared to IRE. c) A durable increase in BBB permeability is indicative of cell death, whereas lower voltages maintain higher cell viability but do not induce as much disruption. d) The magnitude of BBB disruption after IRE pulses increases as voltage is increased from 100 V/cm to 500 V/cm.

6 Chapter 6: Nanofiber networks as basement membrane mimics for In Vitro Blood Brain Barrier Models.

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6.1 Abstract

Current basement-membrane mimics used for modeling endothelial and epithelial barriers *in vitro* do not faithfully recapitulate key *in vivo* physiological properties such as basement membrane thickness, porosity, stiffness, and fibrous composition. Here, we use networks of precisely arranged nanofibers to form physiologically relevant basement membranes for a microfluidic blood-brain barrier (BBB) model. Our nanofiber networks form ultra-thin (1 μm – 3.2 μm thick) , ultra-porous (nearly 90% porosity) fibrous basement membrane-mimics. We show that these

nanofibers networks enable close contact between endothelial monolayers and supporting cell types such as pericytes and astrocytes, which are known to regulate barrier tightness through signaling. Cytoskeletal staining reveals barrier formation on the nanofiber membranes. We anticipate that our nanofiber networks will improve barrier modelling with implications for drug discovery, cell migration studies, and barrier dysfunction modeling.

6.2 Introduction

Physiological barriers are found throughout the body, and their integrity is key for maintaining healthy functions[403]. These barriers, established by monolayers of endothelial and epithelial cells, restrict transport to enable different microenvironments on either side of the monolayer. Important physiological barriers include the alveolar–capillary barrier[404,405], intestinal mucosal barrier[406], skin barrier[407], renal epithelial barrier[408], and the blood-brain barrier [65,66,409-413]. Barrier properties are derived from junctional complexes between adjacent cells that regulate transport between cells (paracellular transport), and from specialized active and passive transporter proteins that regulate transport across the cell membrane (transcellular transport)[65]. Dysfunction of barriers can have significant consequences and have been implicated for a many serious conditions, such as Alzheimer’s disease[414], irritable bowel syndrome[415], pulmonary edema[416], and chronic kidney disease[417], and Atopic dermatitis[418]. Physiological barriers also often present significant blockades for therapeutic agents. Limited drug permeability across these barriers renders many promising drugs/therapies ineffective. For example, nearly 100% of large drugs and 98% for small drugs cannot penetrate the BBB[419]. Barrier properties are also key for immune cell trafficking, cancer metastasis, nanoparticle transport, and other processes.

Although barrier properties are maintained by endothelial and epithelial monolayers, another key aspect of physiological barriers is the basement membrane (BM) [54]. The basement membrane is thin layer of extracellular matrix (ECM) that provides support for endothelial and epithelial cells, while also separating these cells from supporting cell types. The basement membrane *in vivo* is about 50-100 nm thick and composed of fibrous extracellular (ECM) matrix proteins such as collagen IV, fibronectin, and laminin[54]. The basement membrane is formed by nanometer-diameter fibrils of ECM in a dense network. Due to the thin and fibrous nature of the BM, endothelial and epithelial cells are regulated by cell-cell crosstalk from cell types opposite the basement membrane. The specialized endothelial cells that comprise the blood-brain barrier (BBB), for example, gain their unique transport-restricting properties close contact with adjacent cell types across the basement membrane (Figure 6.1a). *In vivo* and *in vitro* studies have shown that pericytes and astrocytes can regulate endothelial barrier function. *In vivo*, brain endothelial cells make direct contact with pericytes through peg and socket connections that cross the BM (Figure 6.1a)[420]. Astrocyte endfeet surround capillaries in the brain and regulate endothelial behavior through the release soluble factors such as VEGF, TGF β , GDNF, and bFGF. Specifically, supporting cell types such as pericytes and astrocytes can upregulate endothelial tight-junction expression to create tighter barriers. Together, endothelial cells, astrocytes, pericytes, and neurons form what is known as the neurovascular unit (NVU).

Due of the importance of physiological barriers, many *in vitro* barrier models have been developed for investigating barrier properties[421]. These models rely on endothelial or epithelial monolayers cultured on engineered basement membrane constructs that are designed to mimic the *in vivo*

basement membrane. Although numerous basement-membrane mimicking materials have been developed, to date these membranes still have significant limitations[55]. The ideal basement membrane model would have the following properties: thickness of ~100 nm, fibrous construction, high porosity, optical transparency (for imaging), and be biocompatible and bioactive[55]. Table 6-1 summarizes the advantages and disadvantages of several engineered BMs. The most widely used *in vitro* basement membrane model are track-etch polycarbonate (PC), polyester or polyethylene terephthalate (PET), or polytetrafluoroethylene (PTFE) [195,401,422-431]. These semi-permeable membranes are commercially available with cylindrical pores of 0.4, 1, 3, 5, or 8 μm -diameter. These membranes are the industry standard for decades and are employed in transwell inserts (manufacturers: Corning, Falcon, etc), and can also be integrated into microfluidic devices by bonding these membranes between upper and lower microfluidic channels. For barrier models, endothelial or epithelial cells are cultured on one side of the membrane, and unless migration is desired, a small pore size (typically 0.4 or 1 μm) is used to ensure cells remain on a single side of the membrane[426,430]. These membranes, however, lack physiological relevance in key aspects. Firstly, these membranes are generally 10-12 μm thick (or 30 μm for PTFE), over 100 times thicker than the basement membrane *in vivo*. This thickness prevents direct cell-cell contact across the membrane and limits the transport of soluble factor transport between adjacent cell types. Secondly, these membranes have inherently low porosity, in part because the pore spacing is random. Track-etch membranes sold as “high porosity” typically have maximum porosities of around 15% for 0.4 and 1 μm pore sizes and have poor imaging properties. Conventional track-etch membranes have porosities as low as 0.5%. Low porosities decrease the cell-cell crosstalk and reduce diffusion of drugs and molecules across the membrane. Finally, the flat, sheet-like

construction of these membranes cannot mimic the fibrous nature of the basement membrane, are far too stiff for physiological relevance[432], and are not bioactive[433]. Furthermore, track-etched membranes can also obscure transendothelial electrical resistance (TEER) measurements[434]. TEER is used to measure tight junction integrity (cell-cell adhesion), however cell-substrate adhesion can cause additional electrical resistance that inflates TEER measurements and leads to less consistent results between studies. Finally, track-etched membranes can obstruct brightfield imaging (PC is translucent) and confocal imaging due to autofluorescence. Due to these limitations, new basement models are required to improve in vitro barrier modeling.

Several basement membranes are under development as alternative to track-etched membranes, such as nanofabricated SiN membranes [435-437], electrospun nanofibers [433,438,439], patterned materials such as PDMS and mylar [440-443], and native ECM membranes such as collagen and collagen vitrigel membranes [432,444-448]. Nanofabricated silicon nitride membranes can be extremely thin (50 nm to 1 μ m), have evenly distributed pores, are and optically transparent[435-437]. However, these membranes require expensive microfabrication processes, are non-fibrous, are not naturally degradable, and can result in cell attachment problems due to the SiN [435]. Anodic aluminum oxides have also been investigated for cell models[449,450]. Similarly, photolithographically-patterned Mylar[441] and PDMS[404,440,442] membranes have been fabricated with desirable properties such as relatively high porosity (up to ~50%), controlled pore size/location, moderate thickness (generally 5-50 μ m, but as low as 2 μ m) and are optically transparent. However, these membranes are non-fibrous and require microfabrication. To achieve fibrous membranes, electrospinning techniques have been used [433,438,439]. However, these

membranes have disadvantages such as large thicknesses ($> 10 \mu\text{m}$), and randomly distributed fibers and pores. Native extracellular matrix (ECM) membranes have also been developed[432,444,451], such as vitrified collagen membranes[445-448], but these membranes have low porosity due to the vitrification process and are rather thick ($\geq 10 \mu\text{m}$). These experimental membranes show improvements to conventual membranes; however, they have yet to gain widespread use. Improved membrane models are needed to reduce the undesirable properties of currently available membranes.

Table 6-1 Table of basement-membrane mimics used for in vitro modeling.

Membrane Types	Desirable Properties	Undesirable Properties	Ref
Track-etched PC, PET, PTFE	Commercially available (0.4, 1, 3, 8 μm pores) Transparent (PET, PTFE) Biocompatible	Non-fibrous Thick ($\sim 10\text{-}12 \mu\text{m}$) Low porosity ($\sim 0.5\% - 15\%$) Translucent (PC) Stiffer than <i>in vivo</i>	[430] [195,401,422-431]
Silicon Nitride	Ultra-thin (50nm -1 μm) to facilitate cell-cell contact High porosity (approaching 50%) Structured pore geometry and size ($\sim 400\text{nm}$ square pores) Transparent	Non-fibrous Poor cell adhesion to SiN Requires nanofabrication Stiffer than <i>in vivo</i>	[435-437]

Collagen or Vitrified Collagen	Fibrous Native ECM	Pore size determined by fabrication. Low porosity due to small pores Thick ($\sim >10\mu\text{m}$)	[432,444-448]
Patterned PDMS/Mylar	Transparent Flexible (PDMS) Can be highly porous	Generally thick ($5\mu\text{m}$) Non-fibrous Fragile Requires nanofabrication	[404,440-443]
Electrospun nanofibers	Fibrous Variable pore sizes Relatively high porosity Can be bioactive Can have good mechanical strength	Relatively thick ($> 10\mu\text{m}$) Random arrangement of pores Wide pore size distribution Not transparent	[433,438,439]
STEP nanofibers	Ultra-thin (down to $\sim 1\mu\text{m}$) Ultra-porous (up to 90%) Transparent Fibrous No Nanofabrication or Cleanroom Excellent biocompatibility	Distributed pore sizes More fragile compared with other membranes.	This Work

*PC, Polycarbonate; PET, polyethylene terephthalate; PTFE, Polytetrafluoroethylene

In this work, we introduce nanofiber networks fabricated with the STEP technique for basement membrane mimicking networks that offer several advantages compared to other membranes. Firstly, STEP membranes are ultra-thin, can be fabricated close to physiologically relevant thicknesses. STEP membranes can be fabricated with 1-3 μm thickness, which is 3-10x thinner than commercial track-etched membranes. Secondly, STEP membranes are highly porous. Membrane porosity depends on fiber diameter and spacing, but porosities over 80% can be achieved, which is well beyond the porosity of other reported membrane types (generally $<50\%$). Because our STEP membranes are thin and highly porous, significant crosstalk can be achieved between cells cultured on adjacent sides of the membrane as these cells can make physical contact across the membrane, while still confining cell growth to their respective sides of the membrane.

Thirdly, STEP membranes are fibrous, just like the native basement membrane. Fibrous membranes are more relevant than commercial membranes that are flat plastic sheets with cylindrical pores. Likewise, our fiber networks are more compliant than plastic sheets. Fourthly, we anticipate that minimal cell-substrate contact will reduce transendothelial electrical resistance (TEER) inconsistencies. Another advantage of the STEP membranes is that they can be integrated in both microfluidic and transwell experiments. Incorporating STEP membranes into microfluidic devices will enable the study of induced shear stress on individual or both sides of the membrane's co-culture, which is important for tight barriers. Finally, STEP membranes are made of polystyrene, making them highly biocompatible and excellent for cell adhesion. They can be coated with ECM to create a natural surface for cells growth. Polystyrene fibers also could promote easy translation into commercial usefulness as complete polystyrene devices, as PDMS devices are not commercially useful.

Our results demonstrate that STEP membrane can be used for barrier models and offers significant advantages over existing membranes. We demonstrate the utility of our nanofiber membranes by modelling the Blood-brain barrier (BBB) using human endothelial cells and pericytes in monoculture and contact co-culture models. Our nanofibers can be incorporated into existing Transwell plates or incorporated into microfluidic devices for dynamics experiments. Ultra-thin, ultra-porous, fibrous networks fabricated by the STEP technique will provide a more physiologically relevant basement membrane than alternative membrane fabrication methods, leading to improved models of the BBB and other in vitro barrier models for a vast range of applications.

6.3 Results

6.3.1 Nanofiber membranes are embedded within a PDMS microfluidic device

To create our microfluidic model, shown in Figure 6.1b-c, we fabricated two patterned polydimethylsiloxane (PDMS) channel layers that enclosed the nanofiber scaffold and membrane. PDMS layers were formed from laser-cut and solvent bonded acrylic molds. The lower PDMS layer was achieved by pressing two acrylic mold together to pattern both sides of the PDMS (see methods). Upper and lower channels were fabricated to be 220 μm tall, 3 mm wide, and 18 mm long. At the intersection of the upper and lower channels, a laser-cut plastic scaffold with nanofiber membrane was embedded, having a nanofiber membrane spanning the 2 mm-diameter circular hollow region. For comparison studies, conventional track-etched PET or PC membranes were bonded to identical laser-cut scaffolds. The scaffolds and membranes were sealed within the device with liquid PDMS glue. Finally, a PDMS well layer was plasma bonded to the top to enable easy fluid handling.

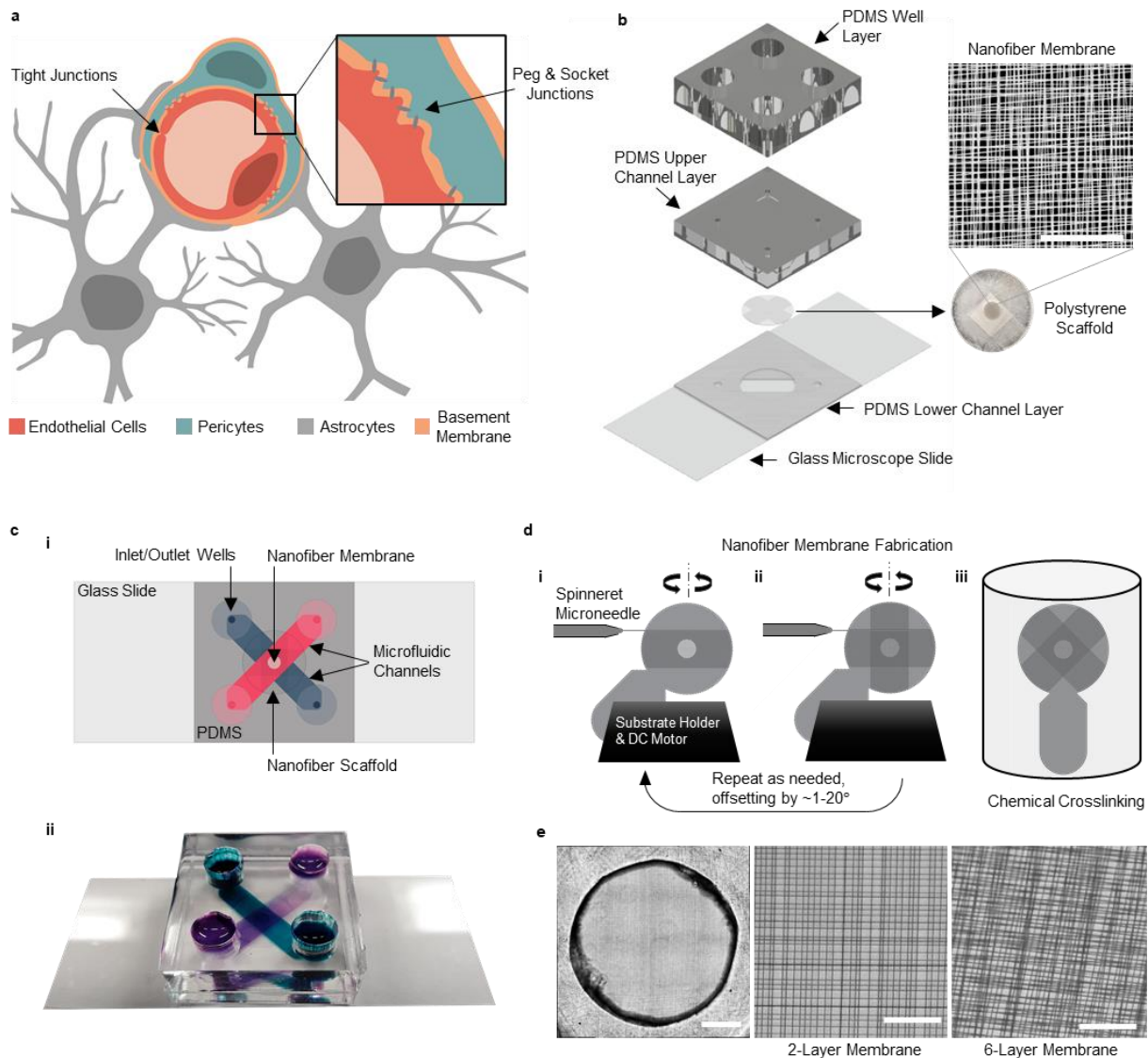


Figure 6.1 A microfluidic BBB model contains a physiologically relevant nanofiber basement membrane.

a) The blood-brain barrier (BBB) is formed by specialized endothelial cells supported by pericytes and astrocyte endfeet across a 50-100 nm thick basement membrane. Pericytes regulate

endothelial cells through physical contact at peg and socket junctions, while astrocytes likewise provide regulation via soluble factors. b-c). A three-layer PDMS microfluidic BBB chip contains two orthogonal channels separated by a dense nanofiber network that forms a basement membrane for co-culture BBB models. d) Polystyrene networks are fabricated using a non-electrospinning technique known as Spinneret-based Tunable Engineered Parameters (STEP). Polystyrene pre-polymer is dispensed from spinneret microneedles and wrapped around a rotating polystyrene scaffold (i). The scaffold can be re-oriented to spin orthogonal fibers (ii), and spinning can be repeated to deposit additional fiber layers. The nanofibers are then chemically crosslinked for stability (iii) e) Nanofibers are spun as uniform networks across the 2-mm circular membrane region (left), and the number of fiber layers regulates pore size. Two-layer (center) and six-layer (right) nanofiber networks are pictured. Scale bars a) 50 μm and e) 500 μm , 50 μm , and 50 μm , respectively.

6.3.2 Ultra-thin, ultra-porous nanofibers membranes are fabricated by the STEP method

To replicate the ultra-thin and highly porous *in vivo* basement membrane, we used the non-electrospinning Spinneret-based Tunable Engineered Parameters (STEP) method previously described [237,238] to deposit dense networks of nanofibers with highly controlled fiber diameters and fiber spacing. The STEP method (Figure 6.1d) uses liquid prepolymer dispensed from a microneedle to deposit nanofibers around a rotating substrate. This method enables a high level of

control of fiber diameters (100 nm – 10 μm) and fiber spacing (as low as $\sim 3 \mu\text{m}$). The nanofibers are polystyrene, and thus highly biocompatible. To create the crosshatch nanofiber pattern, plastic scaffolds are rotated ~ 90 degrees to deposit orthogonal fibers, and layers can be stacked to create membranes of various thicknesses and pore sizes. Figure 6.1e shows phase contrast images of 2-layer, and 6-layer nanofiber membranes. The nanofibers are then chemically fused at their intersections to improve the stability of the membranes.

Conventional membranes for barrier modeling are 0.4 μm PET membrane, shown on the right in Figure 6.2a. Although widely used, these membranes have extremely low porosity of less than 0.5%. Low porosity limits cell-cell interactions across the membrane and also creates an unnatural barrier to molecular transport that could decrease the sensitivity of transport studies. Higher porosities can be achieved with polycarbonate (PC) membranes and larger pore sizes (1, 3, 5 μm diameter, etc). For comparison, we analyzed the porosity of a PC membrane with 3- μm diameter pores. These PC membranes still have less than 15% porosity, and PC membrane are not optically transparent and thus limit imaging capabilities.

