Engineering the Development of Neuromuscular Circuitry On-Chip

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ENGINEERING THE DEVELOPMENT
OF NEUROMUSCULAR
CIRCUITRY ON-CHIP

By
Ines Khiyara
B.S. University of Maine, 2020

A THESIS
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science
(In Biomedical Engineering)

The Graduate School
The University of Maine
December 2022

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The project depicted is sponsored by the National Institute of Health (NIH). Support for this research is provided by the National Institutes of Health, project no. NIH 1R15NS111377-01A1.
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Thesis Co-Advisors: Dr. Rosemary L. Smith and Dr. Scott D. Collins

An Abstract of the Thesis Presented
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Neuromuscular development happens in a complex interconnected network of biochemical pathways. This complicated embryonic development follows a strong, functional, and precise neuromuscular network that has interested both scientists and engineers who seek to better understand neuromuscular diseases. These disorders can be inherited or acquired, and their severity and mortality can vary. Researchers first studied the neuromuscular network from an organismal perspective, and more recently from an embryological, cellular, molecular, biochemical, and genetic perspective. From these studies, the fundamental principles of motor neuron pathfinding to muscles are widely understood, but the molecular drivers of specific nerve-muscle pairing remain unknown.\textsuperscript{1,2,3} Although \textit{in vivo} experiments provide a precise depiction of the living tissue environment, manipulating \textit{in vivo} variables to recreate the neuromuscular development in the laboratory is difficult, and the results are frequently intricated by many uncertainties and unquantifiable variables which make it problematic to draw significant and relevant conclusions.\textsuperscript{4,5,6} \textit{In vitro} experiments, on the other hand, provide a more controlled, precise, and repeatable motif, but they often lack biological realism. In this thesis, a novel \textit{in vitro},
three-dimensional co-culture microfluidic system is presented that seeks to mimic parameters that influence the complex physiochemical and developmental environments found in vivo. This system enables the culture of embryonic stem cells in two adjacent chambers, where they are supplied with their individual, requisite media and morphogens to develop into two distinct types of tissues, either motor neurons or muscle fibers while enabling chemical communication through interconnecting microchannels. Experiments are underway to investigate the effects of skeletal muscles on differentiating motor neurons into specific motor neuron columnar identity and neuromuscular junction formation. Results are expected to increase our fundamental understanding of developmental processes, neural development, and neuromuscular junction formation in order to research and model degenerative diseases.
ACKNOWLEDGEMENTS

With much appreciation and my most sincere gratitude, I would first like to thank both of my graduate advisors Dr. Rosemary Smith and Dr. Scott Collins for giving me the opportunity to continue my education at the University of Maine these past two and a half years. Dr. Smith was always there to give guidance and help when I needed her most. I would also like to thank my other committee member and undergraduate advisor, Dr. Tilbury, whose expertise and conversations over the past five and a half years have taught me so much.

Many thanks to my lab colleagues and members of the Micro-Instruments and Systems Laboratory (MISL), Dr. Phaneendra Chennampally, Jonathan Bomar, Nick Reeves, Avery England, Kailey Bell, and Molly Olzinski for various trainings, and for helping me with the experiments and microfabrication.

Special thanks to Dr. Jared Talbot whose developmental biology class has allowed me to develop a greater understanding of the biology involved in this research.

I would like to express my gratitude to Mike Call for always being available to help and fix equipment when it would go down, and to Dr. George Bernhardt for your help with safety and materials. Thank you to Tracy, for all your assistance and patience with purchase orders, shipping, and paperwork.

I would like to extend my most profound thanks and gratitude to my colleague and friend, Kailey Bell, for our numerous insightful discussions, the countless late nights in the laboratory together, and for your significant contribution to the image analysis of this work. I sincerely appreciate your efforts and unrelenting support for this work.
Thank you to Joseph Dagher for your immense patience with me and for all your help with formatting this thesis. I cannot thank you enough for all you do that brings happiness to my life.

Further, I would like to thank my family and friends for their endless love and support of my goals and for enduring this journey with me. To my mom who also studied abroad and had to go through learning a new culture and language, her experience has given me the strength to do the same. To my dad who has always shown me what perseverance and dedication can do. You all have always believed in me and pushed me to do my best. Without your support, patience, and motivation, I would not be where I am today.

En outre, je voudrais remercier ma famille et mes amis pour leur amour, pour leur soutien dans mes objectifs, et pour avoir surmonté les tribulations de ce parcours à mes côtés. À ma mère, qui a également étudié à l’étranger, a dû apprendre une nouvelle langue et culture, ton expérience m’a renforcée et m’a inspirée à faire la même chose. À mon père, qui m’a toujours montré les bénéfices de dur labeur et la persévérance, tu as toujours eu confiance en moi et m’a inspirée à me surpasser. Sans votre soutien, patience, et motivation, je ne serais pas où j’en suis aujourd’hui.
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CHAPTER 1: INTRODUCTION

1.1. Motivation

Neuromuscular diseases, which impact the function of muscles because of issues with the nerves or muscles in the body, are caused when neuromuscular development and structure are incomplete or degenerated.\textsuperscript{7–9} In order to comprehend the diseases of the neuromuscular system, researchers examine the intricate embryonic and neuromuscular development.\textsuperscript{7–11} From an organismal perspective, these disorders have received substantial research in which the fundamental principles of motor neuron pathfinding to muscles are extensively studied; nevertheless, the neuromuscular network has only recently been examined from an embryological, cellular, molecular, and biochemical perspective, which would help identify the molecular drivers of specific nerve-muscle pairing.\textsuperscript{1,2,3} Most of the research has attempted to offer an adequate representation of the neuromuscular development environment in living tissues, however, manipulating \textit{in vivo} variables in the lab is challenging, making it difficult to draw meaningful and pertinent conclusions.\textsuperscript{4,5} On the other hand, \textit{in vitro} experiments offer a more managed, accurate, and reproducible pattern, but they sometimes lack biological realism. Therefore, this thesis takes an integrative approach to studying developmental biology and disease modeling. The main goal of this research is to accurately simulate an \textit{in vivo}-like milieu in an \textit{in vitro} study and, therefore, there is a need for a system with acute control of the microenvironment to study developmental biology and disease modeling. In recent years, a new technology known as microfabricated microfluidics has proven to be effective in providing fine control over the spatial and temporal chemical environment within a three-dimensional cell culture in order to effectively attempt to mimic \textit{in vivo} settings throughout
cell and tissue development. This method is implemented in this thesis to coculture two different types of cells in three dimensions with the objective to

(a) investigate the influence of released signaling molecules from one type of cell on the other;

(b) study the mechanisms of mutual signaling between motor neurons and muscle cells that drive the differentiation of specific neural subtypes; and

(c) generate morphogens to differentiate embryonic stem cells into spatially organized neuromuscular tissue, complete with neuromuscular junctions.

This coculture microdevice can help further the understanding of neuromuscular development and the formation of neuromuscular junctions.

1.2. Background

1.2.1. Neuromuscular junctions and neuromuscular diseases

A neuromuscular junction (NMJ) is a specialized synapse in the peripheral nervous system and represents the connection between a motor neuron terminal and a skeletal muscle fiber. The structure of a neuromuscular junction can be divided into three parts: the presynaptic terminal (motor neuron terminal); the synaptic cleft, which is the space that separates a neuron and its target cell; and, finally, the postsynaptic membrane, which is the membrane of the muscle fiber (Figure 1).
Figure 1. A magnified view of a single synapse representing the neuromuscular junction.