In contrast to conventional track-etched membranes, our STEP nanofiber membranes have extremely high porosities as demonstrated by the SEM image in Figure 6.2a. We show two possible nanofiber membrane configurations: two layers of orthogonal 600-nm diameter fibers spaced approximately 4 μm apart (2-Layer membranes), and six layers of stacked 600-nm diameter fibers also spaced 4 μm apart in each layer (6-Layer membranes). Analysis of the z-projected pore size shows that average pore size for the 2-layer membranes is $14.0 \pm 8.2 \mu\text{m}^2$ and the average 6-layer membrane pore size is $2.5 \pm 2.5 \mu\text{m}^2$. Given these pore size, our 2-layer membranes have

roughly equivalent pores sizes as 5- μm diameter track-etched pores (pore size $\sim 19.6 \mu\text{m}^2$), and our 6-layer membranes have pores sizes roughly between 1- μm and 3- μm diameter track-etched pores (pore sizes $0.8 \mu\text{m}^2$ and $7.1 \mu\text{m}^2$). The Z-projected (2D) porosity (see methods) was calculated to be 50% for the 2-layer membranes and 37% for the 6-layer membranes. Both nanofiber membranes considerably outperform the standard track-etched membranes. However, true porosity is a 3D property (volume of empty space to total volume). Assuming membranes have nominal thicknesses based on the fiber diameters (fiber thickness are additive – minimal flattening during chemical fusion), membrane porosity is independent of the number of stacked nanofiber layers, but is rather dependent only on average fiber diameter and average fiber spacing. For both the 2-layer and 6-layer scaffolds, which have 600 nm fibers spaced 4 μm apart, the theoretical porosity for both membranes is 88 %. This represents a 220x increase over the 0.4 μm pore PET membranes and a 6.5x increase over the 3- μm PC membranes. At nearly 90% porosity, these membranes are near the limit of what might be experimentally possible, and higher porosity membranes are not likely to significantly increase transport across the membranes. High porosity membranes have the potential to increase the crosstalk between adjacent cell types to physiologically relevant levels.

In addition to the ultra-high porosities of our nanofiber membranes, our nanofiber membranes are also significantly thinner than convention membranes. Conventional track-etched PET and PC membranes range from 10 to 12 μm in thickness, over 100x the thickness of the basement membrane in vivo. Our nanofiber networks offer a several-fold decrease in thickness. Our 2-layer membranes are nominally 1.2 μm thick, while our 6-layer membranes are nominally 3 μm thick.

The chemical-fusion process can cause the nanofiber intersection points to melt and decrease the overall membrane thickness below nominal levels, but at the cost of also reducing the porosity of the membrane. At $< 3\ \mu\text{m}$ thick and highly porous, our membranes can enable significant cell-cell interactions across the membrane.

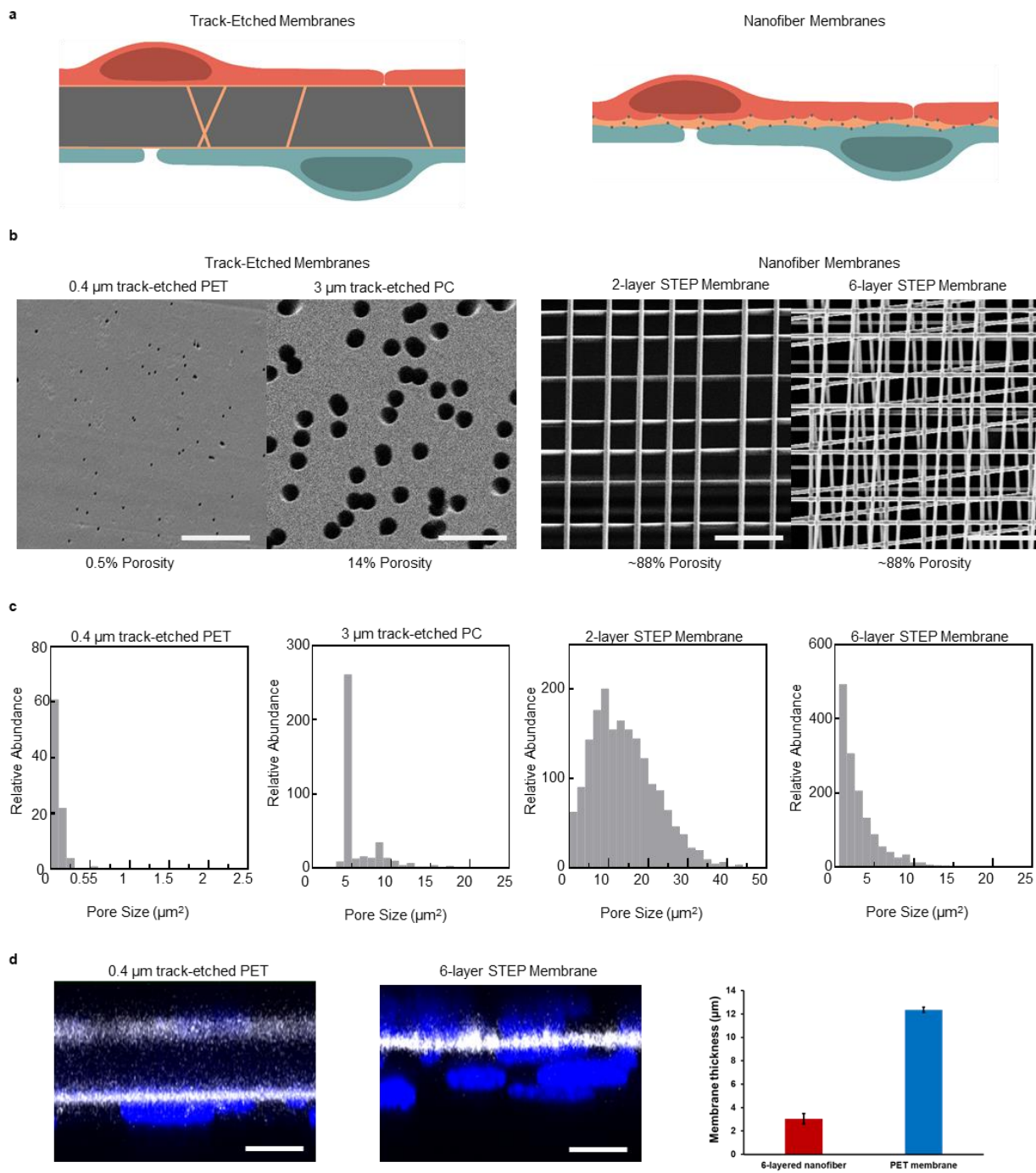


Figure 6.2 Ultra-thin, ultra-porous nanofiber networks offer significantly improved properties compared to track-etched membranes.

a) Schematic representations of track-etched vs nanofiber membranes. Nanofiber membranes are thinner and more porous to better mimic in vivo physiology. b) SEM images reveal the pore structure of i) 0.4- μm diameter track-etched PET membranes, ii) 3- μm diameter track-etched PC membranes, iii) 2-layer nanofiber membranes, and iv) 6-layer nanofiber membranes. 2- and 6-layer membranes formed with 600-nm diameter fibers spaced approximately 4 μm apart. Track-etched membranes have randomly located pores of uniform size whereas the nanofiber membranes have precisely-organized pore structures with a greater distribution of pore sizes. Track-etched membranes have very low porosity of less than 15%. In contrast, our nanofiber networks have porosities of nearly 90%. c) Pore size distributions show nanofiber membranes have a wider pore size distribution than track-etched membranes. The 6-layer membranes have an average pore size significantly lower than the 3- μm diameter track-etched membrane. The pore size, thickness and porosity of the nanofiber membranes can be controlled by fiber spacing, diameter, and the number of fiber layers. d) The nanofiber membranes are significantly thinner than conventional track-etched membranes. Six-layer nanofiber membranes are approximately 3 μm thick (right image), whereas conventional PET membranes are 10-12 μm thick (right image). Rhodamine fibronectin (white) indicates membrane surfaces. Scale bar 10 μm .

Table 6-2 Average pore sizes and distribution for Nanofiber and track-etched membranes.

Membrane Type	Structure	Thickness	Pore Size (μm^2)		Z-Projected (2D) Porosity	Calculated 3D Porosity
			Median	Mean $\pm$ STD		
2-Layer Nanofiber	600-nm Nanofibers	$\leq 1.2 \mu\text{m}$	12.98	14.03 ± 8.18	50 %	88 %
6-Layer Nanofiber	600-nm Nanofibers	$\leq 3.0 \mu\text{m}$	1.62	2.45 ± 2.55	37 %	88 %
Track-etched PC	$3.0 \mu\text{m}$ cylindrical pores	$22 \mu\text{m}^*$	4.54	6.07 ± 3.24	13.5 % (11.3%*)	13.5 % (11.3%*)
Track-etched PET	$0.4 \mu\text{m}$ cylindrical pores	$12 \mu\text{m}$ ($10 \mu\text{m}^*$)	0.09	0.11 ± 0.06	0.4 % (0.5%*)	0.4 % (0.5%*)

*According to manufacturer information

6.3.3 Nanofiber membranes enhance trans-membrane molecular diffusion

The combination of high porosity and low thickness of our nanofiber membranes enable significantly enhanced the molecular diffusion across the membrane, as shown in Figure 6.3. To visually demonstrate increase trans-membrane diffusion, we performed time-lapse diffusion experiments using food dye. As Figure 6.3 clearly shows, the 6-layer nanofiber membrane has a significantly increased rate of dye diffusion from the lower channel to the upper channel. The increase is especially pronounced between the $0.4 \mu\text{m}$ PET membrane and nanofiber membrane.

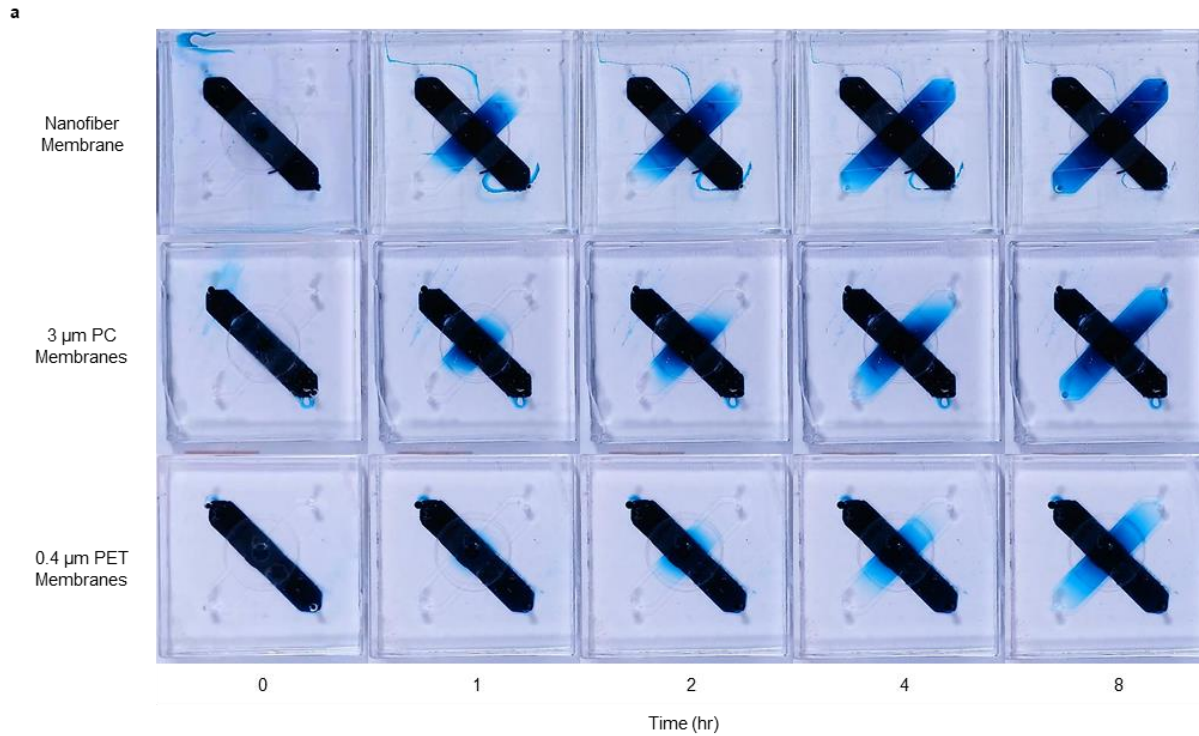


Figure 6.3 Nanofibers enhance trans-membrane diffusion compared to track-etched membranes.

a) Diffusion of food dye from the lower channel to the upper channel show that 6-layer nanofiber membranes (top) enable significantly enhanced diffusion compared with PC (center) and PET (bottom) membranes. During experiments, inlet and outlets were blocked to eliminate fluid flow.

6.3.4 Nanofiber membranes support endothelial monolayer formation

Given the superior properties of our nanofiber membranes compared to track-etched alternatives, we investigated whether endothelial monolayers could be formed on the fiber membranes. We found that two human brain microvascular cell lines were able to form intact monolayers: immortalized human cerebral microvascular endothelial cells (hCMEC/D3) and primary human brain microvascular endothelial cells (HBMECs). Endothelial cells were seeded at high densities on the fibers (see methods), and monolayers formed within 24 hours (Figure 4a, hCMEC cells). Figure 6.4b shows a complete monolayer of hCMECs formed after 72 hours of culture on the fibers. Furthermore, confocal microscopy reveals that endothelial cells are confined to one side of the membrane (See Movie 1). Nanofiber are stained with rhodamine-conjugated fibronectin. Figure 6.4c shows the morphology after 48 hours of culture in static and dynamic (oscillation flow for 24 hours, max shear stress ~ 4 dynes/cm²). Both monolayers show enhanced expression of ZO-1 (tight junctions) at cell boundaries, and significant VE-cadherin expression at cell boundaries. HBMEC cells showed significant localization of VE-Cadherin to cell borders (Figure 6.5c-d).

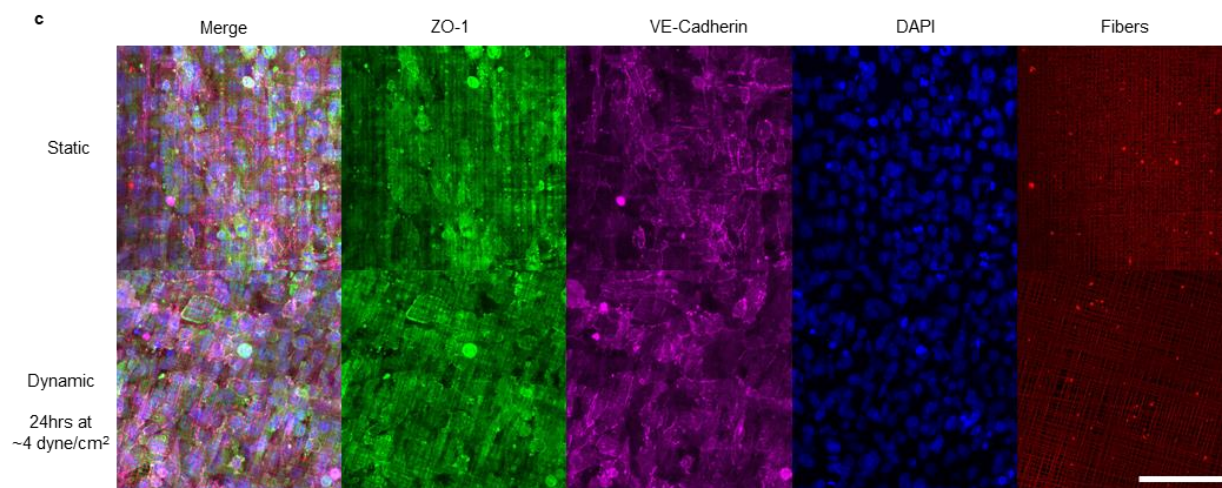
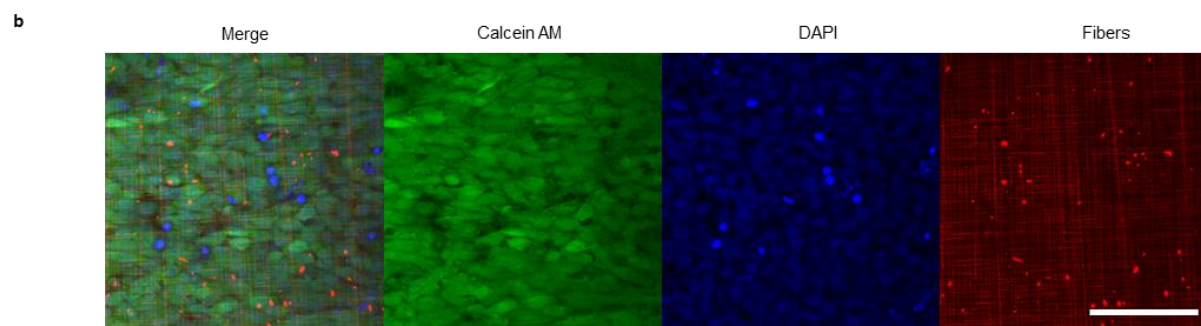
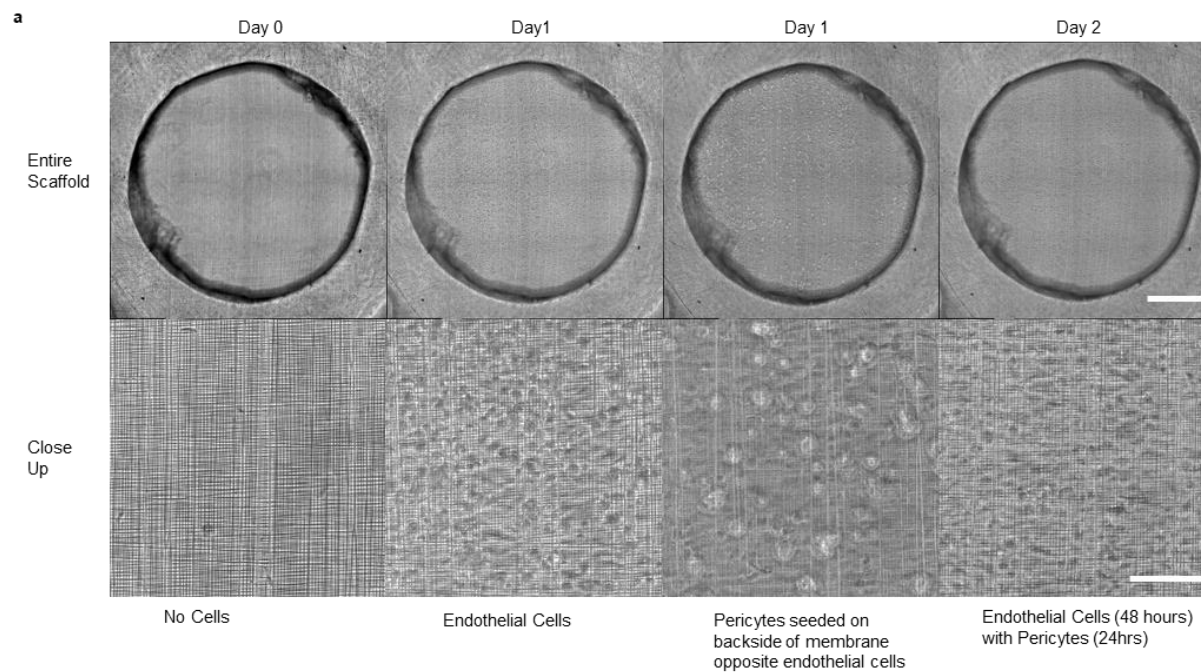


Figure 6.4 Endothelial cells form complete monolayers on nanofiber scaffolds and can be co-cultured with pericytes.

a) Brain endothelial cells (hCMECs) seeded on 6-layer membranes on day 0 form confluent monolayers after 24 hours (day 1). At 24 hours (day 1), pericytes can be added to the opposite side of the membrane. By day 2, pericytes have spread on the fibers to form a stable co-culture BBB model. b) Calcein-AM (green) staining on live endothelial cells (hCMECs) after 72 hours of monoculture show complete monolayer formation on the nanofibers. d) Antibody staining of endothelial cells (hCMECs) for the tight-junction protein ZO-1 and the endothelial adhesion molecule VE-cadherin show localization at cell boundaries indicating barrier formation after static or dynamic culture.

6.3.5 Nanofiber membranes enable cell-cell contact co-cultures for BBB studies.

Due to the low thickness and high porosity of our membranes, we sought to culture pericytes and endothelial cells on opposite sides of the nanofiber membrane to mimic the spatial configuration of these cells in vivo. To perform these experiments, endothelial cells were first seeded onto the underside of the nanofiber membrane and allowed to spread and form a monolayer for 24 hours. Pericytes were then added on the topside of the membrane and allowed to spread for 24 hours, after which devices were fixed and stained. Figure 6.4a shows phase contrast images over the course of an experiment. As shown in Figure 6.6, Pericytes were successfully cultured

opposite endothelial monolayers for both hCMEC and HBMEC cells. As shown, HBMEC cells and pericytes are in very close proximity during co-culture studies (Figure 6c).

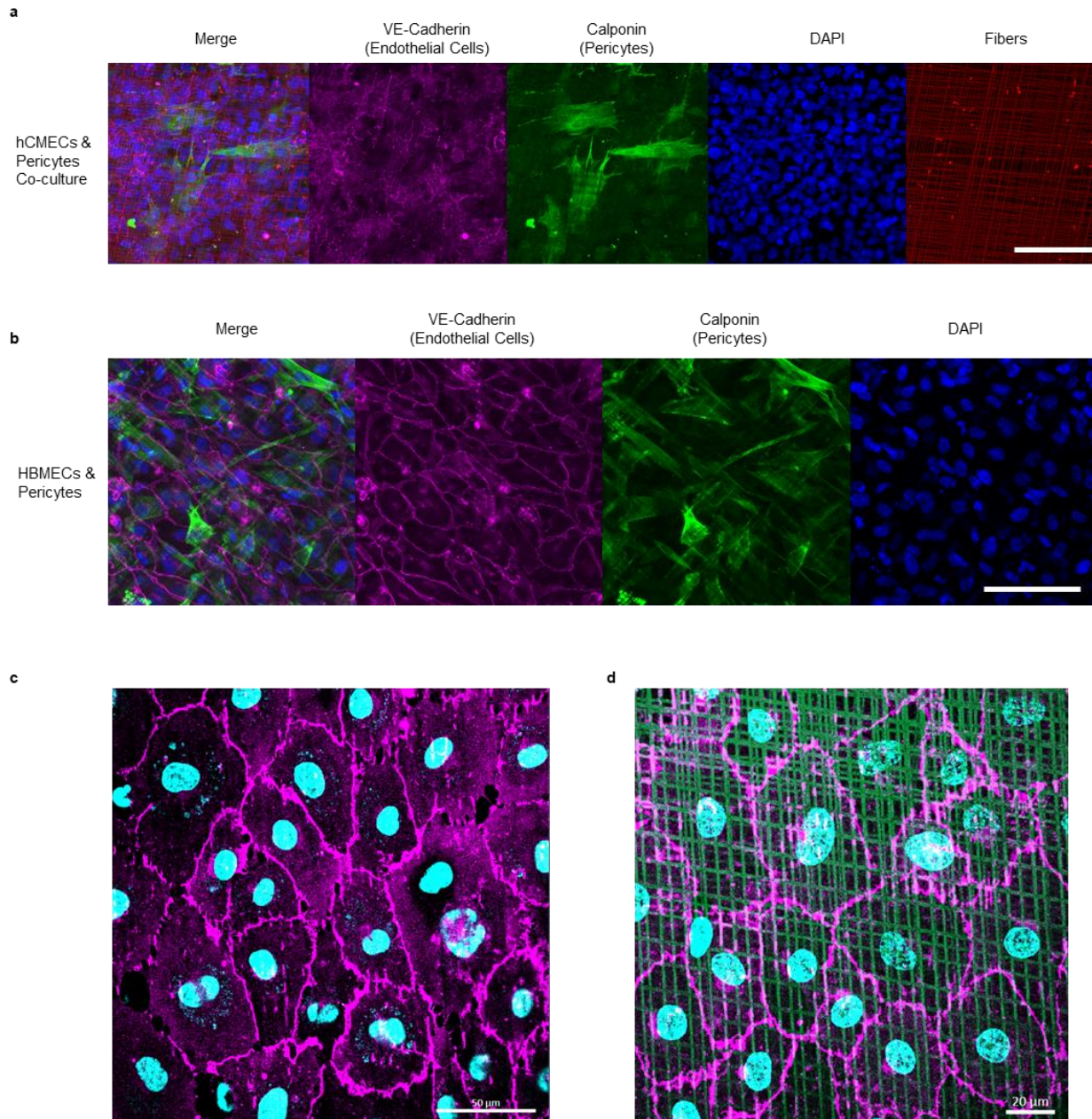


Figure 6.5 Co-cultures of endothelial cells with pericytes enable cell-cell interactions across the nanofiber membranes.

a) Human endothelial cells (hCMECs) stained with VE-Cadherin and primary pericytes stained for calponin demonstrate successful co-culture across the nanofiber membranes. b) Primary human endothelial cells (HBMECs) likewise show characteristic monolayer formation along with pericyte localization. c-d) Higher magnification images showing VE-cadherin (cyan) localization to cell boundaries, and monolayer formation on the nanofiber membrane (right, rhodamine fibronectin shown as green). Scale bars 20 μm .

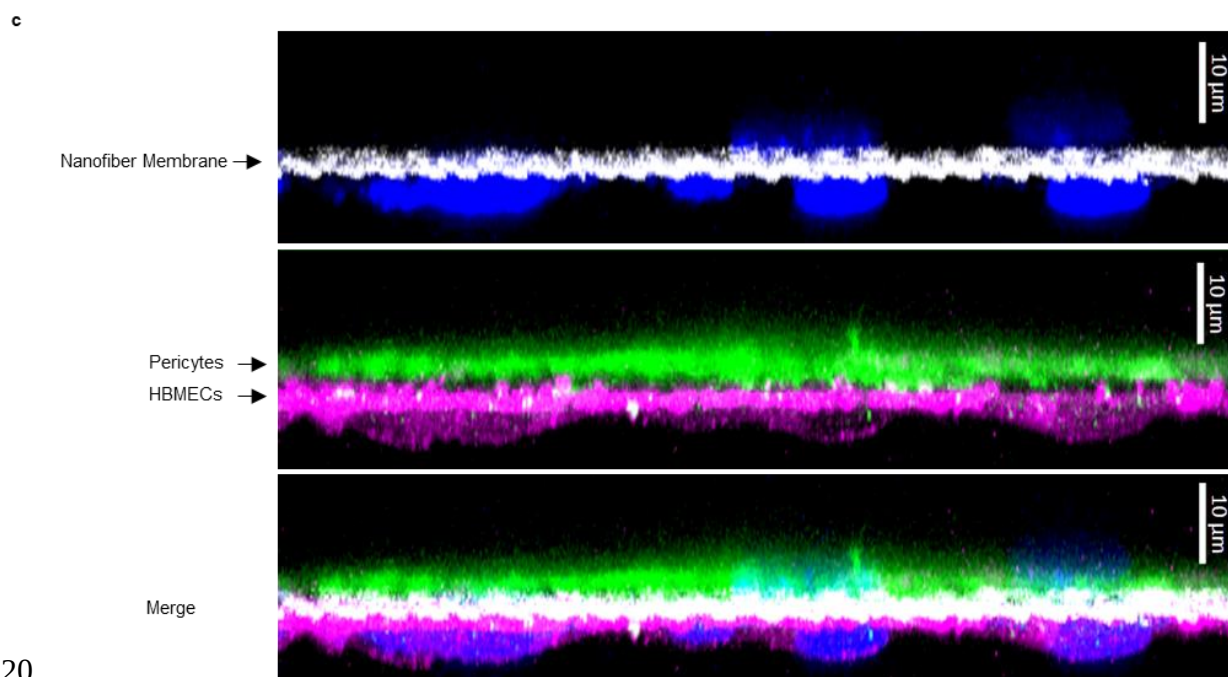
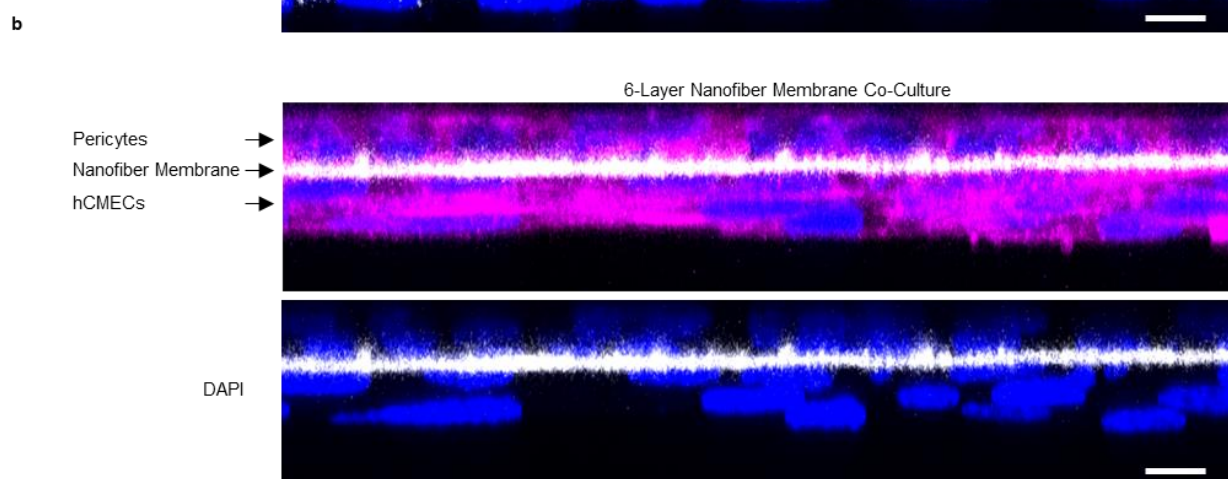
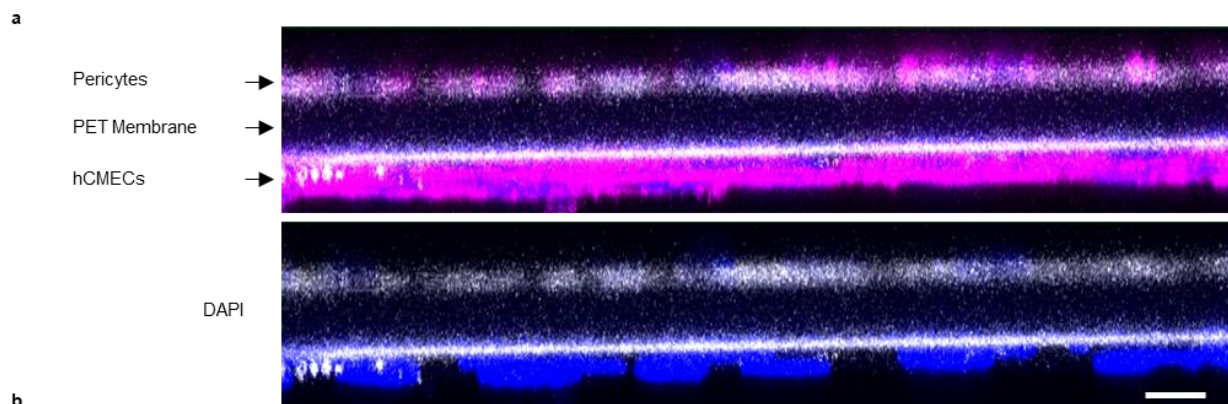


Figure 6.6 Co-cultures of endothelial cells with pericytes enable cell-cell interactions across the nanofiber membranes.

a-b) Z-stack images of co-cultures demonstrates the close-contact of pericytes and endothelial cells across the nanofiber membranes. Actin is pink, and rhodamine-fibronectin on the membrane is pictured was white. a) PET track-etched membranes show significant separation between pericytes (top) and endothelial cells (bottom) which prevents cell-cell interaction. b) In contrast, 6-layer nanofiber membranes are significantly thinner and enable cell-cell interactions across the membrane. Notice that the imaging quality on upper side of the PET membrane is significantly reduced due to the mismatch in refractive index of the plastic. In contrast, there is minimal resolution loss across the nanofiber membranes. c) Z-stack images showing the narrow separation of primary endothelial cells (cyan, VE-Cadherin) and pericytes (green, calponin) in co-culture models.

6.3.6 Transendothelial electrical resistance (TEER) across nanofiber scaffolds

BBB integrity is commonly assessed by transendothelial electrical resistance (TEER) measurements[452,453]. Endothelial monolayers resist electrical current (by restricting Na⁺ and Cl⁻ ion transport) due to tight-junction formation. Thus, by applying a small potential across the monolayer and sensing the electrical current, the resistance of the monolayer can be deduced by fitting the system to a simple electrical model shown in Figure 6.7c. Typical commercial TEER

systems measure resistance values around 12.5Hz, however measuring impedance across a wide frequency spectrum can provide more information. The impedance spectrum can then be fit to the equivalent circuit model and TEER values can be reduced. TEER measurements can be made with two non-polarizable silver-silver chloride electrodes[422,425], or more commonly with four electrodes[431]. Here, we used four stainless steel acupuncture needles (180- μ m diameter) inserted within the device to measure TEER values.

To precisely align the thin electrodes, acrylic molds were fabricated with engraved electrode-guides (See Methods). PDMS was cured around the electrodes, and the electrodes were then removed prior to removing the cured PDMS from the mold. This fabrication method enabled precisely located guides for electrode re-insertion into the PDMS. To improve the uniformity of the electric field, we increased the channel height from 0.22 mm to 1.52 mm for the bottom channel and 1.74 mm for the top channel. This increase helped reduce electric field hotspots around the edges of the membrane, and decreased the electrical resistance of the fluid to provide enhanced sensitivity (Figure 6.7b). Electrical potential is applied across diagonal electrodes and current is sensed across the other diagonal electrodes, similar to previously published methods [454].

One day after adding the endothelial cells the electrical impedance increased, with slight increases in subsequent days. Fitting the impedance spectra, hCMEC monolayers had approximately 10 Ω/cm^2 TEER values after two days in culture on PET membranes.

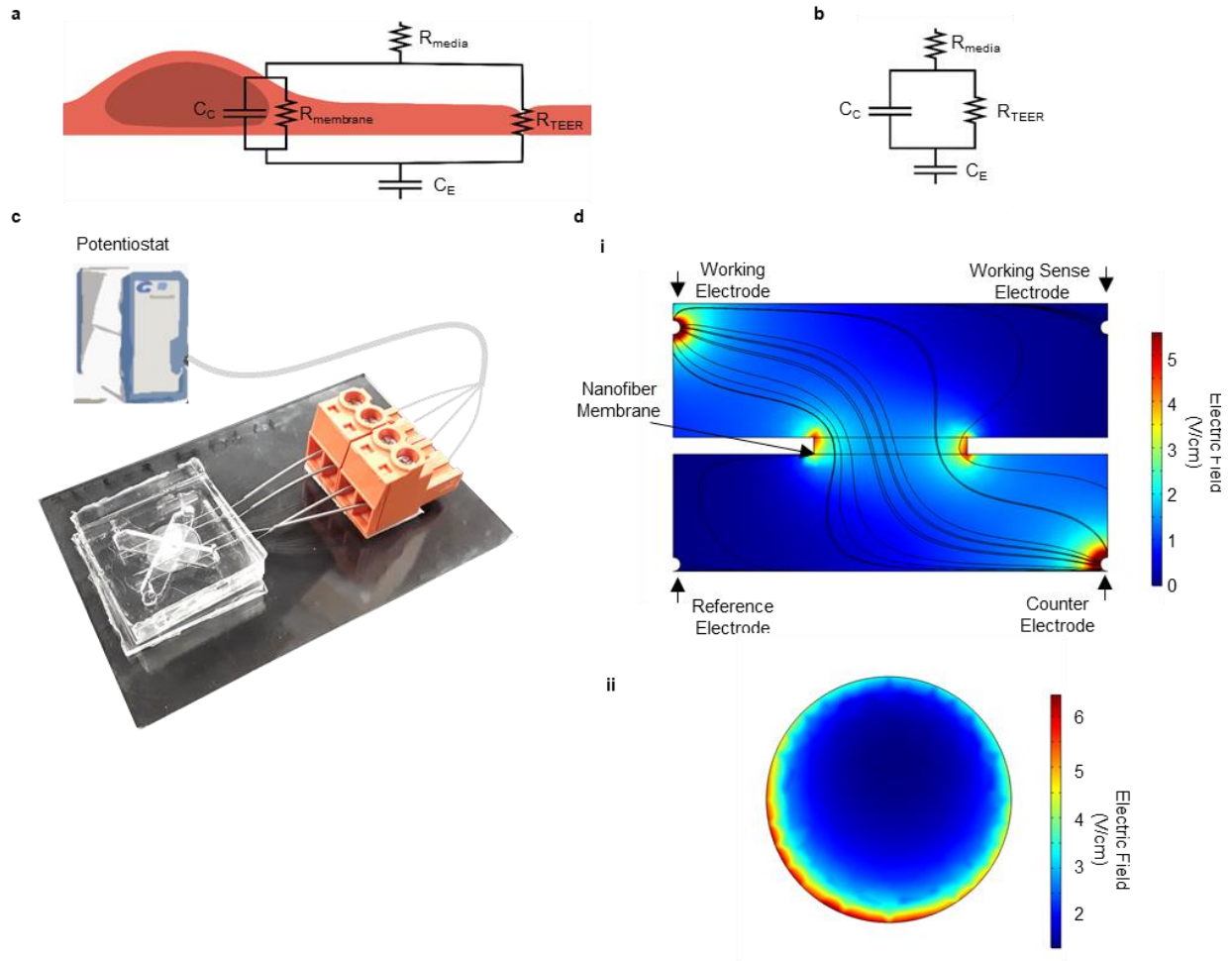


Figure 6.7 Transendothelial electrical resistance (TEER) measurement taken via integrated electrodes monitor barrier formation on the nanofiber membranes.

a) Integrity of the endothelial monolayer can be investigated by analyzing the cell monolayer as a simple circuit. b) the electrical resistance from tight junctions (R_{TEER}) is typically analyzed as a metric of barrier tightness. A simple electrical circuit of capacitors and resistors can represent endothelial barrier formation. Fitting impedance data to the circuit enables a calculation of

transendothelial electrical resistance (TEER). b) Four stainless steel needles integrated within the device measure impedance when connected to a potentiostat. c) A finite element model of the electric field between the working and counter electrodes (1V potential difference) shows current lines passing across the membrane with higher fields near the membrane edges. Impedance is measured by the working sense and reference electrodes. d) Impedance data for hCMEC cells cultured on a PET membrane show barrier formation during the first 5 days of cell culture.