The first part is the presynaptic terminal, also known as the motor neuron or nerve terminal, and it is in charge of producing, storing, and releasing the acetylcholine neurotransmitters (Ach). The myelinated motor neuron loses its myelin sheath to form a branching of nerve endings called nerve terminals or presynaptic terminals, as seen in Figure 1, and their thick membrane is covered with SNAP proteins (syntaxins and synaptosomal-associated protein) and rows of voltage-gated calcium (Ca$^{2+}$) channels. Moreover, the presynaptic terminal contains potassium channels with mitochondria, endoplasmic reticulum, and synaptic vesicles. About 5,000-10,000 molecules of acetylcholine, the neurotransmitter at the NMJ, are encased in each synaptic vesicle, as depicted in Figure 1. The membrane of the synaptic vesicles has synaptotagmin and synaptobrevin proteins. These proteins are required for synaptic vesicle docking and fusion, a process in which the vesicle and presynaptic membrane fuse to form a miniscule
opening that grows larger until the vesicle membrane collapses into the presynaptic membrane and exocytosis occurs.\textsuperscript{7,8,15} This process is caused by an action potential at the presynaptic terminal and the Calcium ($\text{Ca}^{2+}$) channels that trigger the release of neurotransmitters from the synaptic vesicles into the synaptic cleft, which is the second part of the NMJ (Figure 1).\textsuperscript{7,15} This area measures about 50 nm and contains the Ach neurotransmitters that diffuse across the synaptic cleft to activate the postsynaptic or Ach receptors found on the postsynaptic terminal, or the third part of the NMJ.\textsuperscript{15} This part is the thickened portion of the muscle membrane, and it contains several deep indentations called junctional folds. The terminal nerve endings (first part of NMJ) perfectly fit into the junctional folds which contain high numbers of ligand-gated ion channel receptors (postsynaptic receptors) for the neurotransmitter Ach.\textsuperscript{7,15} The binding of Ach neurotransmitters to these receptors in the junctional folds of the muscle membrane causes the ion channels to open, allowing the influx of positive sodium ions from the extracellular fluid into the muscle membrane.\textsuperscript{15,8} This produces a depolarization of that cell called an excitatory postsynaptic potential, also known as the endplate potential, which then creates and sends action potential to the muscular membrane, producing muscle contraction.\textsuperscript{15} The neuromuscular junction operates in a way that the neuron transmits a signal received by the multiple dendrites from the brain or spinal cord to the muscle fiber through the axon, causing the muscle to contract.\textsuperscript{7,8,15} Therefore, the neuromuscular junction performs an important role by bridging the gap between the nervous system and the muscular system and by providing a channel of communication between the two systems.\textsuperscript{7,8,15} Scientists are interested in neuromuscular studies because diseases can emerge if any of the signaling processes of communication or response are disrupted.\textsuperscript{7,8} These diseases are called
neuromuscular diseases and can be widely defined as any disorder that compromises the ability of muscles to contract, either directly through pathologies of the voluntary muscle or indirectly through pathologies of the nerves or neuromuscular junctions. These diseases impact the function of muscles because of issues with the nerves, muscles, or links between the two in the human body. They can be hereditary or acquired, and their severity and mortality can vary. The most common symptoms of neuromuscular diseases are muscle weakness, muscle pain, and spasms that affect the arms and legs more than other parts of the body. Some examples of neuromuscular disorders are myopathies, which are when there is a problem with the muscle, of which one well-known example is muscular dystrophy; neuromuscular auto-immune conditions; and neuropathies, which are when there is a problem with the neuron, which is the focus of the research, as a major objective is to determine how to make the motor neuron send the contraction signal to the muscle at the correct place and time. An example of this is amyotrophic lateral sclerosis (ALS) which predominantly affects the nerve cells (neurons) in charge of regulating voluntary muscle movements such as walking and talking. ALS is the most common adult neuromuscular disease caused by loss of motor neurons and muscle atrophy, which leads to progressive neurodegeneration. It is estimated that 12,000-15,000 people in the US are living with ALS. While the identification of therapies for ALS is an area of active research, existing treatments for ALS are ineffective. Drug screening for ALS has traditionally had low throughput due to the use of genetically engineered mouse models. Additionally, in vitro drug screening attempts have been conducted on incomplete models of the neuromuscular junction.
1.2.2. Motor Neuron and its development and identification

Motor neurons begin to develop early in embryonic development, and they are known as multipolar neurons in terms of their structure. This means that motor neurons have a nucleus, a cell body, and multiple dendrites that receive the signal and pass it down through the single axon that extends from the cell body down to where it ends at the branch-like axon terminal where the synapse can be found to connect to its target cell (Figure 2). There are two main types of motor neurons: the upper motor neurons, which travel from the brain to the spinal cord, and the lower motor neurons, which subtypes in the spinal cord are of interest, travel from the spinal cord to the muscles of the body.\(^2\)\(^,\)\(^3\)

**Figure 2.** Motor Neuron structure. Image Retrieved from “Diffuse Axonal Injury (DAI) - Charlie Waters Law”.

1.2.2.1. Motor Neuron Diversity

Figure 3 shows the motor neuron subtypes organization in the spinal cord as well as the set of homeodomain transcription factors for each specific motor neuron subtype found in the table. Motor neuron subtypes grow axons together along major nerve pathways
to the muscles and are arranged into longitudinal columns along the rostro-caudal axis of the spinal cord.\textsuperscript{2}

In Figure 3, the medial motor neurons in blue are projecting to the axial muscles (dm, dermomyotome). These are found in the Medial Motor Column (MMC) and this column covers all rostrocaudal (R-C) levels of the spinal cord.\textsuperscript{2,3} Next, the neurons located in the Hypaxial Motor Column are in red (HMC), and they innervate the body wall muscles (bw), and the neurons in the Preganglionic Motor Column in yellow (PGC) that innervate the sympathetic ganglia (sg). Both columns (HMC and PGC) occupy more lateral positions exclusively at thoracic levels.\textsuperscript{2,3} Finally, the Lateral Motor Column (LMC) that covers the brachial and lumbar spinal cord levels is shown, and it is further divided into lateral (LMC\textsubscript{L}) and medial (LMC\textsubscript{M}) divisions. The LMC\textsubscript{L} has neurons innervating muscles of the dorsal limb bud (dlb) and the LMC\textsubscript{M} has neurons innervating muscles in the ventral limb bud (vlb).\textsuperscript{2-5} As previously detailed, the table in Figure 3 lists the different sets of homeodomain transcription factors for each motor neuron subtype.\textsuperscript{2,21} It is shown that the

**Figure 3.** Motor Neuron Subtype Diversity. Reproduced from Ref [2].
genes Isl1, Isl2, and Lhx3 are expressed in MMC, HMC and LMC both express Isl1 and Isl2, while LMC expresses Isl2 and Lhx1, and PGC only expresses Isl1. For the purpose of this research, LMC is the focus, as one of the motivations is to develop in vitro specific subtypes of limb motor neurons because they are the ones that innervate or connect to skeletal muscles found in the limbs. These neurons are also relevant when studying developmental and neuromuscular diseases.

1.2.2.2. Specification of Limb-Innervating Motor Neurons

Figure 4 demonstrates the motor columns and motor pool organization along the rostro-caudal axis. It is known now that motor neurons that send axons to the limb muscles are contained within the LMC. These motor neurons are specified by Hox6-8 at brachial level, and by Hox10-11 at lumbar level of the spinal cord, but they are not specified by Hox9 orthologs at thoracic level, as shown in Figure 4.³ At brachial and lumbar levels, LMC neurons are also marked by high levels of the transcription factor FOXP1, which is
an effector of Hox proteins in motor neurons, and at thoracic levels these neurons inhibit
the expression of FOXP1.\textsuperscript{5,21,22} As previously discussed, PGC and HMC motor neurons are
both found at thoracic levels and are specified by Hox 9 while motor neurons within the
MMC are generated at all rostrocaudal levels of the spinal cord and are specified by Hox6-
8, Hox9 and Hox10-11.\textsuperscript{21} To sum up, the combinations of Hox genes and Lim
homeodomain factors are responsible for motor neuron subtype specification.