6.4 Discussion

Here we have shown that precisely aligned nanofiber networks can be used to create physiologically relevant basement-membrane mimics. Current basement membrane models lack physiological relevance, such as unrealistic thicknesses (generally >100 times greater than *in vivo* thickness), low porosity (generally < 15%, but often as low as 0.5%), and sheet-like (flat) rather than fibrous construction. Thus, currently available semi-permeable membranes used for barrier modeling lack key properties that would enable more accurate barrier models, especially cell-cell contact co-culture models. To overcome the limitations of conventional barriers, we use an advanced, yet simple non-electrospinning technique known as STEP to fabricate dense networks of highly ordered polystyrene nanofibers.

Nanofiber network properties, such as fiber diameter spacing, fiber diameter, fiber orientation, and number of fiber layers can be precisely controlled to create membranes with

variably thickness and porosity. Here we use dense networks polystyrene nanofibers to create ultra-thin ($\sim 1\text{-}3\ \mu\text{m}$), ultra-porous (nearly 90% porosity), fibrous basement membranes for co-culture barrier models. To our knowledge, our nanofiber membranes significantly exceed the highest reported porosities for membranes used in barrier modelling. While the described 2-layer and 6-layer nanofiber membranes form basement membranes with superior properties, it may be possible to further optimize these membrane properties by exploring additional fiber diameters, especially smaller diameter fibers. Nanofibers as small as 100-nm in diameter can easily be fabricated with the STEP method. Incorporating smaller diameter fibers could produce membranes that nearly reach *in vivo* membrane thicknesses. However, due to the fragile nature of 100-nm diameter fibers, we chose 600-nm diameter fibers for this study due to their added strength. Using large diameter fibers as structural supports for smaller diameter fiber networks may enable strong yet extremely thin basement membrane mimics.

We integrated our nanofiber membrane into a microfluidic barrier model. Microfluidic models have become increasingly prominent in barrier research, as these devices enable cell confinement, complex geometries, and fluid shear stress. We fabricated our device from PDMS. However, absorption and adsorption of molecules by PDMS makes it unfavorable for drug transport studies[455]. Since our nanofibers are polystyrene, an entirely plastic device could be designed and assembled for commercial use. Furthermore, our nanofibers can be integrated into conventional transwell plates, enabling use with current well plate-based equipment and rapid adoption. As a proof-of-concept, we modified a conventional 24-transwell plastic insert to accept the nanofiber scaffold (See Supplemental Information). However, due to the delicate nature of the

fibers and the advantages of microfluidics, we chose to perform our experiments in the microfluidic device. One key advantage is the ability to add fluid shear stress, which is known to be a critical mechanical stimulus for improving barrier tightness via cytoskeletal changes and tight-junction formation[456]. We found that static culture conditions produced good barriers, however adding oscillating shear flow was investigated. Although 3D hydrogel models of the BBB show significant potential[457,458], the added complexity of these devices has thus far limited their commercialization. Due to the incorporation of gel the device, measurement of absolute barrier permeability (TEER or molecular diffusion) is generally not possible or very difficult. Furthermore, these models are not able to collect the effluent from the models to measure the concentration of a molecule or drug that was transported across the membrane. Consequently, 2D shear models with engineered membranes will continue to remain key to research efforts for BBB modeling and other barrier modelling.

We cultured endothelial monolayers on the nanofiber scaffolds and demonstrated that the monolayers express key junctional proteins such as ZO-1 and VE-Cadherin. Confocal imaging shows that endothelial cells were constrained to only one side of the nanofiber membrane. Furthermore, we were able to co-culture pericytes on the opposite side of the membrane and we show that our highly-porous and thin membrane enables close contact between endothelial cells and pericytes.

Using integrated electrodes, we measured TEER values across the nanofiber scaffold, and found that TEER values increased after seeding endothelial cells across the membrane. TEER values for the BBB *in vivo* have been measured to be $> 1000 \Omega/\text{cm}^2$ [459]. Most *in vitro* models

fall significantly below this value. Our TEER measurements are considerably lower compared with other reports[66,412], however this may be due the geometry of our device and the small size of our nanofiber membrane. Importantly, both PET and our nanofiber membranes showed similar TEER values during the first three days of cell culture. Future experiments will reveal if cell-cell interactions can increase TEER values more substantially in the nanofiber device. Our BBB model could be further improved by including human-induced pluripotent stem cells (hiPSC) that have been shown to have higher TEER values and would enable patient-specific models[460]. Likewise, alternative attachment factors such as Matrigel could be explored to further increase barrier tightness.

We anticipate that TEER values taken across nanofiber membranes will yield more consistent results compared to track-etched membranes. It has been suggested that the inconsistency in TEER values reported in literature may be due to differences in track-etched membranes and not actually reflect the tightness of endothelial junctions[434,461]. For example, track-etched pore diameter and density significantly influence the electric field current paths [434]. Furthermore, due to the flat sheet-like nature of track-etched membranes, cell-substrate adhesion can influence TEER values by artificially inflating values. These sources of error are consistent with reports that surface roughness of the track-etched membranes and pore size influence TEER values[430]. We suggest that our highly porous nanofibers may eliminate most of the error from both cell-substrate adhesion and from distortions in the current paths due to the highly porous nature of our membranes.

Our nanofiber networks have significant advantages for studying cell and nanoparticle migration across the BBB due to their controllable pore size and high porosity. Conventional track-etched membranes are unsuitable for nanoparticle transport studies due to the adhesion of nanofibers to the membranes and within track-etched pores[444]. Our highly porous membrane should minimize interference of the membrane to nanofiber transport. Likewise, modelling cancer metastasis and immune cell migration across the BBB is of significant importance for researchers[462], and our models should enable high-levels of migration due to the high porosity and low membrane thickness. The larger pore sizes (generally $\geq 3\mu\text{m}$ pores) used for cellular transmigration studies have the disadvantage of allowing endothelial cells to cross the membrane and form a second monolayer on the opposite side of the membrane[426,430]. Our thin, highly porous membranes may offer advantages for such studies.

The majority of pericytes are confined to one side of the membrane, however, in some instances it appears that pericytes can migrate across the membrane pores and interact with the endothelial monolayer more closely (see upper right pericyte in Figure 6.5b). Previous studies have shown that migratory astrocytes that cross track-etched membranes for pores as small as $3\mu\text{m}$ in diameter[430]. Migratory pericytes or astrocytes across the membrane clearly is not consistent with the *in vivo* configuration, but is more realistic for our thin membranes.

In our experiments, we limited the nanofiber membrane area to a circle of 2-mm diameter (0.031 cm^2). We chose a small membrane area to reduce manufacturing time of each membrane and constrain the microfluidic chip to a small size. However, our membrane size is small compared with transwell plates (0.143 cm^2 for some commercial 96 transwell designs). Future work could

increase the size of the nanofiber scaffolds using the current methods up to about 3 cm² (1-cm circle). Larger membrane areas, however, require longer fabrication times and are inherently less strong. Large nanofiber membrane would likely need additional support to prevent the delicate fibers from being disrupted by liquid handling and other experimental procedures. Additionally more advanced configuration such as spun fibers around a cylinder could be constructed.

An important consideration when using these nanofiber membranes is that due to the highly porous nature of these membranes, even slight pressure gradients across the network can cause significant trans-membrane fluid flow. In fact, our highly porous nanofiber membranes act more like open channels than walls/barriers. This characteristic makes fluid handling more challenging with nanofiber membranes compared with track-etched membranes. For instance, transwells are generally inverted while seeding endothelial cells on the bottom of the membrane. A similar method for seeding cells on the bottom of a nanofiber membrane would not work, as the liquid would pass through the membrane rather than forming a droplet on top. This channel makes microfluidics a natural choice, since fluid handling is performed within the chip and the entire device can be inverted.

We believe that nanofiber membranes overcome many of the existing limitations and will lead to improved *in vitro* barrier models. The lack of tight barriers in co-culture BBB *in vitro*, and inconsistency of reported TEER values in these models, are in part due to lack of physiologically-relevant basement-membrane mimicking membranes that prevent cell-cell interaction and remove effects of cell-substrate adhesion resistance in TEER values.

6.5 Conclusion

Using an advanced, yet straightforward fabrication technique, we have generated ultra-thin, ultra-porous networks of polystyrene nanofibers that function as *in vitro* basement membranes and significantly increase the physiological relevance of commonly used semipermeable membranes. We constructed a microfluidic model of the blood-brain barrier using human endothelial cells and pericytes, and demonstrated monolayer formation and cell-cell contact across the nanofiber membrane. Our nanofiber membranes can improve the realism of physiological barriers for *in vitro* endothelial and epithelial barrier models and offer new opportunities to modeling human physiology, drug discovery, cell migration studies, and barrier dysfunction studies.

6.6 Methods

6.6.1 Nanofiber fabrication

Dense nanofiber membranes were fabricated according to the previously published Spinneret-based Tunable Engineered Parameters (STEP) method[237,238], schematically illustrated by Figure 1e. Nanofibers were spun around scaffold supports that were laser-cut from plastic coverslips (Fisher Scientific, 0.22mm thick) and contained a hollow region 2 mm in diameter over which the nanofiber membrane was fabricated. Liquid prepolymer was dispensed from a microneedle and nanofibers were spun around a rotating and translating scaffold substrate. The scaffold was then re-oriented and spun again to create orthogonal fiber. The 600 nm-diameter fibers were prepared using a 7 wt % solution of polystyrene (MW: 2000 000 g/mol; category no.

829; Scientific Polymer Products, Ontario, NY, USA) in p-xylene (X5-500; Thermo Fisher Scientific, Waltham, MA, USA). To decrease pore sizes, multiple nanofiber layers were spun across the scaffold. Nanofibers were then chemically fused together. To improve the throughput of the spinning process, and to eliminate the need to remove unwanted fibers from one side of the scaffold, we secured two scaffolds back-to-back by epoxying their stubs together. Prior to integrating them in the devices, the scaffold stub was carefully cutoff.

6.6.2 Microfluidic fabrication

BBB integrity Microfluidic device was fabricated from two polydimethylsiloxane (PDMS) layers that enclosed a plastic nanofiber scaffold. PDMS was mixed 1:10 base to cross-linker as was cured in acrylic molds at 50°C for 4 hours. Acrylic molds assembled from laser cut and solvent bonded layers of acrylic. The lower microfluidic channel and the indentation for the nanofiber scaffold were patterned using a top and bottom acrylic mold pressed together by binder clips. Access holes for the inlet and outlets of the lower channel were punched in the lower PDMS membrane using a 1.5 mm-diameter biopsy punch, and the PDMS layer was plasma bonded (Harrick Plasma) to a glass slide. The upper microfluidic channel was similarly formed in an acrylic mold, and once cured inlet and outlet ports were punched in the upper layer using a 1 mm-diameter biopsy punch. Finally, an top well layer was cured in an acrylic mold and 6 mm diameter holes were punched with a biopsy punch. Upper and lower microfluidic channels were 0.22 mm high, 3 mm wide, and 18 mm long. Liquid PDMS glue was used to bond the nanofiber scaffold between the upper and lower channel layers. Liquid PDMS was spun at 3000 RPM for 1 minute on a glass slide and de-gassed upper PDMS layers were placed channel-side down on the PDMS

layer to absorb the PDMS. Liquid PDMS was loaded into a 1ml syringe connected to a dispensing needle. A small amount was spread on the lower PDMS layer where the indentation for the scaffold was located. The nanofiber scaffold stub was then carefully cut-off, and the scaffold was placed fiber-side down on the lower PDMS layer. The plastic scaffold containing the nanofiber network (or PET membrane for comparison) was then placed in the circular indentation and adhered in place with liquid PDMS glue.

TEER devices were fabricated using a similar method, however two changes were made to enable TEER measurements. Firstly, the channel height was increased from 0.22 mm to 1.52 mm and 1.74 mm for the lower and upper channels respectively. Secondly, acrylic molds were fabricated with laser-engraved electrode guides. (See SI for more information about device molds) Electrodes were inserted into the molds while PDMS was cured such that electrode guides were patterned within the PDMS layers. This method enabled precise placement of the electrodes within the PDMS devices: two in the upper channel and two in the lower channel. Electrodes were epoxied in place, and a 4-pin block terminal was used to connect the device to the potentiostat.

6.6.3 Track-etched membranes

To enable a comparison between the nanofiber membranes, track-etched Polyethylene terephthalate (PET) or polycarbonate (PC) membranes were also incorporated into the microfluidic device. PET membranes were removed from 6-transwell plates (Corning, #3450) and according to the manufacturer were 10 μm thick, had 0.4 μm diameter pores at 4×10^6 pores/ cm^2 . PC membranes (Millipore Sigma, TSTP02500) were also tested and according to the manufacturer were 22 μm thick, had 3.0 μm pores with a porosity of 11.3%. Both PET and PC membranes were carefully

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cut into ~4x4mm squares and bonded to the laser-cut nanofiber scaffolds using liquid PDMS glue. Liquid PDMS was spun at 3000 RPM on a glass slide. One side of the plastic scaffold was placed in contact with the glue, and then removed. The PET or PC membrane was carefully placed across the 2 mm hollow region, and then cured at 55°C for 2 hours.

6.6.4 Cell Culture

Human endothelial cells and pericytes were used to model the BBB. Cell culture was performed according to supplier protocols. Human cerebral microvascular endothelial cells (hCMEC/D3, EMD Millipore) were maintained in EngoGrow™-MV Complete media supplemented with 1 ng/mL FGF-2 (MilliporeSigma), and 1% penicillin/streptomycin (Life Sciences). Primary human brain microvascular endothelial cells (HBMECs, Cell Systems) were cultured in Cell Systems Medium supplemented with 5mL CultureBoost™. Primary human brain vascular pericytes (HBVP, ScienCell) were cultured in pericyte basal medium supplemented with 10 ml of fetal bovine serum, 5 ml of pericyte growth supplement, and 5 ml of penicillin/streptomycin solution. Cells were incubated at 37 °C and 5% CO₂.

6.6.5 Fluorescence microscopy and cell staining.

Antibody staining was performed to investigate cell morphology. Cells were fixed and stained within the microfluidic devices. Cells were fixed in 4% paraformaldehyde for 15 minutes, washed with PBS, and permeabilized in 0.1% Triton-X-100 in PBS for 15 minutes. Cells were then washed with PBS and blocked with 5% normal goat serum in PBS for 15 minutes. Cells were incubated with primary antibodies mixed in antibody dilution buffer consisting of PBS

supplemented with 1:100 w/v Bovine serum albumen (BSA) and 1:333 v/v Triton X-100. Endothelial cells were stained for ZO-1(ZO-1 Polyclonal Antibody, Invitrogen # 40-2200, 1:100) VE-Cadherin (CD144 (VE-cadherin) Monoclonal Antibody (16B1), eBioscience, #14-1449-82, 1:100), and/or CD31 (Human CD31/PECAM-1 Antibody, R&D Systems, #BBA7, 1:33). Pericytes were stained for calponin (Abcam, Recombinant Anti-Calponin 1 antibody [EP798Y] (ab46794), 1:200). Cells were incubated with primary antibodies overnight at 4C. Cells were washed with PBS, and secondary antibodies (Alexa Flour 488, 555, or 647 at 1:400 in antibody dilution buffer) were added to the device for 45 minutes in the dark at room temperature. Finally, cells were washed with PBS, incubated with 300 nM DAPI in PBS for 10 minutes, and then flushed with PBS for imaging.

For live cell imaging, cells with Cell Tracker Green/Deep Red, Calcein-AM, and/or Nucblue. Calcein-AM staining was performed with 4 µl/ml Calcein-AM in cell culture media and incubated for 15 minutes. Two drops of Nucblue were added to culture media and incubated for 15 minutes. For Cell Tracker experiments, endothelial cells were seeded within the device and allowed to attached and form a monolayer for 24 hours. Endothelial cells were then incubated for 45 minutes in 5 µg/ml CellTracker™ Green CMFDA (Invitrogen, #C2925) in serum-free media within the device. The device was then flushed with media. Pericytes were then passaged and suspended in 1 µg/ml CellTracker™ Deep Red dye (Invitrogen, # C34565) for 45 minutes in serum-free media. The pericytes were then centrifuged twice (1000 RPM for 5 minutes) and added to the microfluidic device. One day after staining the pericytes and endothelial cells, the cells were fixed in place with 4% paraformaldehyde and imaged on a confocal microscope.

Nanofibers were stained with rhodamine-conjugated fibronectin (Cytoskeleton Inc.) at 4µg/ml overnight.

6.6.6 Diffusion Experiments

Diffusion experiments were performed by adding McCormick Culinary Blue Food Color Dye (mixture of Blue 1 (MW: 792.85 Da) and Red 40 (496.42 Da)). To prevent dye flow across the membrane, packing tape was used to seal the inlet and outlet ports of the upper channel while filling the device. To decrease evaporation from the device, the entire device top of the device was then sealed with packing tape. Images were collected every minute for 10 hours at room temperature. Diffusion data was then analyzed in ImageJ to determine the time constants of diffusion.

6.6.7 Experimental methods

To prepare the microfluidic devices for experiments, vacuum-degassed devices were sterilized with ethanol and then washed with PBS. The devices were incubated overnight at 37 °C with 4 µg/ml of human fibronectin in PBS. For imaging experiments, rhodamine-conjugated fibronectin (Cytoskeleton Inc) was used at the same concentration. The following day, devices were flushed with endothelial media and incubated for another hour at 37 °C. Endothelial cells were passaged according to supplier instruction and suspended at 1×10^7 cells/ml (2,200 cells/mm²) for the hCMEC cells or at 5×10^6 cells/ml (1,100 cells/mm²) for the HBMECs cells in cell culture media. Before pipetting the cells into the lower channel of the device, the inlet and outlet of the upper channel were blocked with PDMS-clogged pipet tips to prevent fluid flow across the

membrane. Cells were then added to the lower channel and the devices were immediately inverted and incubated at 37 °C for three-four hours. The devices were then placed right-side up, the pipet tips removed, and additional media was added to the device wells. Devices were incubated overnight to allow endothelial monolayer formation. The following day, pericytes were passaged according to supplier instructions and resuspended in pericyte media at 5×10^6 cells/ml (1,100 cells/mm²). PDMS-clogged pipet tips were used to block the lower channel inlet and outlets, and the cell suspension was added to the upper channel. After incubation for three-four hours, the pipet tips were removed, and additional media was added. Pericytes were allowed to spread and interact with the endothelial cells for 24 hours, after which time the device was fixed with 4% paraformaldehyde for imaging.

For TEER devices having an increased channel height, the cell suspension densities were adjusted to maintain 2,200 cells/mm² or 1,100 cells/mm² for the hCMEC and HBMECs/pericytes respectively. For consistent TEER measurements, TEER devices were initially incubated with a 50:50 mix of endothelial and pericyte media, and all cells were suspended in 50:50 media before adding to the device.

6.6.8 Porosity and pore size calculations

Pore size distributions for the nanofiber membranes and track-etched membranes were determined by thresholding Scanning Electron Microscope (SEM) images of the membranes using ImageJ's particle analysis menu. Z-projected (2D) porosity values were also calculated from SEM image in ImageJ by comparing the sum of pore areas with the total imaging area. Actual, 3D

porosity values can be estimated based on fiber diameters and fiber spacing. Porosity can be calculated as:

$$Porosity = \frac{V_M - V_F}{V_M}$$

Where V_M is the total volume of the membrane and V_F is the total volume of the fibers. Since both V_M and V_F depend on linearly on the number of layers, the number of layers does not affect porosity values. Rather, porosity is determined by the fiber diameter and fiber spacing. Porosity of our 2-layer and 6-layer membranes were estimated using nominal 600-nm diameter fibers and 4 μm spacing.

6.6.9 Impedance Spectroscopy and TEER measurements

Transendothelial electrical resistance measurements (TEER) were performed using a potentiostat (Gamry, Reference 600). Electrical impedance spectroscopy was performed from 10Hz to 1 MHz with 10 measurements taken per decade. The applied potential was 0.1 V. A four-electrode configuration was used as described elsewhere in the methods. Impedance measurements were performed at 37C every 24 hours. TEER values were determined by fitting a circuit model to the impedance spectrum and finding the best-fit values.

6.7 Supplemental Tables and Figures

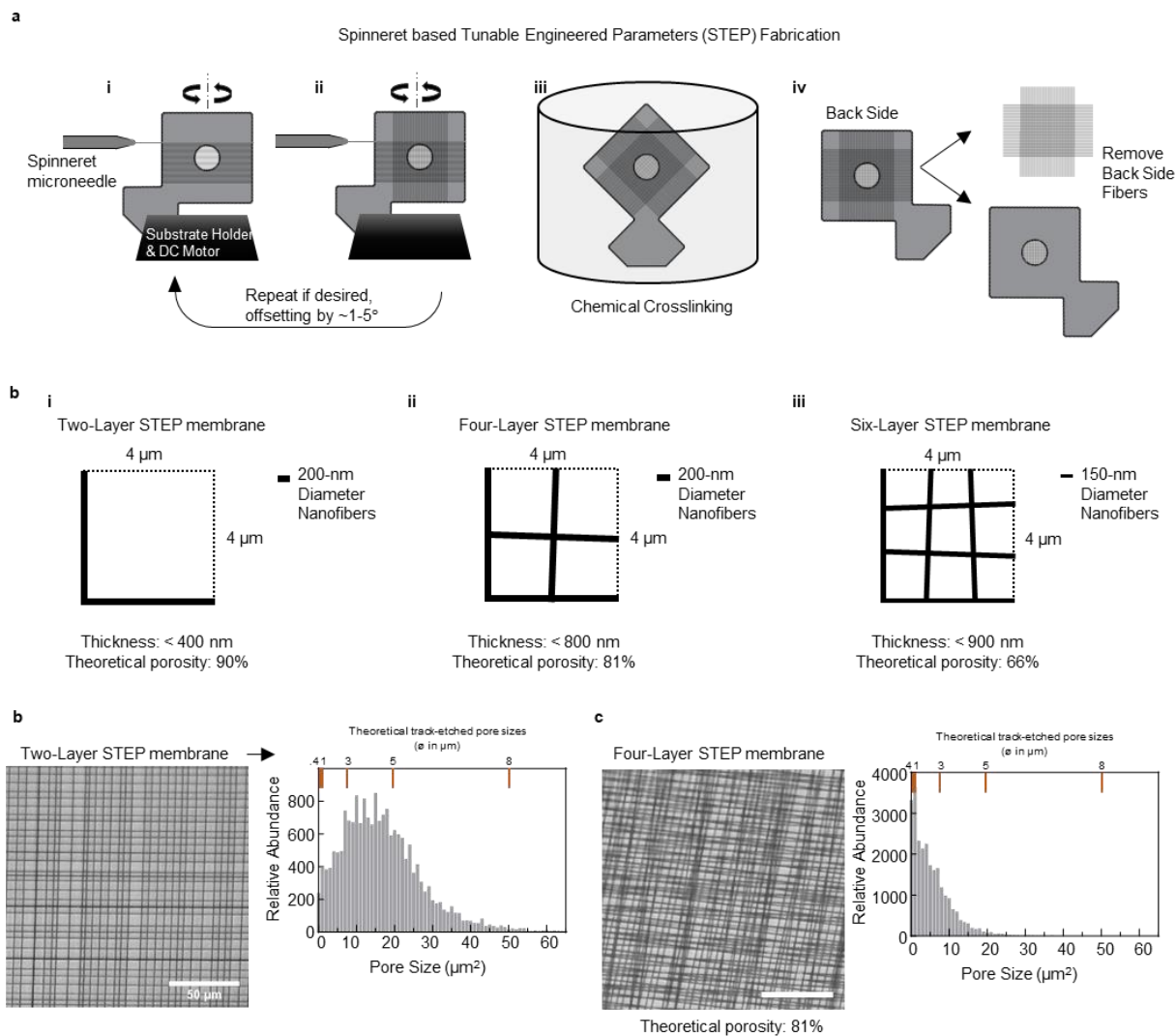


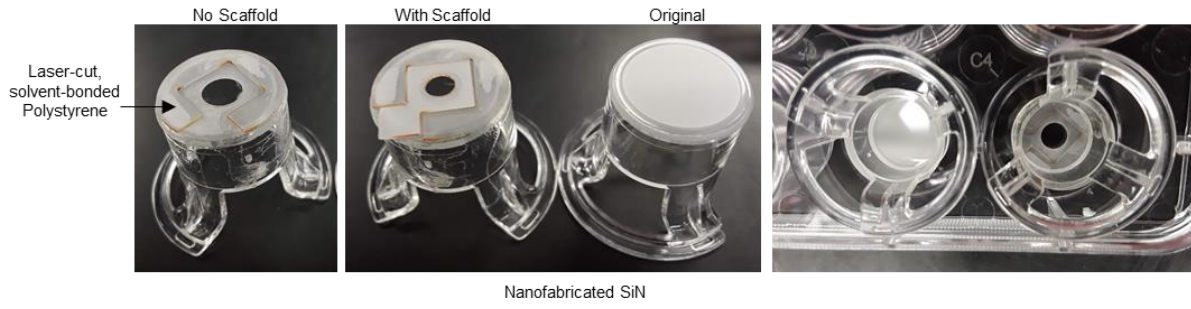
Figure 6.8 Nanofiber Networks.

a) Polystyrene networks are fabricated using a non-electrospinning technique known as Spinneret-based Tunable Engineered Parameters (STEP) fabrication. Polystyrene pre-polymer is dispensed from spinneret microneedles and wrapped around a rotating polystyrene scaffold (i). The scaffold can be re-oriented to spin orthogonal fibers (ii), and this spinning process can

be repeated if desired to create additional fiber layers. The nanofibers are chemically crosslinked (iii), and the back-side fibers are removed (via double-stick tape) before use in BBB models. b) The pore size, thickness and porosity of the membrane can be controlled by fiber spacing, diameter, and the number of fiber layers. For two-, four-, and six- layer scaffolds, ultra-high porosities (>66-90 %) can be achieved with small pore sizes and having thicknesses < 1 μ m.

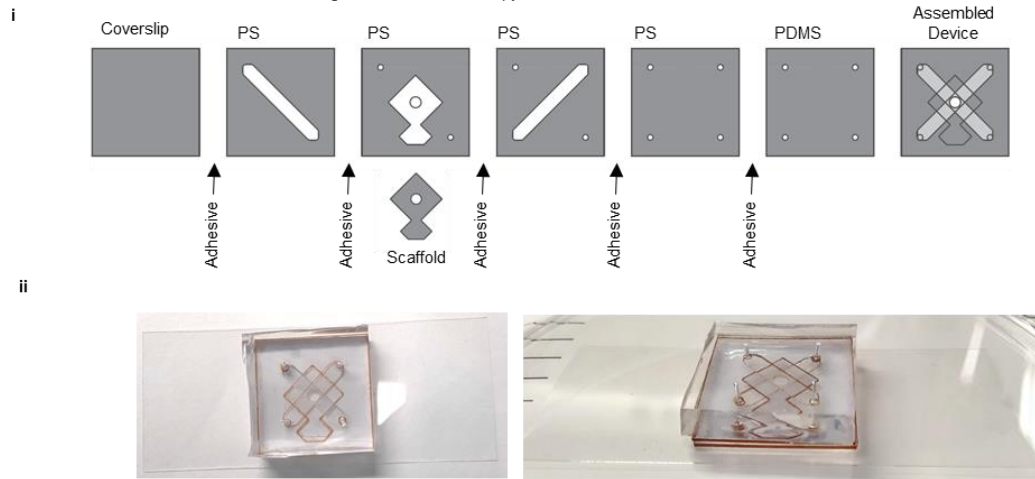
a

STEP membrane integration in Transwell Plates



b

High-Resolution Microscopy Microfluidic BBB Device



c

Electrical Impedance Spectroscopy (EIS) Microfluidic BBB Device

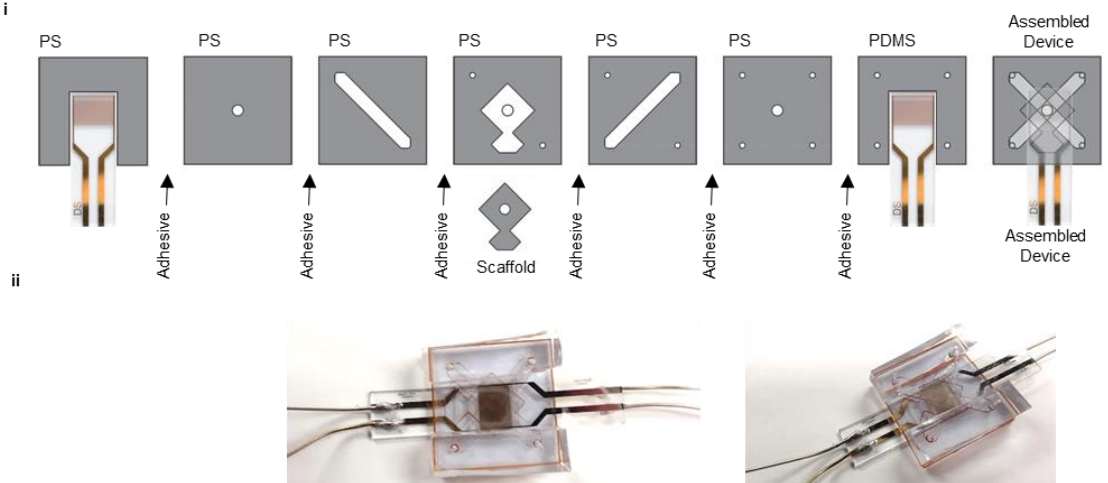


Figure 6.9 STEP fibers can be integrated into Transwells and microfluidic devices.

a) Laser-cut polystyrene layers were solvent-bonded to the bottom of 12-well transwell inserts, to accept the nanofiber scaffolds. Scaffolds were fixed to the transwells with high-vacuum grease. b-c) Nanofiber scaffolds were integrated within two similar microfluidic device; one designed for high-resolution microscopy, and another for electrical impedance spectroscopy (EIS) measurements. Devices were created from laser-cut polystyrene layers (300 μm thick) that were bond with laser-cut, biocompatible acrylic adhesive. A PDMS layer on top of the devices enabled easy interfacing with tubing and pipette tips. The device for microscopy incorporated a glass slide for the base layer, while the device for EIS incorporated two sets of interdigitated gold electrodes above and below the fiber network to monitor barrier tightness.

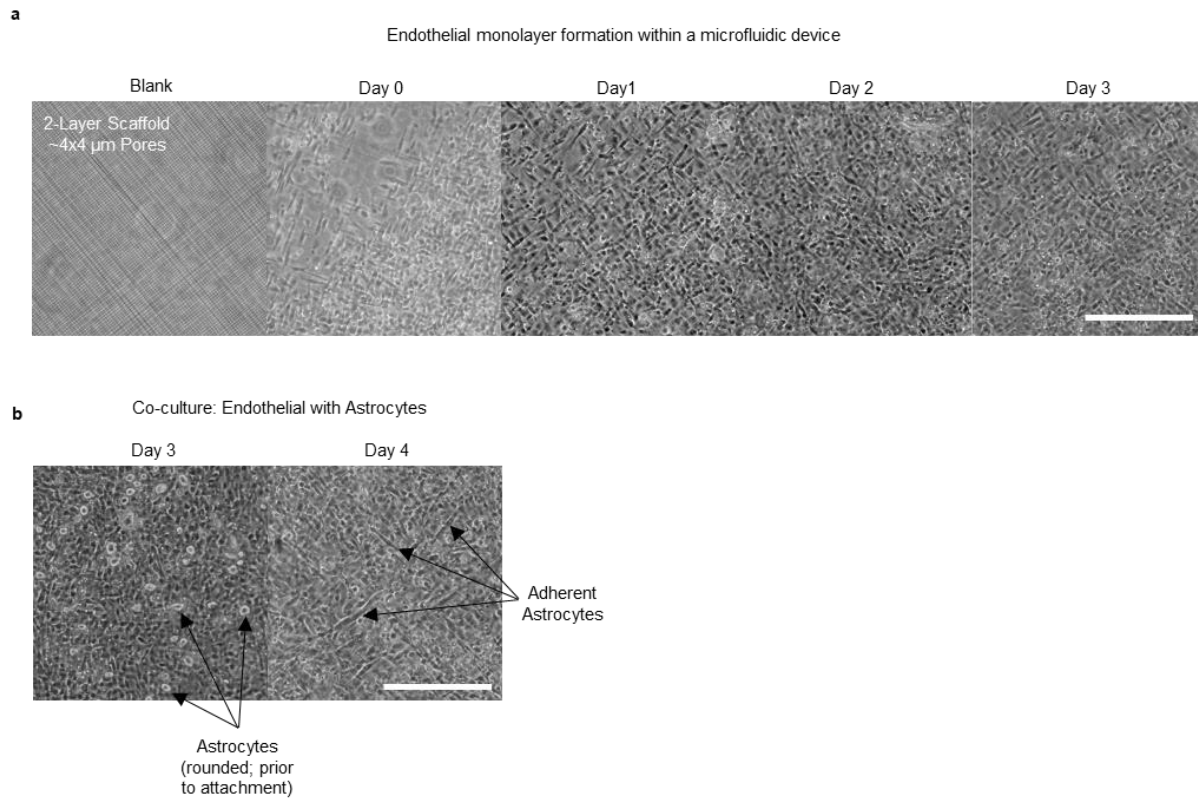


Figure 6.10 Human endothelial cells form confluent monolayers on STEP scaffolds.

a) A blank fiber network within a microfluidic device was seeded with endothelial cells at high-density on Day 0. The endothelial cells quickly established a monolayer by days 2 and 3. b) Supporting cell types can be added to the opposite side of the membrane. Astrocytes, added at on Day 3, are visible as rounded cells in the image, and spread on the monolayer 24 hours later.

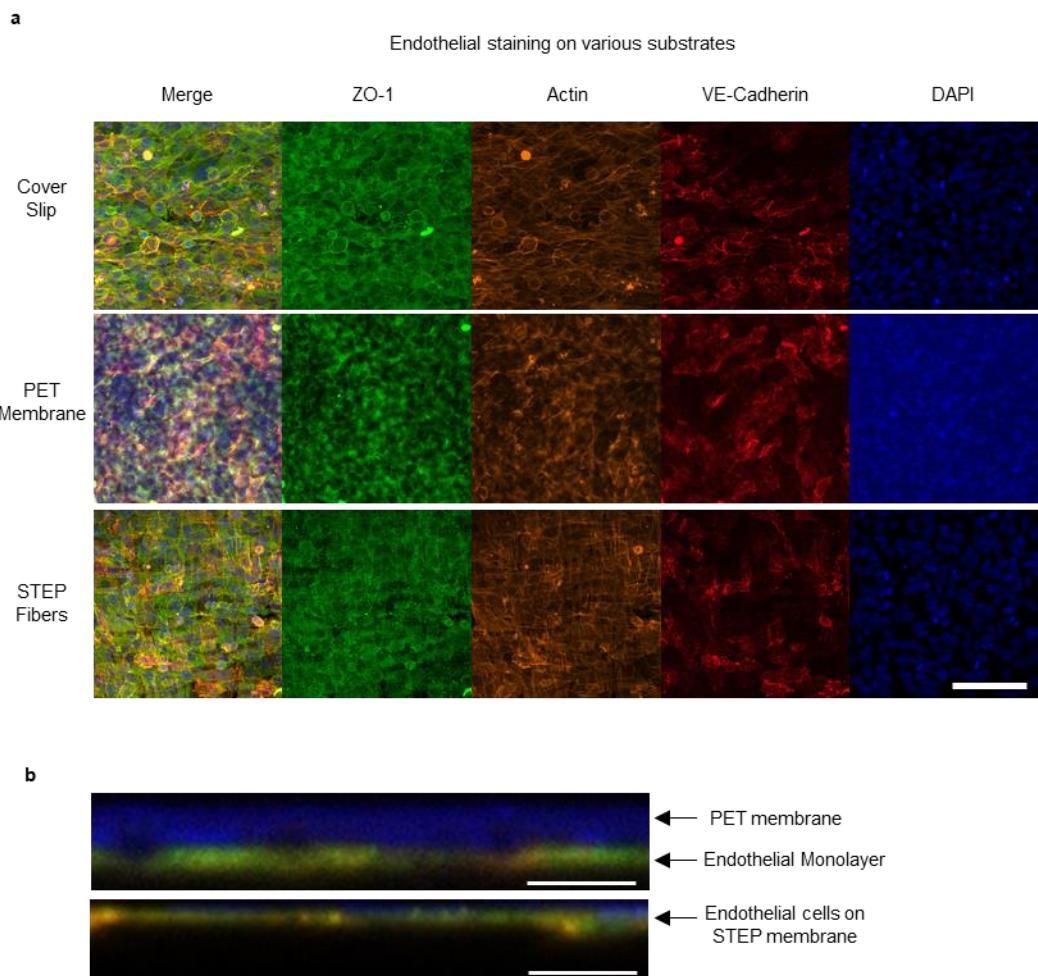
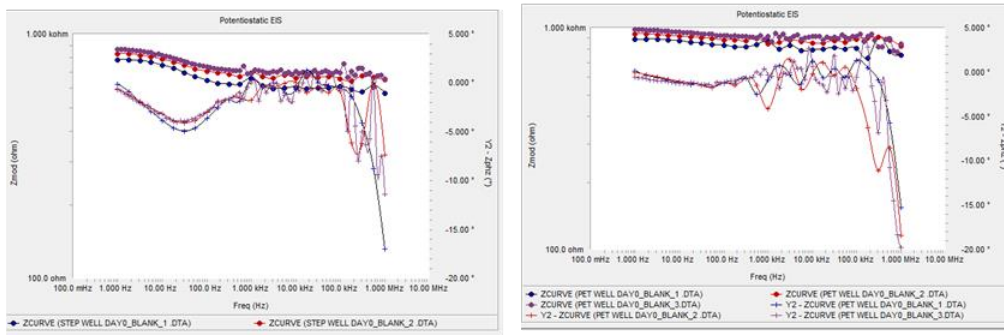


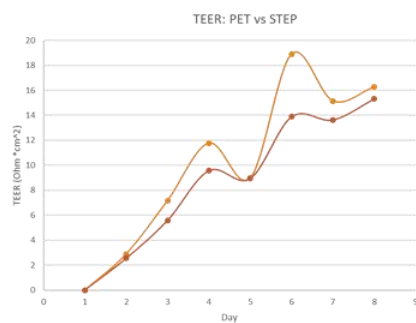
Figure 6.11 Cytoskeletal staining reveals endothelial morphology.

Endothelial cells stained for key markers such as tight junctions (ZO-1), VE-Cadherin, and Actin reveal endothelial cell morphology between substrates.