1.2.2.3. Guidance Pathways for LMC

![Figure 5](image)

**Figure 5.** Motor axons (LMC\textsubscript{L}, LMC\textsubscript{M}) navigating into the limb. Reproduced from
Ref [3].

Considering the focus on LMC neurons, it is crucial to determine the set of
homeodomain transcription factors. As previously mentioned, LMC is divided into two
main divisions, LMC\textsubscript{L} and LMC\textsubscript{M}, and this is due to their different transcription factors,
which are affected differently by retinoic acid (RA) signaling\textsuperscript{2-4} Figure 5 is showing motor
axons from LMC\textsubscript{L} and LMC\textsubscript{M} neurons navigating into the limb. The axons co-extend to
the base of the limb before bifurcating into nerve branches that target either the dorsal or
ventral muscles. The dorsal limb muscles are innervated by LMC\textsubscript{L} neurons, which express Isl1\textsuperscript{−}, Lhx1\textsuperscript{+}, Isl2\textsuperscript{−} and Hb9\textsuperscript{High} transcription factors, while the ventral limb muscles are innervated by LMC\textsubscript{M} neurons which express Isl1\textsuperscript{+}, Lhx1\textsuperscript{−}, Isl2\textsuperscript{−}, Hb9\textsuperscript{low}.

1.2.2.4. LMC Motor Neurons Identification

In the flow diagram displayed in Figure 6, it is shown that Hb9, a critical determinant of MN differentiation, comes from the sonic hedgehog (Shh) signaling in the dorsoventral axis and Hox6 coming from the Fibroblast Growth factor (FGF) signaling for the rostrocaudal axis. The merging activities of these two MN-specific transcription factors direct the expression of FOXP1 and then the expression of RALDH2 (retinaldehyde dehydrogenase 2), which is required for retinoic acid synthesis in LMC, and leads to the induction of Lhx1 that directs expression of the guidance receptor EphA4. This receptor directs the motor axons of the lateral LMC neurons toward the dorsal limb. Similarly, Isl1 directs expression of the guidance receptor EphB1 for medial LMC neurons towards the ventral limb.

Figure 6. Flow diagram that defines the identity of lateral LMC neurons (LMC\textsubscript{L} and LMC\textsubscript{M}). Image Modified from Ref [15].
1.2.3. **Skeletal muscle cells**

Mammalian skeletal muscles are parts of the musculoskeletal system which include nerves, tendons, connective tissues, bones, and blood vessels, and they are largely connected to the skeleton's bones by tendons. They have evolved to perform a diverse set of functions, including locomotion, breathing, and protecting internal organs. Skeletal muscle cells are frequently referred to as muscle fibers since they are much longer than those found in other types of muscular tissue. The muscle tissue of a skeletal muscle is striated, giving it a striped look because of the way the sarcomeres are organized. In this work, mammalian skeletal muscles are used as the postsynaptic terminal of the neuromuscular junction. Myoblast differentiation from stem cells and fusion into multinucleated cells are two features of the highly regulated process that creates and regenerates skeletal muscle. First, there are three germ layers that arise during embryonic development as a result of gastrulation: ectoderm, mesoderm, and endoderm. Skeletal muscles are of mesodermal origin, and their development begins when somites differentiate into the dermomyotome (lateral somite), concurrent with neuron differentiation. Through the use of transcription factors involving the Pax genes, dermomyotome cells are referred to as muscle progenitor cells (MPC), and these progenitor cells delaminate and migrate to create the myotome, where they are identified as myoblasts that differentiate into myotubes or myofibers, as shown in Figure 8. Members of the Pax genes family with paired box/homeodomains regulate the contribution of MPC to different tissue types and are crucial for development. Pax3 and its paralog Pax7, and the MyoD family of transcription factors have been associated with the myogenic determination and differentiation. For instance, cells derived from the ventral lateral lip
of the dermomyotome form the hypaxial myotome, which later develops into the limb, diaphragm, and tongue muscles with myoblasts expressing MyoD, while cells from the dorsal medial lip (DML) of the dermomyotome form the apaxial myotome, which eventually forms the axial trunk muscle with myoblasts expressing Myf5. In the absence of both Pax3 and Pax7, skeletal muscle is severely lacking, and later in embryonic and fetal development, myogenesis is stopped.\textsuperscript{26} Moreover, skeletal muscle development is controlled by extracellular signaling molecules released by the environment, such as Wnt, Bone morphogenic Protein (BMP), and Shh.\textsuperscript{25,27,28,29} Lastly, after the myoblast stage, MPCs exit the cell cycle and migrate to their final location where they differentiate into myocytes that fuse to form multinucleated myotubes. The secondary myotubes, which make up the majority of the muscle fibers, are created by a second wave of myogenesis later on in the development process.\textsuperscript{26} These muscle fibers' nuclei become peripheral as they develop (Figure 8).

\textbf{Figure 7.} Primary gene expression at each stage of the differentiation of ESCs into skeletal muscle fibers.

The limb progenitor cells serve as an example of this process; following the cell cycle exit, these cells move peripherally to the limb buds, where they eventually develop into limb skeletal muscle that expresses LIM1.\textsuperscript{25,28}
1.3. Microfluidic technology

*In vitro* models and experiments have evolved from 2D cell culture to 3D cell culture in a Petri dish to microfluidics technology which is an emerging discipline uniting the principles of fluid dynamics, microelectronics, material science, physics, chemistry, and biology. Various materials can be manufactured into miniature chips with microscale channels and chambers in order to control and manipulate small volumes of fluids (10^{-6} liters – 10^{-12} liters). Such platforms can be produced using a variety of techniques to achieve the desired size, shape, and geometry. The “organs-on-chip”, a fascinating concept, has recently arisen where multiple on-chip compartments provide diverse systemic organ tissues derived from organoids or dissected tissue. To replicate blood traveling between different organs, the compartments are connected by microfluidic channels. This method is considered to be quite effective in identifying drug metabolism mechanisms as well as the impact of drug byproducts or upstream secretions on numerous downstream organs. However, these technologies are not capable of manipulating the spatial chemical topography of cells, making them unsuitable for developmental studies, which are of interest to our research. As a result, we elaborate on this concept in this thesis to create an on-chip replica of the spatiotemporal neuromuscular chemical environment. This allows researchers to track how motor neurons (especially limb-innervating neurons) and skeletal muscle cells co-differentiate. Microfluidic chips are used in this research for cell examination and cell culture, but this technology is also used in cell diagnosis as well as for the creation of nanoparticles, drug encapsulation, delivery, and targeting. More details on microfluidics materials and fabrication can be found in Appendix A.
CHAPTER 2: DEVELOPMENTAL STUDY OF LMC MOTOR NEURONS IN A COCULTURE DEVICE