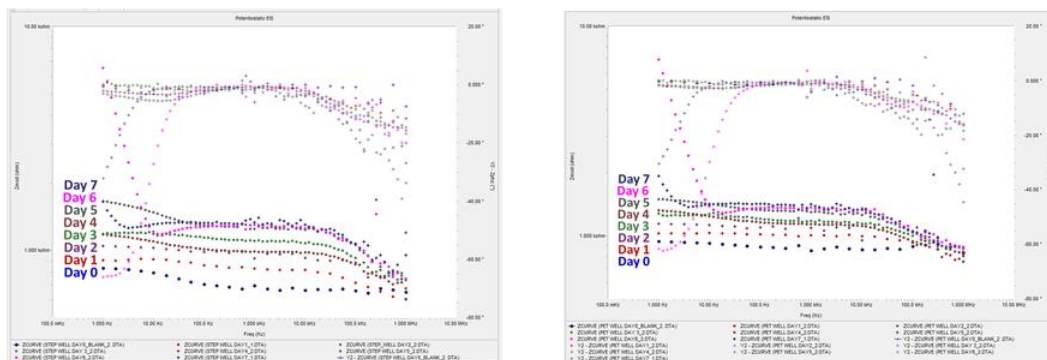
a



b



c



d

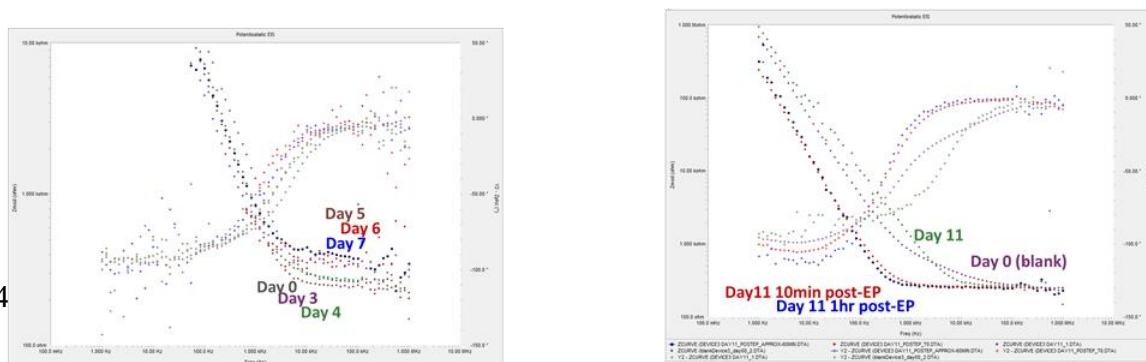


Figure 6.12 Electrical Impedance Spectroscopy from Tranwell and Microfluidic Experiments.

a-c) EIS data from transwell experiments. a) EIS measurements of transwells with STEP (left) and PET (right) before cell-seeding shows that STEP membranes have lower electrical resistance than PET membranes, reflective of the higher porosity of these membranes. b) Transendothelial electrical resistance (TEER) measurements show very similar values between the STEP and PET membranes. c) Impedance Spectra for the first 7 days after cell seeding. Increasing resistance can be seen by the rising trendlines. d) Microfluidic BBB devices show increased electrical resistance during the first 7 days. d) Electroporation changes the impedance spectra.

7 Chapter 7: Cell shape and cytoskeletal structure affect electroporation lethality

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Keywords: Pulsed Electric Fields, Electroporation, Cytoskeleton, Calcium, Actin, Microtubules.

ATTRIBUTION: Aniket Jana is a co-first author of this chapter. Edward Jacobs assisted with the writing of the Methods section.

7.1 Abstract

Pulsed electric fields that cause membrane permeabilization by electroporation have found applications in a variety of fields, yet the impact of cell shape has not yet been fully understood. For most electroporation applications, cell survival and recovery post-treatment is either desirable, as in gene transfection, electrofusion, electrochemotherapy, or is undesirable as in tumor ablation and cardiac ablation. A complete understanding of how cell shape and cytoskeletal morphology

affect cell viability post-electroporation may lead to improved electroporation methods. In this study, we use nanofiber networks within a microfluidic device to generate highly elongated cells with precisely controlled orientation with respect to the electric field. We show that post-electroporation cell viability is highly dependent on cell orientation with respect to the electric field. Additionally, the conductivity of the electroporation buffer significantly impacts viability outcomes in elongated cells. Using fibers of varying diameter, we show that in addition to cell shape, cytoskeletal morphology influences cell viability post-electroporation. Cells on larger diameter fibers had more established cytoskeletons and showed higher viability post-electroporation compared with cells on narrow fibers that had less developed cytoskeletons. We show that cell survival for elongated cells is contrary to the predictions of the standard pore model of electroporation applied with accepted model properties. Our results suggest the standard pore model of electroporation cannot be applied to highly elongated cells without modifications to the model. Furthermore, an improved understanding of cell shape and cytoskeletal structure may lead to improved methods for improving cell viability or death after electroporation techniques by engineering cell morphology and choosing suitable electroporation conditions.

7.2 Introduction

Pulsed electric fields (PEFs) treatments are increasingly popular in both the laboratory and clinic due to their ability to increase cell membrane permeability for therapeutic or otherwise desirable purposes. PEFs induce membrane disruption via a process known as electroporation - high magnitude electric fields induce a transmembrane potential that destabilizes the lipid bilayer and initiates the formation of membrane pores or defects, that enable uncontrolled diffusion across

the cell membrane. Increased membrane permeability can be either reversible or irreversible. For reversible electroporation (RE), cells regain intact membranes and survive. However, high electric fields or high pulse numbers can induce irreversible electroporation (IRE), where membrane disruption overwhelms the cells' ability to maintain homeostasis and leads to cell death. Reversible electroporation is desirable for applications where post-electroporation viability is important, such as gene transfection [78-83], electrofusion [70,71], and electrochemotherapy. Cell death by irreversible electroporation reduces the efficiencies of these techniques. Contrarily, irreversible electroporation has shown great promise for inducing cell death during tumor and cardiac ablation [37-41]. For these applications, complete cell death of the treated tissue is desired. Thus, carefully selecting electroporation parameters for the desired post-treatment cell viability is critical for achieving optimal outcomes.

Membrane disruption during electroporation is driven by an induced transmembrane potential (ITP). An induced transmembrane potential (ITV) causes membrane disruption by electroporation. Electroporation theory and experiments show that pore formation occurs when ITP values exceed a critical threshold ($\sim 0.2\text{-}1\text{ V}$). [9-15] Electroporation mediated molecular transport is limited to regions above the threshold for electroporation[463]. Computational methods are often used to investigate electroporation effects on cells. These models are generally based on well-established electroporation principles [9,14,84,228,464]. Electroporation is conventionally thought of as a membrane phenomenon, however recent research demonstrates that profound cytoskeletal disruption can occur after electroporation [231]. The cytoskeleton is structurally and functionally

linked with the cell membrane, and thus cytoskeletal disruption may be induced directly by the electric fields, or as a secondary response to membrane disruption.

Several studies have investigated how cell size, shape, and orientation affect viability after electroporation. Cells of larger sizes have been shown to be more sensitive to electroporation[465,466], and hemispherical cells have been reported to be more likely to survive than ellipsoid cells with high aspect ratios[465]. Several studies have investigated elongated cells[463,467-470]. Likewise, the response of elongated cells has been shown to be dependent on cell orientation with respect to the electric field. One study suggests that elongated cells are less affectively permeabilized by electroporation compared to spherical cells[468]. A recent report suggests that 1 μm pulses can achieve homogenous electroporation on elongated, randomly-oriented cells, whereas shorter and longer pulses preferentially electroporation cells of a particular orientation[471]. Electroporation is also influence by media conductivity[469,472-474], media composition (especially calcium concentration)[475], and pulse parameters (pulse duration, polarity, number, and frequency).. Many of these studies focus solely on reversible electroporation and use membrane impermeant dyes or calcium dyes to investigate dye uptake. Fewer studies, however, seek to quantify cell viability after pulses. Despite previous studies, a clear experimental and computation analysis of cell viability for highly elongated cells post-electroporation has yet to be presented.

Standard electroporation procedures are performed with cells in suspension or adherent to a flat, 2D surface or a randomly-oriented 3D fibrous environment (as in hydrogels). When suspended, the cell cytoskeleton is altered, and cells take on a roughly spherical shape. Adherent cells, either

in 2D or 3D generally take on random shapes and orientation. Because precise control of cell shape, structure, and orientation with-respect-to the electric field is not possible with these systems, understanding the effects of these factors is challenging. To overcome these challenges, we use networks of polystyrene nanofibers to precisely control cell shape.

In a previously published study, we found that cell viability was not well predicted by simple computational models[476]. In this study, we further investigate these discrepancies using an improved microfluidic device. Our central scientific premise is that the impact of cell morphology (shape & cytoskeletal structure) on cell survival after electroporation is not well understood. The precise control of cell shape and orientation enabled by nanofiber scaffolds provides a convenient and expedient platform for investigating a wide variety of factors (morphological and experimental) on cell viability. We seek to link specific morphological characteristics with cell susceptibility to electroporation, which will enhance fundamental knowledge and be widely useful for improving electroporation techniques where cell viability is desirable (gene transfection, electrofusion, electrochemotherapy) or where cell viability is undesirable (tumor ablation, cardiac ablation).

7.3 Results

7.3.1 A microfluidic device encloses two nanofiber scaffolds that control cell shape and orientation for controlled electroporation.

We fabricated a polydimethylsiloxane (PDMS) microfluidic device to contain two nanofiber scaffolds between three embedded needle electrodes (Figure 7.1a). The microfluidic device encloses two nanofiber scaffolds. Nanofibers were spun with the non-electrospinning Spinneret-based Tunable Engineered Parameters (STEP) method, schematically illustrated in Figure 7.1b. Briefly, liquid pre-polymer is dispensed from a microneedle and spun around a rotating and translating substrate. To achieve orthogonal fibers, the substrate (scaffold) is then reoriented and re-spun. For this study, 250-nm diameter nanofibers were spun approximately 16- μm apart and were fused with orthogonal 2- μm diameter fibers spaced approximately 300 μm apart. To complete the nanofiber network, the fibers were chemically fused together to create rigid intersection points. Prior to integrating the scaffolds into the device, the stubs were removed.

Two scaffolds were integrated within the device enable two fiber orientations to be tested simultaneously: One scaffold was orient such that the 250-nm diameter fibers were aligned with the electric field while the second scaffold was orient such that the 250-nm diameter fibers were perpendicular to the field (Figure 7.1c). Cells seeded within the device naturally spread on one fiber causing an elongated spindle shape, or on two fibers causing an elongated rectangular shape.

We electroporated elongated cells within the device using ten, 100 μs square electrical pulses delivered at 1Hz. Finite element modelling of the electric field within the scaffold region

of interest revealed that for every 1 V applied, and average electric field of 1.1 V/cm was generated across the nanofibers. We performed experiments at 500, 1000, 1500, 2000, 2500, and 3000 V/cm to cover the entire range of cell behavior.

To test the effect of pulsation buffer composition and conductivity, we tested four pulsation solutions: regular growth media, calcium-free DMEM, HEPES buffer and a low-conductivity sucrose solution. Pulsation buffer properties are presented in Table 7-1.

Table 7-1 Pulsation buffer properties

Pulsation Solution	Composition	Electrical Conductivity (mS/cm)	pH	Calcium Concentration (mM)	Osmolarity (mOsm)
Growth Media	DMEM + 10% FBS + 1% PS	12.8	7.1	1.95 mM	355
Calcium-Free DMEM	DMEM, no calcium (Gibco 21068028).	14.5	7.4	< 0.05	356
HEPES Buffer	HEPES, Sucrose, Water [477]	0.975	7.2	~ 0.0	
Low-Conductivity Sucrose Solution	Sucrose, NaCl, Water	0.0998	7.3	~ 0.0	

DMEM, Dulbecco's Modified Eagles Medium; FBS, Fetal Bovine Serum

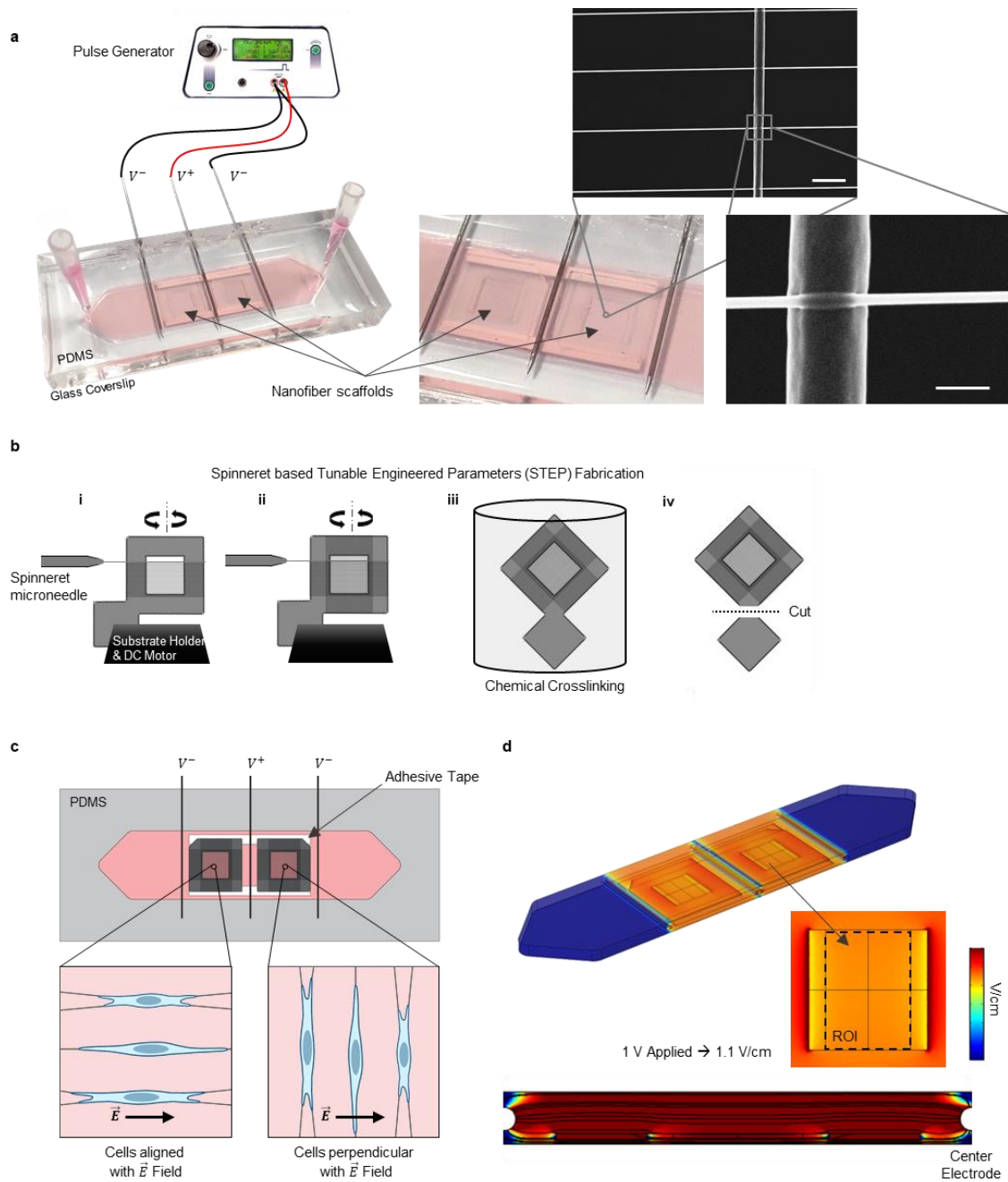


Figure 7.1 A microfluidic device with enclosed nanofiber networks precisely control cell shape and orientation.

a) Two nanofiber scaffolds are contained within a single PDMS channel and electroporated with needle electrodes connected to a high voltage generator. b) the symmetry of the scaffold enables networks of any orientation to be fabricated without changing the electric field within the nanofiber regions. c) Adhesion of cells to nanofiber networks enables cell shape and orientation with respect to the electric field to be controlled. In addition to the traditional adherent and suspended cell shapes, nanofiber-attached cells take on spindle and 2-fibers. Cell orientation for spindle and 2-fiber cells can be controlled with nanofiber direction.

7.3.2 Cell orientation has a significant effect on post-electroporation cell viability.

Our analysis of cell viability for four pulsation buffers with cells aligned and perpendicular to the electric field are presented in Figure 7.2. We found the cell orientation has a profound impact on post-pulse cell viability. When cells are electroporated in regular media, cells in the parallel orientation have significantly higher cell viability compared to cells electroporation perpendicular top the electric field. Plotting the difference in average viability between cells in the perpendicular and parallel show that the difference reaches a maximum near 1500 V/cm. Impressively, there is nearly a 75% difference in average viability between the two orientations at 1500 V/cm. Similar trends prevail when cells are electroporated in calcium-free DMEM. In calcium-free DMEM, cells in the parallel orientation show significantly higher rates of post-pulse viability compared to cells in the perpendicular orientation, with a maximum difference well over

50% at 1500 V/cm. Electroporation of cells in HEPES buffer shows identical trends, however the difference in viability between the two orientation is less pronounced, with a maximum difference of around 40%. Interesting, electroporating cells in low-conductivity sucrose solution results in opposite viability trends: cells in the perpendicular orientation show significantly higher post-electroporation viability compared with cells in the parallel orientation.

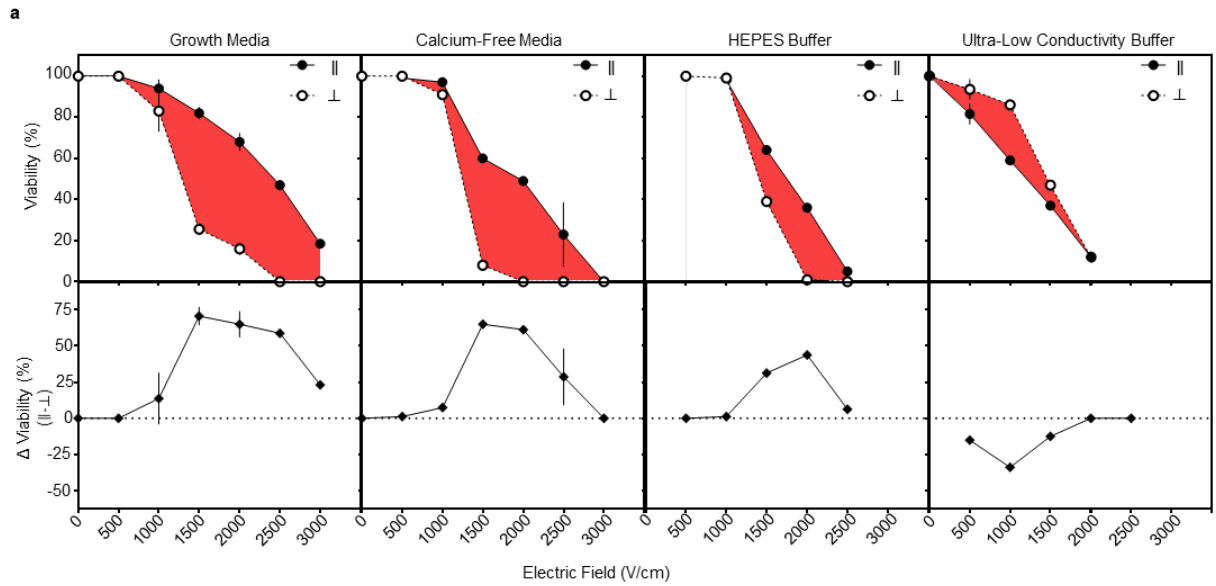


Figure 7.2 Cell orientation to the electric field significantly affects viability and is dependent on pulsation buffer conductivity.

a) Percent viability of U251 cells after electroporation for cells aligned (||) with the electric field or perpendicular to the electric field ($\perp$). Both regular media and calcium-free media show significantly higher viability for cells aligned with the electric field, reaching a maximum difference over 60% at 1500 V/cm compared with cells oriented perpendicular to the field. Both calcium-free media and growth media show a dramatic decrease in viability at 1000 V/cm in the perpendicular orientation. Electroporation in HEPES buffer, having ~10-fold lower electrical conductivity, results in a higher viability for parallel cells, but the maximum difference is less than regular and calcium-free media. Contrary to these trends, electroporating cells in low-

conductivity sucrose solution (~100-fold less conductive than media) reverses the viability trends: cells perpendicular the field have a higher viability.