2.1. Approach

The neural tube is the embryonic precursor to the central nervous system, which is made up of the brain and spinal cord. In the ventral part of the growing neural tube, motor neurons are produced in response to chemical gradients of morphogens such as RA, Shh, BMP, etc.\(^{36,37}\) Along the dorsoventral (D-V) axis of the neural tube, these morphogens cause the expression of transcription factors in a distinctive way, resulting in domains of cell type-specific regions.\(^{1,38}\) As seen in Figure 8 (a) there are 11 different types of neurons, including sensory neurons, interneurons, and MNs, that differentiate along the D-V axis in response to morphogens.\(^{36,39,40}\) Motor neurons make up a very modest portion of all conceivable neuronal subtypes in the neural tube. The majority of neurons are interneurons (Figure 8 (a)) that control movement and integrate sensory and motor information.\(^{41}\) The establishment of the different types of ventral cells is significantly influenced by the Shh concentration gradient coming from the notochord (NC) at various time points. The dorsal cell type is determined concurrently by opposing gradients of BMP and Wnt coming from the roof plate (RP)\(^{42-44}\) (Figure 8 (b)). As previously described in Chapter 1 and seen in Figure 8 different Hox genes are expressed in the brachial (Hox6), thoracic (Hox9 orthologs), and lumbar (Hox10) regions of the neural tube, dividing the MNs along the anterior-posterior (A-P) axis into different columns (median motor column: MMC, hypaxial motor column: HMC, lateral motor column: LMC, and preganglionic motor column: PGC).\(^{21,40,45-47}\) Motor columns are further subdivided into motor pools, which are made up of all individual motor neurons that innervate a specific muscle.\(^{47}\) Only one motor neuron can innervate a single muscle fiber, yet that one motor neuron can innervate several
muscle fibers. Motor columns can be identified by their differential and combined expression of LIM-homeodomain proteins, Hox proteins, and other transcription factors in addition to their muscle targets. Moreover, the concentration gradients of RA in the anterior region, fibroblast growth factor (FGF), and growth differentiation factor (GDF) in the posterior region control the expression of many Hox genes along the A-P axis\textsuperscript{21,37} (Figure 8 (b)). FGF and GDF are mostly produced in the caudal area by Hensen's node and presomatic mesoderm, whereas somites and paraxial mesoderm are responsible for RA signaling (round spherical tissues flanking the neural tube during embryonic development).\textsuperscript{37,40,45,48,49} Hox genes have developed a combinatorial coding where an anterior-posterior identity is decided by the co-expression of a particular protein or a transcription factor because of the ephemeral nature of the morphogen signal. In addition to governing MN differentiation and settling positions in the spinal cord, Hox gene expression also affects muscle patterning and development.\textsuperscript{50-52}
Figure 8. Application of neural tube patterning to the microdevice.

a) Neural tube cross-section showing concentration gradients (BMP/Wnt and Shh) along the dorsoventral axis, resulting in the development of sensory neurons (DI1), dorsal interneurons (DI2-6), ventral neurons (V0-3), and motor neurons.

b) Anterior-posterior patterning of the neural tube showing MNs into different columnar identities; LMC (lateral motor column), MMC (median motor column), HMC (hypaxial motor column), and PGC (preganglionic motor column). The neural tube's posterior region has a gradient of Wnt, FGF, and GDF, while the anterior region exhibits an opposing gradient of RA.

c) Representation of in vitro cell culture microdevice to study the differentiation of MNs into LMC identity with and without the presence of skeletal muscles (somites around the neural tube).
As mentioned previously, MNs in this column are particularly involved in innervating the limb skeletal muscle, and in the case of a motor neuron disease such as ALS, these motor neurons are the first to experience apoptosis.\textsuperscript{16,18} There has been a lot of research on the variables affecting motor neuron subtype development both \textit{in vitro} and \textit{in vivo}. However, most researchers have reported being challenging to induce significant \textit{in vitro} yields of LMC motor neurons, one of the MN subtypes, in homogenous cultures.\textsuperscript{4,5} This includes Dr. Demers from our lab who recapitulated organized spinal tissue on-chip from mESCs and showed that not all neuron types were found to be present in the artificial neural tube by day 7, the one subtype particularly missing and of interest was the LMC motor neurons.\textsuperscript{13} Other results have shown significant yields of LMC MNs in the presence of skeletal muscles in the muscle chamber but when the muscle chambers contained mESCs supplied with MN differentiation medium, it produced far fewer of these neurons (LMC).\textsuperscript{53} This suggested that the MNs are directed toward a particular subtype (LMC) by signaling molecules from skeletal muscles.

Instead of using mESCs in the muscle chamber, the aim of this study is to examine the differentiation of motor neurons, specifically limb-innervating neurons with and without the presence of skeletal muscle cells, by recreating the spatiotemporal neuromuscular chemical environment on-chip. In an attempt to generate and comprehend the developmental and differentiation processes of LMC MNs, the microdevice in Figure 8(c) can culture different types of cells (mouse embryonic stem cells and skeletal muscles) separately in a 3D microenvironment while still enabling intercellular communication through the interconnecting microchannels.
2.2. Methods

2.2.1. Microfluidic Device

The goals of using a microfluidic system are to direct stem cell development into the neuromuscular system, identify and monitor the progression of MN development towards LMC neurons, and later study the mechanisms of signaling that drive differentiation of specific neuron subtypes. As previously mentioned, microfluidic devices can provide precisely controlled environments for the study of cell-cell and cell-extracellular matrix (ECM) interactions, soluble factors, and mechanical forces, as well as single-cell handling, with real-time observation and analysis. These devices have channel and chamber dimensions comparable to biological cells and tissue environments which make it possible to study developmental biology.\textsuperscript{35} As seen in Figure 9, our experimental design has two adjacent chambers, one with mouse embryonic stem cells (mESCs) and the other with skeletal muscles (C2C12). The two chambers need to be isolated because they are supplied with different media specific to each type of cell through diffusion ports to allow the stem cells to differentiate into motor neurons and drive skeletal muscle cells into muscle fibers. The muscle chamber has posts with a 20 \( \mu \text{m} \) diameter and two alternating heights of 45 \( \mu \text{m} \) and 100 \( \mu \text{m} \), and each post is 100 \( \mu \text{m} \) apart. These posts are used in the muscle chamber only to help contain the skeletal muscle fibers (by having them grow around each post). Moreover, these chambers are connected by microchannels to offer chemical communication and a path for axon development. These microchannels are 1 mm long, 10 \( \mu \text{m} \) wide, and have alternating depths of 45 \( \mu \text{m} \) and 55\( \mu \text{m} \). The purpose of selecting these dimensions was to enable rapid axon travel (~2 days) to the muscle chamber. There are four inlets in total, two receiving the muscle medium and two for the
motor neuron differentiation medium (Figure 9). Individual fluidic channels located beneath the chambers (7mm x 500 μm x 375 μm) are used to supply the diffusion ports of each chamber with the appropriate chemical mixtures (either C2C12 or ADFNK media) received from the inlets as seen in Figure 9. The media flow out of the outlets and get collected in a petri dish as seen in Figures 9-10. Pictures of the actual microchip can be found in Appendix I.
Figure 9. 3D representation of the coculture microdevice.

(a) Bottom view of the microdevice showing only the cell chambers for MNs and muscle cells.
(b) Top view of the microdevice showing the inlets for both types of cells where the medium flows in through their respective fluidic channels as well as the outlets where the medium flows out.
2.2.2. Cell Culture Methods – Prior to Experiment Day

Cell culture processes make up the majority of this research, therefore, understanding the nature of it and its procedures is critical. Cell culture is the process of growing cells in controlled environments, usually away from their native milieu. The cells of interest can then be maintained under carefully monitored conditions after being removed from living tissue. There are two types of cell culture processes: primary cell
culture and subculture of cells. What differentiates the two is that cells collected from
tissues or animals are known as primary cell culture, while cell subculture, also known as
passaging of cells, entails expanding a portion of the primary culture for use in subsequent
experiments. More details on cell culture processes can be found in Appendix B.

Subculture of cells is the most used process in this thesis, and it involves retrieving
and thawing a vial of cells from the liquid nitrogen tank. Then, to separate the cells from
the medium, the mixture of thawed cells and medium was mixed with fresh media specific
to the type of cells used, and then centrifuged for 5 minutes. After centrifugation, the cells
stuck to the bottom of the tube with the medium on top of the cell pellet. To protect the
bottom cell pellet, the medium was cautiously sucked without disturbing it. Next, the cell
pellet needs to be broken by gentle mechanical agitation to facilitate the dispersion of the
cells in the medium. This was done by softly dragging the centrifuged tube across the hood
grill. Fresh medium was then added to the separated cells and mixed a few times before
being placed to a dish for proliferation.