7.3.3 Cell aspect area and cell spread area impact cell outcomes.

To better understand the factors impacting the post-electroporation viability of elongated cells, we analyzed the z-projected spread area and aspect ratio (length to width) of cells for experiments that contained populations of both live and dead cells post-electroporation. Figure 3 shows the results of these analyses. We find that in some case, cell spread area and cell aspect ratio significantly influence the outcome of electroporation. Cells treated in HEPES buffer that are in the parallel orientation show that both spread area and cell aspect ratio significant influence post-pulse viability. Similar trends are seen in calcium-free media, however cell aspect ratio is not significantly different. Although aspect ratio and spread area do not show significance for cells in the perpendicular orientation, our results suggest that the influence of these factors may be reversed: lower spread area may actually increase the chances of survival post-electroporation.

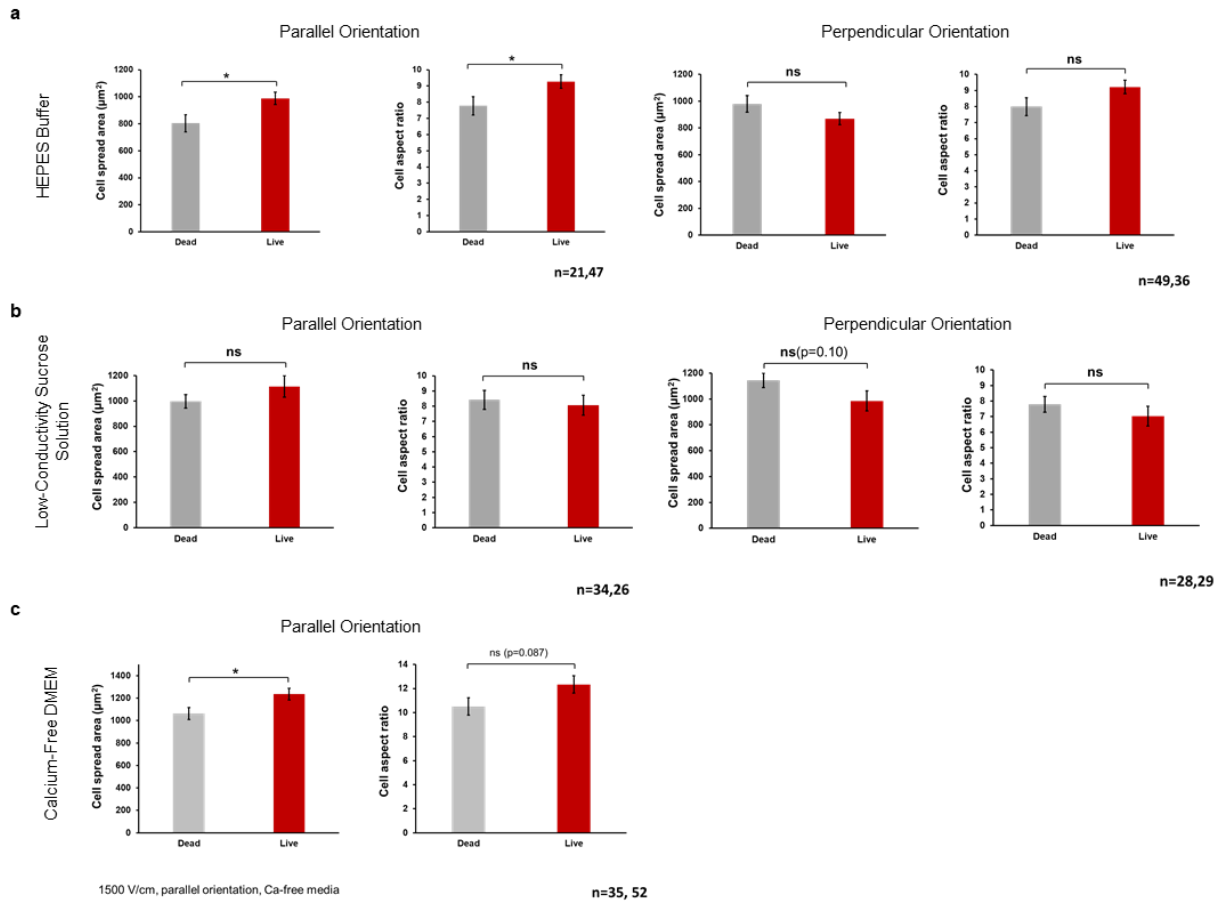


Figure 7.3 Cell aspect ratio and z-projected spread area influence cell viability post-electroporation.

Pre-electroporation cell aspect ratios (length to width) and spread area were compared for cells that lived and died after treatment when treated in a) HEPES buffer, b) low-conductivity sucrose solution, and c) calcium-free DMEM. For HEPES buffer and calcium-free DMEM show significant differences in the spread area of cells for cells in the parallel orientation. Cells with higher spread area generally show a greater likelihood of post-electroporation survival. Trends for the perpendicular orientation do not show significance but suggest that correlation between

spread area and viability may be reversed in these cells. Cell aspect ratio is also significantly different between live and dead cells in the parallel orientation when treated in HEPES buffer.

7.3.4 Computational models of electroporation do not predict experimental results.

We created realistic 3D finite element models to simulate membrane disruption using standard pore models of electroporation. We performed time-dependent simulations to model the evolution of induced transmembrane potential and pore density during the high-voltage pulse. Figure 7.4 shows the results of these simulations for an applied electric field of 1000 V/cm applied parallel and perpendicular to the cell orientation and for three extracellular media conductivities. As can be seen, cell orientation has a significantly more profound impact on ITV and pore density than extracellular media. For cells in the parallel orientation, high ITV values are found near the cell protrusions. Contrarily, high ITV values are found near the central sidewalls for cells in the perpendicular orientation. Decreasing the extracellular media conductivity increase the ITV on the cell membrane for cells parallel with the field but does not have a noticeable impact for cells in the perpendicular orientation. Plotting the average ITV across the cell membrane for all conditions (Figure 7.4b) demonstrates that ITV is significantly elevated in the cells oriented in the parallel orientation compared with the perpendicular orientation.

Plotting the average pore density across the cell membrane shows that for both orientation there are small regions with extremely high pore densities: in the parallel orientation at the

extremities of the protrusions and at the widest sections of the sidewalls in the perpendicular orientation. Lower pore densities surround these regions of very high pore densities. When average pore densities are plotted for all simulated conditions (Figure 7.4c), cells in the parallel orientation show a higher average pore density by several orders of magnitude compared to cells in the perpendicular orientation.

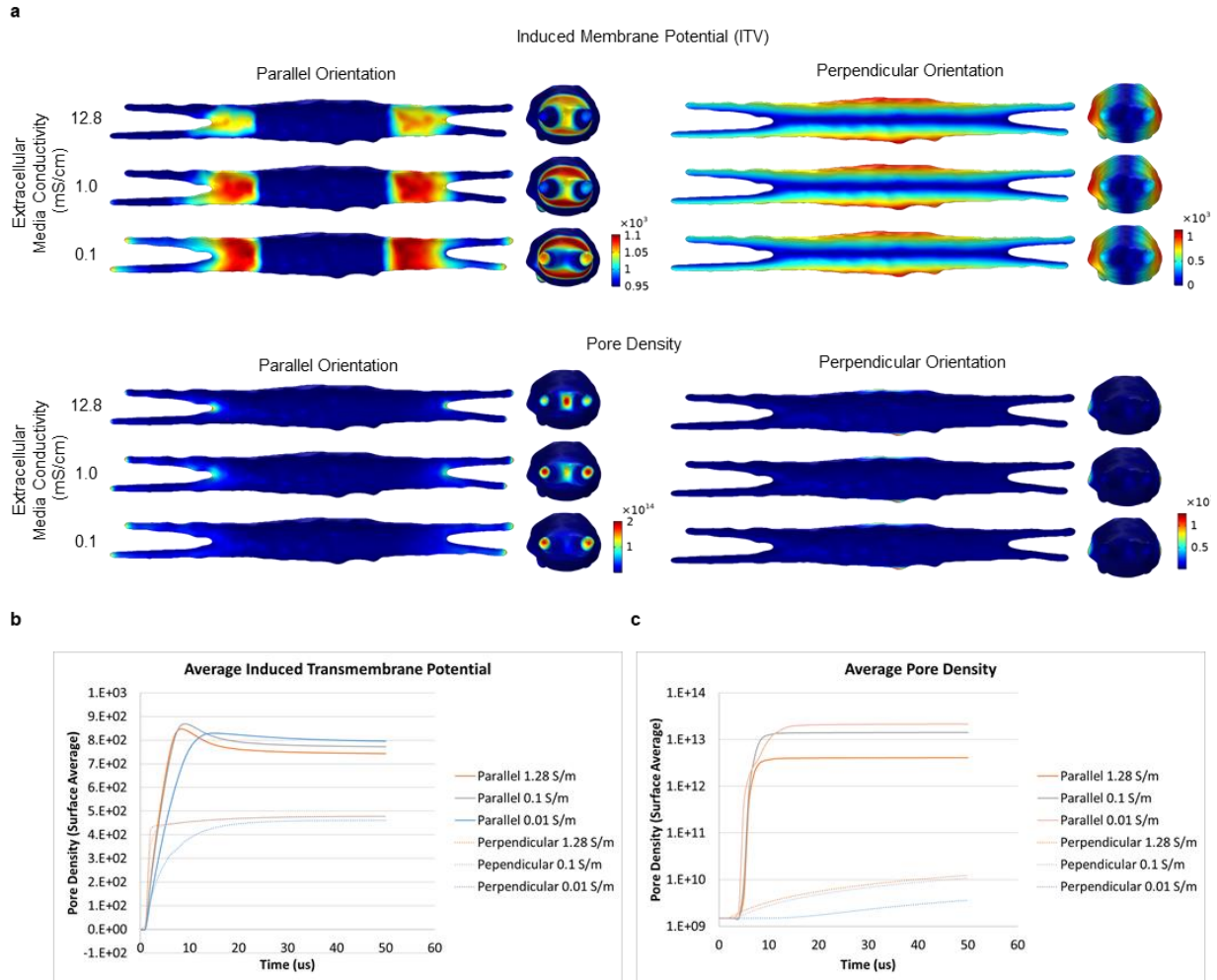


Figure 7.4 Finite element models of electroporation indicate greater membrane disrupt on cells aligned with the field.

a) COMSOL models of the induced transmembrane potential (ITV) indicate that cells in the parallel orientation have high ITV values near the cell protrusions at the extremities, while cells in the perpendicular orientation have high ITV values near the central sidewalls of the cells. b) Likewise, cells in the parallel orientation also show higher pore numbers. c) Comparison

between the average ITV (left) and average pore number, N , (right) show that the parallel orientation has higher values, indicative of more membrane disruption. This result does not agree with our experimental data on cell viability post-electroporation. c) COMSOL models of the induced transmembrane potential (ITV) indicate that cells in the parallel orientation have higher average ITV values compared to cells in the perpendicular orientation. Likewise, models predict higher average pore density on cells in the parallel orientation compared with cells in the perpendicular orientation.

7.3.5 Cell shape may influence transfection efficiency.

Transfection of genes into cells is an important laboratory technique used for a wide variety of applications. Electroporation is an increasingly popular method for transferring genes into cells. The high electric fields generated during electroporation drive negatively charged DNA plasmids into contact with the cell membrane, and the membrane disruption due to the electric field enhances the uptake of the DNA. For transfection, long millisecond pulses are generally used to enable time for electrophoretic movement of the DNA.

To investigate if cells could be transfected via electroporation while attached to nanofibers, we electroporated cells with a single 20 ms pulse at 500 V/cm in 50 $\mu\text{g/ml}$ of the mammalian expression vector for expression of GFP pEF-GFP from AddGene. Figure 7.5 shows that cells can be transfected while adherent to nanofibers in both spindle (1-fiber) and 2-fiber configurations.

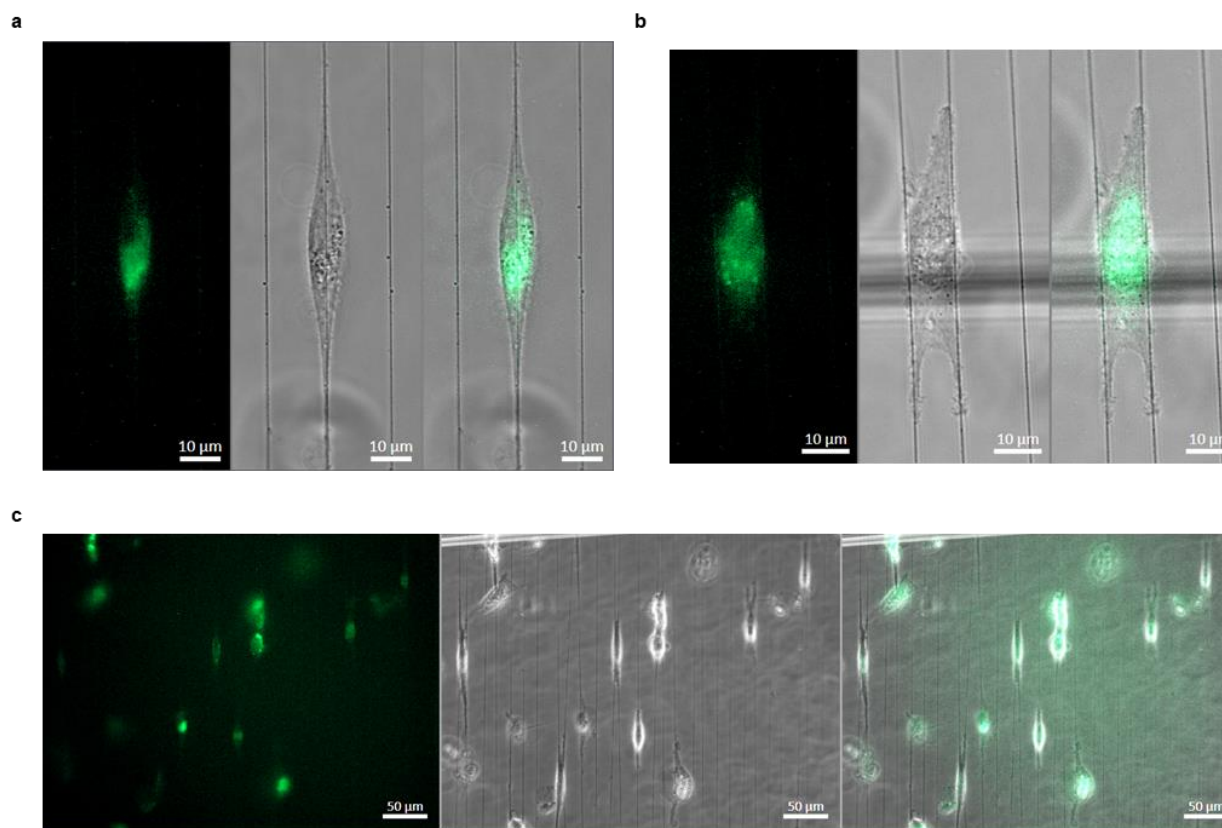


Figure 7.5 Proof-of-concept transfection of elongated cells on nanofibers.

a) Cells electroporated with a GFP plasmid show expression after 16 hours for both a) spindle and b) 2-fiber cells parallel with the electric field. c) high expression levels are seen for cells electroporated on nanofibers. Cells were electroporated with a single 20 ms pulse at 500 V/cm.

7.4 Discussion

Cell viability post-electroporation was found to be dependent on both cell orientation and pulsation buffer conductivity. For media conductivities that significantly exceed the cytoplasmic conductivity of the cells (~ 2.5 mS/cm), we found that there is a profound effect of cell orientation on post-electroporation viability: Cells elongated in the direction of the electric field have significantly higher rates of survival compared to cells in the perpendicular orientation, with the maximum differences in viability reaching around 75%. Since regular media and calcium-free media show analogous trends, it seems unlikely that calcium entry into the cells is driving the difference in viability. Similar trends are found when electroporating in HEPES buffer, which has a conductivity comparable to that of the cell cytoplasm. However, in HEPES buffer the influence of cell orientation is slightly less pronounced. Finally, when cells were treated in a pulsation buffer with a conductivity much lower than that of cytoplasmic conductivity, the viability trends reverse: cells the perpendicular orientation show higher rates of survival compared to cells in the parallel orientation, with a maximum difference around 35%.

The dependance of post-pulse viability on pulsation buffer conductivity can be intuitively understood by considering the cell and pulsation buffer as two resistors in series. Considering just the parallel orientation, when the extracellular buffer conductivity is higher than the cell, electrical current is more likely to pass through the media and be shunted around the cell. However, when the cytoplasmic conductivity is significantly higher than the extracellular media, electric current is more likely to cross the cell membrane and travel through the cells. In this case, the elongated cells as an electrical “wire” where current enters one end and exits at the far end. Comparing these

situations, it is intuitive that increased current travelling across the cell membrane will lead to more membrane disrupt and adversely affect cell viability.

Our computation models of electroporation show that according to the standard pore models, cells in the parallel orientation should have significantly higher ITV and pore densities compared to cells in the perpendicular orientation for all extracellular media conductivities. Future work is warranted to reconcile the difference between model predictions and experimental results. The disagreement may arise from several sources such as differences in molecular transport, different levels of nuclear permeabilization, and cytoskeletal structure.

We show that cells can be transfected with plasmid DNA while elongated on nanofibers. Since orientation significantly affects cell viability after electroporation, transfecting cells on nanofibers to regulate cell shape and orientation may yield improvements in transfection efficiencies. One significant drawback to transfection by electroporation is that high transfection efficiencies are hampered by low cell viability post-electroporation. By electroporation highly elongated cells, high transfection efficiencies and high viabilities might be attained.

7.5 Conclusions

In this study we have analyzed post-electroporation viability of highly elongated cells with precise orientations relative to the electric field and compared experimental results with computation predicts based on current electroporation models. Contrary to model predicts of ITV and pore density, our experimental results show that cell viability is higher for cells aligned with the electric field compared with cells perpendicular to the electric field for all pulsation buffers

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tested accept very low-conductivity sucrose buffer. In a low-conductivity sucrose buffer, the influence of cell orientation reverses. Our results suggest that changes to the standard pore model of electroporation and/or parameter values need to be made to accurately model evaluate the effect of electroporation of highly elongated cells. Taken together, our results suggest that controlling cell orientation with respect to the electric field may offer advantages for electroporation techniques where high cell viability or high cell death are desired.

7.6 Methods

7.6.1 Nanofiber fabrication

We used a non-electrospinning method known as Spinneret-based Tunable Engineered Parameters (STEP) to fabricate precisely designed nanofiber scaffolds[476]. Briefly, liquid prepolymer was dispensed from a microneedle and fibers were spun around a rotating and translating polystyrene scaffold. 250- μm diameters were deposited approximately 16 μm apart, and orthogonal to 2- μm diameter large support fibers. Some experiments tested larger, 2 μm diameter fibers spaced approximately 16 μm apart.

The nanofiber scaffolds were laser cut from plastic cover slips (Fisher Scientific), with hollow rectangular region of 3.5 x 3.5 mm across which the nanofiber networks are suspended. The outer dimensions of the nanofiber scaffold are 7 x 7 mm, with an attached stub for mounting during the fabrication process. The plastic scaffolds are symmetric, such that two nanofiber scaffolds can be integrated into the same device yet have different orientation of the fibers without losing the symmetry of the device.

7.6.2 Microfluidic device fabrication

The microfluidic device consisted of a single channel 1 mm high, 9 mm wide, and 38 mm long, with a total volume of 325 μl . The device was comprised of a Polydimethylsiloxane (PDMS) channel (volume of $\sim 350 \mu\text{l}$) bonded to a glass cover slip. PDMS, mixed 10:1 base to cross-linker, was cured at 80C for 2 hours within an acrylic mold. The mold was fabricated from stacked and bonded layers of acrylic and Polyethylene (PET) sheets. Plastic sheets were laser-cut and bonded together with Weld-on 4. The mold laser-etched electrodes guide channels that enable electrodes to be inserted during PDMS curing, but removed before peeling off. This method provided electrode guides in the PDMS for precise and consistent electrode placement. See SI for additional information about the mold. After curing, the PDMS was peeled off the mold, and inlet and outlet holes were punched with a 1-mm biopsy punch. Three needle electrodes (Acupuncture needles, diameter 0.45 mm) were inserted into the PDMS mold using the guide channels, each spaced exactly 9 mm apart. Securing the nanofiber scaffolds to the glass cover slip was accomplished using two strips of biocompatible 3M™ Microfluidic Diagnostic Tape (#9965, Double Sided White Tape, 89 μm thick). The adhesive strips (16 mm x 1.25 mm) were spaced 5.5 mm apart and centered on the glass coverslip. The scaffolds were carefully adhered to the tape. Prior to placing the scaffold, a surgical scissor was used to carefully cut off the stub from the scaffold, leaving only the square region. With adherent scaffolds, the cover slips and PDMS layers with inserted electrodes were placed in a plasma cleaner (Harrick Plasma) and plasma treated for 2 minutes. The PDMS was then immediately bonded to the cover slip to enclose the scaffolds. To seal the

electrodes and prevent leakage, the electrodes were sealed in place with household silicone sealant. Assembled devices were then placed under vacuum until use.

7.6.3 Cell Culture

U251 malignant glioma cells (Sigma Aldrich) were maintained in Dulbecco's Modified Eagle Media (DMEM, Sigma Aldrich) without MgCl and supplemented with 1% penicillin/streptomycin (Life Sciences) and 10% fetal bovine serum (Atlanta Biologicals). Cells were kept at 37°C and 5% CO₂ and passaged regularly at 80 – 90% confluency. To passage, the cells were removed from the flask with 0.25% trypsin and centrifuged at 160 x g for 5 minutes. The cells were resuspended in fresh media and passaged 1:4 into a new flask with fresh media.

7.6.4 Buffer composition

Four buffers were tested in the study: regular growth media, calcium-free DMEM, HEPES buffer, and a low-conductivity sucrose solution. Solution conductivities, pH, and osmolarities are presented in Table 7-1. Regular growth media was comprised of Dulbecco's Modified Eagle Media (DMEM, Sigma Aldrich) without MgCl supplemented with 1% penicillin/streptomycin (Life Sciences) and 10% fetal bovine serum (Atlanta Biologicals). No calcium DMEM (Gibco 21068028) was measured to have less than 50 µM calcium. HEPES buffer was composed of 10 mM HEPES, 250 mM sucrose, and 7.5mM NaCl, as described previously[477]. Low-conductivity sucrose solution was composed of deionized water with 8.5% w/v sucrose and 1% v/v calcium-free DMEM. The HEPES buffer and low-conductivity sucrose solution were titrated to 7.2 using 1M HCL or NaOH.

7.6.5 Experimental Procedure

The devices were taken from the vacuum and immediately filled with 500 μ l of ethanol at an angle to prevent the formation of bubbles in the device and on the scaffolds. Ethanol was flushed out of the device with 1000 μ l of phosphate buffered saline (PBS, info), and then filled with 500 μ l of 4 μ g/ml human fibronectin in PBS. The devices were incubated at 37°C 5% CO₂ for 1 hour. T25 flasks of cells were trypsinized with 0.5 ml of 0.25 % Trypsin for 2 minutes, suspended in 4.5 ml of DMEM media, and centrifuged at 160 x g for 5 minutes. The supernatant was aspirated and the pellet was resuspended at 30,000 cells/ml. For each device, 500 μ l of the cell suspension was pipetted into the channel. The device was incubated at 37°C and 5% CO₂ for 2 hours to allow the cells to adhere and spread on the fibers prior to the start of experiments.

The microfluidic devices with adherent cells were imaged with phase contrast 10x objectives on a Zeiss Axio Observer inverted microscope or a Leica DMI8 inverted microscope. Brightfield tile-scans were collected for the fiber layers on both the parallel and perpendicular oriented scaffolds within the device. Imaging was performed at room temperature. The devices were then taken off the microscope and prepared for electroporation. For experiments involving growth media, devices were directly connected to a high-voltage square wave electroporation system (BTX 830). For experiments involving calcium-free DMEM, HEPES buffer, and low-conductivity sucrose solution, the device was flushed with 1ml of these solutions and then connected to the electroporation system. The central electrode was connected to the positive output of the generator, while the other two electrodes were connected to the negative output. Ten, 100 μ s electrical pulses were delivered at room temperature to produce electrical fields of 500 to 3000

V/cm across the nanofiber scaffolds. Following electroporation, devices were maintained at room temperature for 5 minutes. For electroporation experiments in growth media, cells were then transferred to an incubator at 37°C and 5% CO₂ for incubation overnight. For experiments involving calcium-free DMEM, HEPES buffer, and low-conductivity sucrose solutions, the devices were slowly perfused with 1 ml of growth media at 250 µl/ml with syringe pump (Harvard Apparatus). Devices were perfused at a slow rate so as not to dislodge recovering/dead cells from the nanofibers. was flushed with 1ml of these solutions and then connected to the electroporation system.

The following day (approximately 14-16 hours after electroporation) devices were stained with 15 µl/ml of propidium iodide (PI), 4 µl/ml of Calcein-AM green, and 2 drop/ml of NucBlue. The addition of the dye solution to the device was performed by slowly perfusing 350 µl of dye solution at 100 µl/min so as not to dislodge any dead/dying cells from the fibers. Phase contrast and fluorescence tile-scans of the fiber regions were collected at the 10X magnification.

7.6.6 Image Analysis

Tilesan images were stitched in ZEN or LAS X software. Prior to analysis, the region-of-interest (ROI) for analysis was defined by measuring 0.5 mm from the edge of both electrode-facings inner sides of the scaffold and excluding data within this area of the scaffold (due to the non-uniform field in these regions). In the remaining scaffold region, cells were sequentially numbered in ZEN or LAS X. Cells were numbered in the pre-pulse tile scans, including only spindle cells (adherent to 1 fiber) and 2-fiber cells that were not touching other cells or the larger orthogonal support fibers. The numbers were then copied to the corresponding post-pulse tile scan and adjusted if

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necessary. Due to the low cell density on the nanofibers, nearly all cells in the pre-pulse image could be definitively associated with their corresponding cell in the post-pulse images. However, in a minority of cases, cells appearing in the pre-pulse image did not appear in the post-pulse image. Previous timelapse data indicate that live cells are firmly attached to fibers even after severe electroporation[476]. Consequently, cells that did not appear in the post-pulse images were assumed to have died and been washed off the fibers.

Pre-pulse images were used to determine cell shape (length, width, area) and cell attachment type (spindle or 2-fiber). Cell shape parameters were evaluated in ImageJ by tracing the cell and evaluating the cell length and width using ImageJ's Analyze menu. Post-pulse images were used to determine the viability of cells. Live cells showed strong Calcein-AM levels (bright green) and had typical cell shape/morphology (no blebs, smooth membranes, spread on fibers, fiber deflection for 2-fiber cells). Contrarily, dead cells had strong PI levels, no or minimal Calcein-AM levels, showed large blebs, were low-contrast, and had shapes and morphologies that indicated cell death. See SI for example images of live and dead cell.

Viability results were calculated by examining each cell from the pre-pulse images to determine if the cell was viable post-pulse. A single scaffold typically contained 30-90 cells for analysis.

7.6.7 Electroporation Pulses

Electroporation pulses were applied using a BTX 830 (Harvard Apparatus). Ten, 100 μ s square wave pulses were delivered at 1Hz. Our COMSOL models showed that for every 1V applied, the average electric fields across the scaffold region of interest was 1.1 V/cm. Therefore,

we delivered, 455, 910, 1365, 1820, 2275, and 2730 V to the devices to produce average electric fields of 500, 1000, 1500, 2000, 2500, and 3000 V/cm across the scaffold region of interest. To decrease electrochemical effects and cool the device after pulsing, we designed the device to have half of the total fluid volume outside the electroporation region. We performed experiments at room temperature to minimize thermal damage caused by electroporation. Some experiments were performed with 1 μ s monopolar pulses using a custom pulse generator (Votmed). These 1 μ s pulses were delivered in 10 “trains” of 100 pulses spaced 1 μ s apart, and thus had the equivalent energy delivered as the 100 μ s pulse experiments.

Transfection experiments were performed using a single 20ms pulse or two 10 ms pulses delivered at 1Hz as is standard in transfection applications.

7.6.8 COMSOL Modelling

We implemented a 3D, time-dependent electroporation model on realistic cell shapes in COMSOL to investigate the induced transmembrane potential (ITV) and pore density across cells of highly elongated shapes. Z-stack images from fixed cells stained for actin and microtubule were used to reconstruct accurate 3D models using 3D Slicer. The reconstructed cells were then meshed using 3Matic. COMSOL Multiphysics was used to apply the appropriate physics to a time-dependent model using the AC/DC module. Table 7-2 lists the model and material properties used for simulations. Current conservation was modeled as:

$$\nabla(\sigma_i \nabla V) - \nabla \frac{\partial(\epsilon_i \nabla V)}{\partial t} = 0 \quad 1$$

where σ_i is the conductivity of the material and ϵ_i is the permittivity of the material. To model the membrane, we applied a distributed impedance boundary condition:

$$n \cdot J = \frac{\sigma_m}{d_m}(V - V_{ref}) + \frac{\epsilon_m}{d_m} \left(\frac{\partial V}{\partial t} - \frac{\partial V_{ref}}{\partial t} \right) \quad 2$$

Where n is the unit normal vector to the membrane surface, J is the current flux, voltage V on the outside of the membrane, V_{ref} is the voltage on the inside of the membrane, σ_m is the conductivity of the membrane, d_m is the thickness of the membrane, and ϵ_m is the membrane permittivity. Here Induced transmembrane potential is described as:

$$ITV = (V - V_{ref}) \quad 3$$

Where ITV is the induced transmembrane voltage.

We included the standard electroporation pore model that governs the pore density across the membrane:

$$\frac{dN}{dt} = \alpha e^{\left(\frac{ITV}{V_{ep}}\right)^2} \left(1 - \frac{N}{N_0} e^{-q\left(\frac{ITV}{V_{ep}}\right)^2} \right) \quad 4$$

where N is the pore density on the membrane, N_0 is the equilibrium pore density, V_{ep} is the threshold voltage for electroporation, and α and q are values describing the electroporation process. Pore formation alters membrane conductivity, which can be describes as:

$$\sigma_{ep} = N \frac{2\pi r_p^2 \sigma_p d_m}{\pi r_p + 2d_m} \quad 5$$

where σ_{ep} is the electroporated membrane conductive, r_p is the pore radius, σ_p is the pore conductivity determined as:

$$\sigma_p = \frac{\sigma_e - \sigma_{ep}}{\text{Log} \left(\frac{\sigma_e}{\sigma_{ep}} \right)} \quad 6$$

Dynamic membrane conductivity is evaluated as:

$$\sigma_m = \sigma_{m0} + \sigma_{ep} \quad 6$$

where σ_{m0} is the initial membrane conductivity.

Table 7-2 Parameters for COMSOL model of electroporation.

Parameter	Symbol	Value	Reference
Extracellular media conductivity	σ_e ($\text{S} \cdot \text{m}^{-1}$)	1.28, 0.01	0.1, Measured
Extracellular media relative permittivity	ε_e	80	[277,278]
Cytoplasm conductivity	σ_c ($\text{S} \cdot \text{m}^{-1}$)	0.25	[278]
Cytoplasm relative permittivity	ε_c	70	[200,278,279]
Initial cell membrane conductivity	σ_{m0} ($\text{S} \cdot \text{m}^{-1}$)	5e-7	[278]

Cell membrane relative permittivity	ϵ_{cm}	4.5	[278,280]
Cell membrane thickness	d_m (nm)	5	[278]
Pore Radius	r_p (nm)	0.76	[200]
Electroporation Constant	q	2.46	[200]
Electroporation Parameter	α ($m^{-2} \cdot s^{-1}$)	1×10^9	[200]
Characteristic voltage of electroporation	V_{ep} (V)	0.258 V	[200]
Equilibrium Pore Density	N_0 (mm^{-2})	1.5×10^9	[200]

7.6.9 Transfection Experiments

Proof-of-concept transfection experiments were carried out using the mammalian expression vector for expression of GFP (EF1a promoter) pEF-GFP from AddGene (Plasmid #11154). The plasmid was contained in DH5alpha *E. Coli*, and liquid cultures were prepared overnight from streaked agar plates. The following day, a miniprep kit (GeneJET Plasmid Miniprep Kit, ThermoFisher) or maxiprep kit (PureLink™ HiPure Plasmid Maxiprep Kit) was used to extract and purify the plasmid DNA according the kit protocols.

Extracted plasmid was added to HEPES pulsation buffer at 50 µg/ml, and added to the microfluidic devices just prior to electroporation with a single 20 ms pulse at 500 V/cm. 10 minutes after electroporation the device was flushed with regular media and placed in an incubator overnight. The following day, the devices were imaged for plasmid expression.

7.7 Supplemental Tables and Figures

Proof-of-concept transfection experiments were carried out using the mammalian expression vector

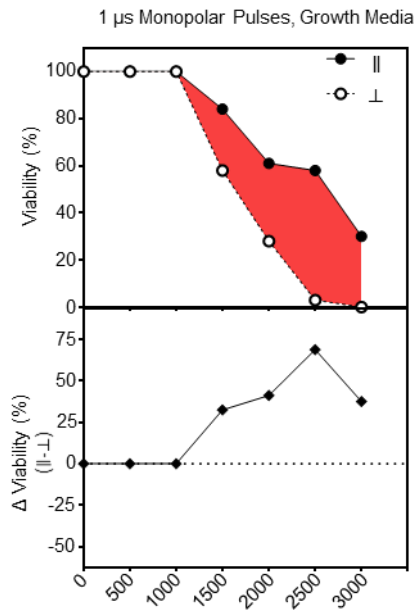


Figure 7.6 Electroporation with 1 μ s pulses shows similar trends as 100 μ s pulses.

a) Electroporation pulses with 1 μ s pulses, delivered in sets of 100 with 1 μ s delays between each pulses, shows similar trends in post-electroporation viability as 100 μ s.

8 Chapter 8: Conclusions and Future Work

8.1.1 Summary

Taken together, these studies shed new light on brain cancer mechanics, and how pulsed electric fields can be used therapeutically to disrupt cell mechanics and open the blood-brain barrier. Cell-level and tissue-level mechanical properties are critical to biological functions, and we show that pulsed electric field (PEFs) and cellular pathology, can significantly change cell mechanics and therefore influence biological function. By developing innovative microfluidic and organ-on-a-chip devices, we have created new methods to investigate cell mechanics for a variety of clinical applications.

In Chapter 3, we used Nanonet Force Microscopy (NFM) to measure single-cell mechanical disruption of glioblastoma cells after reversible electroporation and found significant cytoskeletal disruption. In Chapter 4, we investigated the mechanical properties of brain cancer for various-grade glioma cells and found that low-grade glioma cells were stiffer than healthy and grade IV glioma cells. This result suggests that low-grade tumors that may benefit from specialized treatments that exploit their unique mechanical properties. Blood-brain barrier (BBB) disruption after high-frequency electroporation was investigated in Chapter 5 using a multiplexed microfluidic device. High-frequency electroporation pulses increased in BBB permeability within the first hour after electroporation in a voltage- and pulse-number dependent manner. To improve

our microfluidic modeling of the BBB, in Chapter 6 we describe a novel basement membrane that is ultra-thin and ultra-porous for physiologically relevant barrier modelling. The basement membrane is fabricated using the non-electrospinning Spinneret-based Tunable Engineered Parameters (STEP) technique. Finally, in Chapter 7 we investigate how viability after electroporation is impacted by cell shape and show that both cell orientation (with respect to the electric field) and media conductivity have significant impacts on post-treatment viability.

8.1.2 Future work

The research projects presented in this dissertation provide exciting opportunities for future research to expand the findings of these projects.

In Chapter 3, we show that cell contractility is significantly disrupted by electroporation. Future work investigating how the magnitude of cell contractility (contractile forces) before electroporation impacts cell recovery post-electroporation would be valuable to better understand how the cytoskeleton contributes to cell survival. Additionally, since cell mobility is regulated by the cytoskeleton, future work to investigate the changes in mobility after electroporation are warranted and may have implications for tumor treatments.

In Chapter 4, we found that lower grade astrocytoma cell lines showed elevated stiffness values compared to higher grade gliomas and healthy astrocytes. Future work is warranted to confirm this finding through additional testing with additional cell lines. Testing the stiffness of additional healthy astrocyte cell lines and grade I astrocytoma cells could confirm the results of our study. Future studies could also expand the methodology for analysis (finite element

simulations with a neural network) to include additional experimental parameters and validate that our predicted stiffness values are consistent with alternative techniques such as needle aspiration[478]. Future studies may also be able to resolve the stiffness contributions from various components of the cell, such as the nucleus. Studies of active migration[479] may also provide additional insight about cell migratory potential compared to our experiments that used hydrostatic pressure to deform cells.

In Chapter 5, we introduced a multiplexed microfluidic BBB model. Future work could use this model to investigate a wide range of electroporation parameters to search for optimal IRE and HFIRE parameters. This model could also be enhanced with the addition of pericytes and astrocytes in a tri-culture model.

The nanofiber basement membranes described in Chapter 6 offer many opportunities for future research. In Chapter 6, we validate the nanofibers for use in a blood-brain barrier (BBB) model. However, this model could be extended for additional physiological barriers such as the alveolar–capillary barrier, intestinal mucosal barrier, skin barrier, and renal epithelial barrier. Healthy and disease models could be constructed for each of these organs. These membranes could be used to study immune cell trafficking, cancer metastasis, nanoparticle transport, and other processes. Additionally, the barrier models could be electroporated using the incorporated electrodes. The resulting disruption could be studied by TEER measurements, diffusion measurements, or imaging. Finally, these nanofiber membranes could be refined for commercial use in either transwell plates or microfluidic devices.

In Chapter 7, we show that cell orientation has a significant effect on post-electroporation viability. This work could be expanded by modulating fiber diameter, which regulates the cytoskeleton, to investigate how fiber diameter impacts cell survival. Elongated cardiac myocytes may be tested to determine if those cells show similar trends, since cardiac ablation is a promising application of electroporation.

8.1.3 Conclusion

Pulsed electric fields show significant promise for the therapeutic treatment of primary brain cancers, and microfluidic organ-on-a-chip devices are a promising technology for investigating key biological responses after these treatments. By investigating the mechanics of three key brain cells - astrocytes, pericytes, and endothelial cells – we have quantified the effects of pathology and electric fields to better understand their impact on the brain. It is the author's hope that the research present herein furthers the greater efforts of the neuroscience research community to discover better treatment for debilitating neurological conditions, so that one day no patients are confronted with the dismal diagnosis associated with primary brain cancer and other neurological disorders.



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