Trypsinization consists of removing the adherent cells from the cell culture dish
and is the most frequently used procedure after subculturing but is done prior to the start
of the experiment or another subculture. Phosphate Buffered Saline (PBS) without calcium
(\(\text{Ca}^{2+}\)) and magnesium (\(\text{Mg}^{2+}\)) is used in the dissociation process before trypsin solution to
wash out or remove any medium or serum components from the dish, and resuspend cells
as opposed to Dulbecco’s Phosphate-Buffered Saline (DPBS) with the presence of calcium
and magnesium which may inhibit trypsin activity. The proteins that allow the cells to
adhere to the dish are broken down by trypsin. A detailed procedure on cell culture
procedures can be found in Appendix C.
2.2.2.1. Stem Cells Maintenance and Differentiation

Stem cells have the extraordinary ability to differentiate into a variety of cell types in the body. They can theoretically divide indefinitely to restore other cells as long as the human or animal is still alive, acting as a form of body repair system. When a stem cell splits, each new cell has the option of remaining a stem cell or transforming into a more specialized cell, such as a muscle cell, neuron cell, red blood cell, or brain cell. In this thesis, mouse embryonic stem cells (mESCs) with Hb9::GFP (Green Fluorescence Protein) reporter were derived from B6.Cg-Tg (Hlxb9-GFP)1Tmj/J mice and donated from Dr. Greg Cox Lab, The Jackson Laboratories.

2.2.2.2. Mouse Embryonic Fibroblast and Mouse Embryonic Stem Cell Subculture

Firstly, in a Petri dish (60 mm), mitomycin-c treated mouse embryonic fibroblasts (MEF) were cultured for ~24 to 48 hours, or until 80% confluence (Figure 11), using a specific media composition found in Table 1, using the subculture procedure described above (and detailed in Appendix C). Then, the MEF medium is vacuumed out of the dish and a vial of the thawed mESCs was cultured in the same Petri dish over the feeder layer of MEF. For maintenance and colony formation, the mESCs were supplied with the appropriate media composition (Table 2) every 24 hours for roughly 4 to 6 days until they reach about 80% confluence and show round clusters as seen in Figure 12.
**Table 1.** Components used to make the MEF medium for 100mL

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity for 100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Medium (DMEM) – high glucose (1% Anti-antimycotic (sc3690))</td>
<td>89 mL</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS), ESC-qualified</td>
<td>10 mL</td>
</tr>
<tr>
<td>MEM Non-essential amino acids solution, 10mM</td>
<td>1 mL</td>
</tr>
<tr>
<td>β-mercaptoethanol , 1000X (Cat. No.21985-023)</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

**Figure 11.** MEF feeder cells at 80% confluency as seen in the microscope (10x).
Table 2. Components used to make the mESC medium for 100mL (can last up to one week in 4°C).

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity for 100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knockout DMEM (1% Anti-antimycotic (sc3690))</td>
<td>83 mL</td>
</tr>
<tr>
<td>Knockout serum Replacement (KOSR)</td>
<td>15 mL</td>
</tr>
<tr>
<td>MEM Non-essential amino acids solution, 10mM</td>
<td>1 mL</td>
</tr>
<tr>
<td>Glutamax-I</td>
<td>1 mL</td>
</tr>
<tr>
<td>LIF (10ug/ml stock soln)</td>
<td>100 µL</td>
</tr>
<tr>
<td>β-mercaptoethanol, 1000X (Cat. No.21985-023)</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Figure 12. mESCs at 80% confluency as seen in the microscope (10x).
2.2.3. Experiment Preparation

2.2.3.1. Device Preparation

In order to be prepared for the day of the experiment, various steps need to be done to ensure that cells will happily grow and differentiate in the microdevice channels. Firstly, after the chip is made in the clean room, four caps or inlets and two small circular outlets are glued on the chips using A-100 silicone adhesive (Factor II). A little amount of uncured PDMS is then applied 500 μm from the top and bottom margins of the chambers. This PDMS functions as a temporary adhesive and water barrier between the coverslip and the device. Next, because the coculture microdevices are reusable, they need to be carefully sterilized before beginning cell culture to remove and prevent any contaminants, media, or cells remaining in the chambers that might come into contact with the microdevice, the culture media, or the cells. The devices were sprayed with 70% ethanol and given 30 minutes to dry in the hood while being exposed to UV light. Thereafter, repeated rinses with autoclaved and filtered deionized (DI) water were used to remove any remaining ethanol. Additionally, the fluidic channels must be relatively dry before the experiment starts to ensure any ethanol or water residue is gone as that would interfere with the medium and cell growth. This was accomplished by vacuuming out any residual water droplets from the outlets. In a similar manner, the already diced coverslips that are utilized to seal the cell culture chamber are also sterilized. A more detailed procedure for getting the microdevice ready for the experiment can be found in Appendix E.

2.2.3.2. Stem Cells to Motor Neuron Differentiation

On the day of the experiment, the trypsinization and centrifugation procedures were initiated after the mESC culture dish had been maintained in mESC medium for 4 to 6
days. After centrifugation, the separated cells were combined with fresh mESC medium and put into a dish coated with an attachment factor (AF 1x Gelatin, S006100), where they were cultured for an hour. This is known as the first gravity separation at this stage. Next, about 45 minutes into the hour-long wait for the first gravity separation, a 60-mm dish with the attachment factor (around 1.5mL) is put in a 37°C, 5 % CO₂, and 60% relative humidity incubator. After an hour, the second gravity separation was performed by carefully pipetting and transferring the cells and medium combination from the first dish to the dish that had been coated with AF (after aspirating the AF). The dish from the first gravity separation can now be thrown out, and the second dish will need to spend an extra hour in the incubator. The reason why the gravity separation process is performed is to help separate the MEF cells from the mESCs. As mentioned earlier, mESCs are cultured on top of MEF cells as they are known to be surface-loving cells (or feeder layer) and tend to settle faster than stem cells. At the end of the hour wait, the mixture of cells and medium from the second gravity separation dish was transferred to a tube and centrifuged at 1100 rpm for 5 minutes. This formed a pellet of cells at the bottom of the tube which means the cells and the medium are now separated. The medium was properly discarded, and the isolated cells were mixed with 80-100 μL of Geltrex™ (A1413301), which is an extracellular basement membrane matrix at a concentration of 1x10¹² cells/mL. By culturing the cells in the presence of a new medium composition, ADFNK medium, which contains 3 μM Purmorphamine, an Shh agonist, and 1 μM Retinoic acid, as shown in Table 3, the mESCs have the capacity to differentiate into MNs. The Hb9::GFP reporter transfected in the mESC allows for the detection of differentiating motor neurons by checking for Green Fluorescence Protein (GFP), which may become visible under the
microscope for the first time during the 4th and 5th day of the experiment. Then, the Hb9:GFP expression gradually increases proportionally to the fluorescence intensity and reaches its peak around the 7th day, following which the GFP expression moderately declines as the Hb9 expression decreases in postmitotic MNs until it completely vanishes by the 13th day.

**Table 3.** Components used to make the ADFNK medium for 50 mL.

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity for 50 ml Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced DMEM - F12 (1% Anti-antimycotic (sc3690))</td>
<td>22 mL</td>
</tr>
<tr>
<td>Neurobasal (1% Anti-antimycotic (sc3690))</td>
<td>22 mL</td>
</tr>
<tr>
<td>KOSR (10%)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Anti-antimycotic, sc3690 (1%)</td>
<td>50 μL</td>
</tr>
<tr>
<td>Glutamax (1%)</td>
<td>500 μL</td>
</tr>
<tr>
<td>β-mercaptoethanol , 1000X (Cat. No.21985-023)</td>
<td>50 μL</td>
</tr>
<tr>
<td>Retinoic acid (RA) – 1 μM concentration</td>
<td>5 μL</td>
</tr>
<tr>
<td>Purmorphamine (PM) – 3 μM concentration</td>
<td>15 μL</td>
</tr>
</tbody>
</table>

2.2.3.3. *Myoblasts (C2C12) Culture*

This study uses C2C12 (C2C12 - ATCC® CRL-1772) cells which is an immortalized mouse myoblast cell line that initiates the formation of skeletal muscle fibers, as indicated in Chapter 1. The reason for using this cell line instead of differentiating mESCs to skeletal muscle is because of the timeline; it takes about 21 days for the mESCs
to differentiate into skeletal muscles while it takes 7 days for the mESCs to differentiate into MNs. This difference is too great for us to simultaneously conduct our experiment and load the cells onto the device. The C2C12 cell line has the advantage that it differentiates into myotubes in the presence of a growth medium, eliminating the need for a differentiation medium specific to skeletal muscles. Moreover, the differentiation timeline is similar to that of MNs (7 days) when the seeding cell density is $1 \times 10^4$ cells/mL. The same subculture technique as described under the “cell culture methods” section is used to plate a vial of C2C12 in a 60-mm Petri dish coated with AF. The C2C12 cells are then cultured for 24–48 hours prior to the start of the experiment, or until the cells reach 80% confluency as seen in Figure 13, using the C2C12 growth medium as indicated in Table 4. Unlike mESCs, C2C12 cells do not require the use of feeder cells (mitomycin-c treated MEFs) which makes them very straightforward to maintain and expand, hence, on the day of the experiment, gravity separation procedures are not necessary. On experiment day, C2C12 cells are just trypsinized and centrifuged. The separated cells were mixed with Geltrex™ at $1 \times 10^4$ cells/ml concentration and loaded into the microdevice by blobbing 0.8μL on a coverslip and flipping it over the chamber, concurrently with mESCs. With the supply of the appropriate C2C12 medium (Table 4), the cells are prone to proliferate and differentiate in the microdevice. More details on plating the C2C12 cells can be found in Appendix C.
Table 4. Components used to make the C2C12 medium for 100mL.

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity for 50 ml tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM – high glucose (1% Anti-antimycotic (sc3690))</td>
<td>45 mL</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS), ESC-qualified</td>
<td>22 mL</td>
</tr>
<tr>
<td>Anti-antimycotic, sc3690 (1%)</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

Figure 13. C2C12 cells at 80% confluency as seen in the microscope (10x).
2.2.3.4. **Loading the Cells in the Coculture Device**

This section provides a more detailed explanation of how the cells are loaded into the microdevice chambers. As explained in the previous two sections, Geltrex™ was mixed with the appropriate concentration of mESCs and C2C12 cells, each in one small (0.5 mL) Eppendorf tube. The Geltrex is preserved in a -20°C freezer and transferred to a 4°C fridge the night before the experiment. After the coverslips were sterilized, about 0.2 μL of just Geltrex is pipetted onto the center coverslip, and 30 seconds later, the coverslip was then flipped over the entire region of interconnecting channels, using tweezers. This filled the channels with gel, leaving no room for cells. Immediately, 0.8 μL of the gel mixture (mESCs and Geltrex) is blobbed onto a coverslip for the MN chamber, and 30 seconds later, the coverslip is flipped over before placing it onto the MN chamber. The same process is used for C2C12 chambers. Then, to hold the three coverslips together and avoid uneven drying or bubble formation, 0.8 μL of Geltrex is pipetted on the big coverslip, and after a 30-second pause, the coverslip is then carefully placed on top of all three smaller coverslips as demonstrated in Figure 14. A detailed procedure for loading the cells onto the microdevice is found in Appendix E. Once all of the coverslips are positioned in the right places, the device is put in a 37°C, 5% CO₂, and 60% RH incubator for four minutes in order for the Geltrex to solidify and form the desired 3D matrix for the cells. After four minutes, the device is placed under the hood and the cryotubes (media reservoirs) are twisted on the inlets of the device. The appropriate medium for each chamber is supplied in the tubes, and a cap is placed on top of each tube to seal the reservoir preventing any sort of contaminants from entering the medium tubes, as seen in Figure 10. After a brief length of time, the medium should have flowed out of the two outlets after flowing through
the fluidic channels and delivering the proper medium to each chamber through the diffusion ports.

![Image](image.jpg)

**Figure 14.** Coverslips layout on the device after loading the cells.

Lastly, the device is placed in the incubator, and the media is changed every day for 15 days. As previously explained, it is possible to monitor the mESCs differentiation into MNs by looking for GFP expression in live cells since the mESCs are transfected with the Hb9::GFP reporter. This means that if Hb9 is present implying that mESCs are differentiated into MNs, then GFP fluorescence will be visible under the microscope (usually around day 5 of the experiment). The Hb9::GFP expression gradually increases proportionally to the fluorescence intensity and reaches its peak around the 7th day, following which the GFP expression moderately declines as the Hb9 expression decreases in postmitotic MNs until it completely vanishes by the 13th day. The experiment is then terminated by day 15 of cell differentiation. A multi-step process demonstrating the experiment timeline can be found in Figure 15.
Figure 15. Multi-step process demonstrating the experiment timeline.

1. Grow the mouse embryonic fibroblasts or feeder layers (MEFs) in a Petri dish until 80% confluency (Figure 11).
2. Add the mouse embryonic stem cells on top of the MEFs and grow until 80% confluency (Figure 12).
3. Start the skeletal muscle cells (C2C12) two days prior the experiment and grow until about 80% confluency (Figure 13).
4. Start experiment by loading the cells on the device (Figure 14), then monitor the MN differentiation by looking for Hb9::GFP expression.
2.2.4. Immunocytochemistry and Imaging

In the scientific process of immunocytochemistry, certain proteins, antigens, or markers in a sample of cells are identified using antibodies. There are two sorts of antibodies, based on whether they target other antibodies or directly bind to antigens.\(^{54,55}\) The secondary antibody is designed to have a fluorescence attached to it for visibility, whereas the primary antibody locates the marker, gene, or protein in the cell sample. Secondary antibodies bind to primary antibodies and give them a luminous color, making it possible to see the particular gene of interest from the original antibody.\(^{54,55}\) Only primary antibodies from the same species of the secondary antibody will attach to the secondary antibody.

On day 15 of the experiment, the cells in the chambers were fixed, then various transcription factors were stained using immunocytochemistry. The coverslips are cautiously removed to maintain the spatial dispersion of cells in Geltrex. This is a critical step as the 3D matrix of cells needs to remain in the chambers to proceed with the immunocytochemistry process of the cells kept in the device. If this is not done correctly and the 3D matrix of cells gets ripped off with the coverslip, the device can no longer be stained. Cell samples were fixed by immersing the device (chambers facing down) in 4% paraformaldehyde for 10 minutes prior to primary and secondary antibody staining. The primary antibodies used to identify the limb innervating MNs from the LMC subtype as well as the lateral subdivision were anti-GFP (1:200), FOXP1 (1:100), and Lhx1 (1:100) at appropriate dilution. The secondary antibodies (AlexaFluor) were used at 1:500 dilution, green color for anti-GFP, red color for Foxp1, and magenta color for Lhx1. For staining the nuclei of the fixed cells, Hoechst-33342 at 1:500 dilution was used by diluting
the stain in cell culture media without serum or PBS and soaking the sample for 45 minutes under the hood, followed by three PBS washes. A detailed procedure for fixing and staining the cells in the microdevice can be found in Appendix F.

The lab microscope, Zeiss Axio Observer Z1 inverted microscope with Zeiss MRm monochrome camera, was used to image the stained samples with a 10x objective magnification when imaging one chamber at a time and a 5x objective when imaging the whole microdevice (both chambers and interconnecting microchannels). The overall visual magnification can be determined by multiplying the objective magnification (10x or 5x) by the eyepiece magnification which is also 10x and this gives us a 100x visual magnification (with a 10x objective), meaning that 2mm can be seen for one chamber (1mm).\(^56,57\) The images analyzed were all taken with a 10x objective as only the MN chambers were analyzed. The numerical aperture (NA) is a dimensionless measure of the ability to gather light and resolve fine specimen detail at a fixed object distance. In this case, for 10x objective magnification, the NA is 0.25.\(^56\)

Various imaging techniques were used to capture images used for analysis. Transmitted light microscopy, which is used to check the cells, is just any type of microscopy where the light is transmitted or passed from a source to the opposite side of the lens through the sample. To look at the devices when the HB9::GFP is expressed, an imaging method called fluorescence imaging can aid in the visualization of biological processes occurring in a living or fixed organism. Using this technique, fluorophores can be excited, and the fluorescence signal can then be detected.\(^58\) In this work, fluorescent dyes from a secondary antibody that labels transcription factors (primary antibody) in fixed cells are visualized using fluorescence imaging. A stack of images at different Z-
planes (4 μm apart) was acquired to visualize the cells that grow in a three-dimensional matrix (Geltrex) in the chambers. Finally, the multi-channel fluorescence technique is used to register different emission wavelengths almost simultaneously, allowing us to image different fluorescent dyes representing different transcription factors from the same sample or device.\textsuperscript{57,59} For example, it is able to capture the fluorescent dyes Alexa fluor 488 which has an excitation peak at 499 nm and an emission peak at 520 nm, Alexa Fluor 594 with an excitation peak at 590 nm and an emission peak at 618 nm, and Alexa Fluor 647 with an excitation peak at 650 nm and an emission peak at 671 nm, almost at the same time. In order words, this imaging technique is capable of capturing one image or a Z-stack image with multiple channels that represent each fluorescent dye for each device as seen in Figure 16 (in this example both anti-GFP and anti-Myosin were stained for the green-fluorescent color Alexa Fluor 488, this was not the case for the subsequent devices).

\textbf{Figure 16.} Example of fixed and stained fluorescence images using fluorescence, the multi-channel techniques.
CHAPTER 3: EXPERIMENTS AND RESULTS

3.1. Experimental Outcome Rundown

A summary of all the experiments performed can be found in Table 5. There was a total of thirteen experiments performed and six of them were successful. There are four experiments with C2C12 cells in the muscle chamber, and these devices were fixed at day 14. Then, they were stained with anti-GFP which stains for the Hb9 promoter as after the 13th day of differentiation the hb9::GFP expression disappears, and with anti-Foxp1 which with the combination of hb9 stains for LMC neurons. The last two successful experiments had no C2C12 cells in the muscle chambers, but the muscle chambers were still supplied with C2C12 medium. These devices were fixed at day 15 and stained for the same transcription factors (Hb9, Foxp1) with the addition of Lhx1 which is a transcription factor only expressed in lateral LMC (LMC_L), the subdivision of LMC neurons. Staining for Lhx1 would further prove the presence of LMC MNs, and it would be interesting to compare the amount of LMC_L present as opposed to the other subdivision of LMC which is the medial LMC (LMC_M) MNs. All devices were stained with Hoechst-33342, which stains for nuclei in live and fixed cells. This stain was crucial to quantify the cells in the MN chambers and determine the percentage of MNs and LMC MNs present in these chambers.
Table 5. Rundown of experimental outcomes.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Outcome</th>
<th>Antibody Stains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unsuccessful</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td><strong>Successful (coculture with C2C12 cells)</strong></td>
<td>Anti-GFP, anti-Foxp1, Myosin, Hoechst</td>
</tr>
<tr>
<td>3</td>
<td><strong>Successful (coculture with C2C12 cells)</strong></td>
<td>Anti-GFP, anti-Foxp1, Myosin, Hoechst</td>
</tr>
<tr>
<td>4</td>
<td><strong>Successful (coculture with C2C12 cells)</strong></td>
<td>Anti-GFP, anti-Foxp1, Myosin, Hoechst</td>
</tr>
<tr>
<td>5</td>
<td>Unsuccessful</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>Unsuccessful (no Hb9::GFP fluorescence)</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td><strong>Successful (coculture with C2C12 cells)</strong></td>
<td>Anti-GFP, anti-Foxp1, Myosin, Hoechst</td>
</tr>
<tr>
<td>8</td>
<td>Unsuccessful (contamination)</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td><strong>Successful (control without C2C12 cells)</strong></td>
<td>Anti-GFP, anti-Foxp1, Lhx1, Hoechst</td>
</tr>
<tr>
<td>10</td>
<td><strong>Successful (control without C2C12 cells)</strong></td>
<td>Anti-GFP, anti-Foxp1, Lhx1, Hoechst</td>
</tr>
<tr>
<td>11</td>
<td>Unsuccessful (expired Geltrex)</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td>Unsuccessful (expired Geltrex)</td>
<td>N/A</td>
</tr>
<tr>
<td>13</td>
<td>Unsuccessful (expired Geltrex)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.2. Image Quantification and Analysis

After imaging the devices under the microscope using the various imaging techniques previously described, the images were imported to FIJI (NIH) for analysis. As previously mentioned, as a result of the cells growing in a three-dimensional manner, the devices needed to be imaged using the Z-stack technique to capture the cells at different heights. For this work, two analyses were performed and then compared. For both analyses, the raw Hoechst images (Figure 18 (a)) were processed in FIJI, which means certain operations were performed on the Hoechst images. This crucial intermediate step, which is taken before performing an analysis, helps to identify and accentuate the desired information in a raw image or suppress unimportant signals and noise that would otherwise obstruct accurate analysis. First, some signal enhancement operations were used; a contrast enhancement (saturated 0.35), then a background subtraction which removes uninformative signals not associated with the structure of interest. Without subtracting background noise, the presence of uninformative signals such as the fluorescence generated from molecules that are not the reporter of interest or out-of-focus signals that may emanate from the fluorescent reporter molecules could render subsequent analysis inaccurate or otherwise misleading. The background subtraction method used here is the ‘rolling ball’ subtraction of 10. Then another contrast enhancement (saturated 0.35) was applied before the feature detection operation was conducted through an automated intensity threshold method using the IsoData method consistently throughout all images. Finally, some object segmentation operations were used such as ‘fill holes’ within the objects that have areas of dim signal and ‘watershed’ which can separate the nuclei that share a common border followed by binary operations that can refine the segmented nuclei. The binary operations were erosion followed by dilation which can be useful to remove small unwanted
nuclei produced by spurious signals. Figure 17 shows all the steps for image processing after image acquisition, image pre-processing, and before starting the two analyses.

**Figure 17.** A flow diagram showing the different steps used for image processing in FIJI from the image acquisition stage to the subsequent image analysis methods.

From all these operations, images like Figure 18 (b) were generated. See macros in Appendix G for all the operations used. These operations were carried out to extract useful information from the Hoechst images which helped to quantify the number of cells.
Figure 18. Hoechst images (a) before and (b) after image processing.

The first analysis method used a built-in function on FIJI called Z-project, which analyzes a stack by applying different projection methods to the pixels within the stack. This process handles the whole stack and combines all of the slices in the stack by converting it to a 2D image which is the projection of maximum intensity pixels from each individual slice. The 2D image projection highlights specific data from the stack. This built-in function was used because it reduces computational time in comparison to handling an entire Z-stack. Figure 19 is a representation of how the Z-project built-in function works with the image processing operations.
This script, written in FIJI, consisted of a binary logical structure, in which a box is drawn around each nucleus within the Hoechst (Z-project) image to facilitate the counting of the total number of cells. Then, each box is further examined, and if a green pixel is detected, it will be counted as a cell stained with anti-GFP (expressing Hb9), meaning the cell is a MN. This allows for the total number of MN to be counted. Lastly, if a red pixel (FOXP1-stained) is also detected in the GFP-stained nuclei boxes, this means that the MN is an LMC subtype, therefore, the number of LMC within the MNs can be determined. Knowing the total number of cells, the number of motor neurons and the number of LMC within MNs, the percentage of MNs and LMC MNs was determined. Table 6 lists these numbers for six devices (MN chamber) from all six successful experiments (Table 5) respectively, four of which were coculture devices (MN 1, MN 2, MN 3, MN 4), and two of which were control devices (MN 5, MN 6).
Table 6. MN Z-project image results (a) with and (b) without C2C12 in the muscle chamber.

(a)

<table>
<thead>
<tr>
<th>MN Image</th>
<th>Total Cell Count</th>
<th>MN Count</th>
<th>LMC Count</th>
<th>MN/Total</th>
<th>LMC/MN</th>
<th>LMC/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN 1</td>
<td>79</td>
<td>64</td>
<td>62</td>
<td>81.0%</td>
<td>96.9%</td>
<td>78.5%</td>
</tr>
<tr>
<td>MN 2</td>
<td>856</td>
<td>518</td>
<td>413</td>
<td>60.5%</td>
<td>79.7%</td>
<td>48.2%</td>
</tr>
<tr>
<td>MN 3</td>
<td>783</td>
<td>573</td>
<td>548</td>
<td>73.2%</td>
<td>95.6%</td>
<td>70.0%</td>
</tr>
<tr>
<td>MN 4</td>
<td>802</td>
<td>523</td>
<td>436</td>
<td>65.2%</td>
<td>83.4%</td>
<td>54.4%</td>
</tr>
<tr>
<td>Overall</td>
<td>2520</td>
<td>1678</td>
<td>1459</td>
<td>66.6%</td>
<td><strong>86.9%</strong></td>
<td>57.9%</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>MN Image</th>
<th>Total Cell Count</th>
<th>MN Count</th>
<th>LMC Count</th>
<th>MN/Total</th>
<th>LMC/MN</th>
<th>LMC/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN 5</td>
<td>373</td>
<td>306</td>
<td>295</td>
<td>82.0%</td>
<td>96.4%</td>
<td>79.1%</td>
</tr>
<tr>
<td>MN 6</td>
<td>740</td>
<td>472</td>
<td>463</td>
<td>63.8%</td>
<td>98.1%</td>
<td>62.6%</td>
</tr>
<tr>
<td>Overall</td>
<td>1113</td>
<td>778</td>
<td>758</td>
<td>69.9%</td>
<td><strong>97.4%</strong></td>
<td>68.1%</td>
</tr>
</tbody>
</table>

The disadvantages of this method are undercounting the number of cells because not every slice in the stack is used individually, but a combined image of the stacks is used; and this method is strenuous and inefficient, leading to higher computational times. As a result of these disadvantages, a second analysis method was used.

The second image analysis method handled all the slices in the stack individually, which leads to a more realistic number of cells counted, even though it could be overcounting the cells that are shown in two different slices. This script is much more efficient as instead of generating a bounding rectangle around each nucleus, it generates a mask for each slice of the entire stack for the Hoechst-stained processed images (same image processing methods). Figure 20 (a) shows the mask generated for Hoechst using Z-project for visualization of a single representative image (this was completed for every slice in the stack). Then, that mask was overlaid on the autothresholded image, using IsoData method, for the GFP channel, producing a new image. A few operations were done on this new image; ‘enhance contrast’ (saturated 0.35), an automated intensity threshold method using the Otsu method, and some
object segmentation operations (‘fill holes’ and ‘watershed’), resulting in Figure 20 (b), a second mask (for GFP) which represents the number of the total cells that are MNs. This second mask (GFP) was overlaid on the autothresholded (IsoData method) Foxp1 channel, producing another new image where the same operations were performed (‘enhance contrast’– saturated 0.35, Otsu method, fill holes, and watershed). This resulted in Figure 20 (c) which represents the LMC MNs number. A count of the total cells, MNs, and LMC MNs were generated in Table 7 from the corresponding images in Figure 20. The second analysis can be summarized in Figure 21 with all the operations included.

Figure 20. Masks-generated images for coculture device (Z-Project for visualization only): (a) Hoechst (nuclei) mask, (b) Hoechst mask overlaid on GFP (hb9) image, and (c) GFP mask overlaid on FOXP1 image.
**Figure 21.** Summary of the different steps and operations performed in the second analysis (Mask/Z-stack method)

The same approach was used for the control devices with the addition of Lhx1 stain. First, the Hoechst (nuclei) mask was generated using the same processing methods to keep the analysis consistent as seen in Figure 22 *(a)*. This mask was then superimposed on the GFP (Hb9) channel autothresholded (IsoData method) image. And after performing the same operations as explained above (‘enhance contrast’—saturated 0.35, Otsu method, fill holes, and watershed) Figure 22 *(b)* was produced showing the proportion of MNs to total cells. This led to the creation of a second mask (GFP), which was then applied to the autothresholded (IsoData method) Foxp1 channel to produce Figure 22 *(c)* after the same operations were applied (‘enhance contrast’—saturated 0.35, Otsu method, fill holes, and watershed), which illustrates the LMC MNs value. Similarly, this led to the third mask being generated for Foxp1 which was overlaid on the last channel representing Lhx1 stained cells using the same image processing operations (Figure 22 *(d)*). The relevant images (Figure 22) were used to generate a count of all the cells, MNs, LMC MNs, and LMC\_L MNs as depicted in Table 7.
Figure 22. Masks-generated images for control device (Z-Project for visualization only): (a) Hoechst (nuclei) mask, (b) Hoechst mask overlaid on GFP (hb9) image, and (c) GFP mask overlaid on FOXP1 image, (d) Foxp1 mask overlaid on Lhx1 image.
Table 7. MN Z-stack images results (a) with and (b) without C2C12 in muscle chamber.

(a)

<table>
<thead>
<tr>
<th>MN Image</th>
<th>Number of Slices</th>
<th>Total Cell Count</th>
<th>MN Count</th>
<th>LMC Count</th>
<th>MN/Total</th>
<th>LMC/MN</th>
<th>LMC/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN 1</td>
<td>13</td>
<td>674</td>
<td>503</td>
<td>488</td>
<td>74.6%</td>
<td>97.0%</td>
<td>72.4%</td>
</tr>
<tr>
<td>MN 2</td>
<td>30</td>
<td>8643</td>
<td>4824</td>
<td>3655</td>
<td>55.8%</td>
<td>75.8%</td>
<td>42.3%</td>
</tr>
<tr>
<td>MN 3</td>
<td>22</td>
<td>7759</td>
<td>5568</td>
<td>5245</td>
<td>71.8%</td>
<td>94.2%</td>
<td>67.6%</td>
</tr>
<tr>
<td>MN 4</td>
<td>19</td>
<td>7926</td>
<td>4091</td>
<td>2980</td>
<td>51.6%</td>
<td>72.8%</td>
<td>37.6%</td>
</tr>
<tr>
<td>Overall</td>
<td>84</td>
<td>25002</td>
<td>14986</td>
<td>12368</td>
<td>59.9%</td>
<td>82.5%</td>
<td>49.5%</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>MN Image</th>
<th>Number of Slices</th>
<th>Total Cell Count</th>
<th>MN Count</th>
<th>LMC Count</th>
<th>LMCl Count</th>
<th>MN/Total</th>
<th>LMC/MN</th>
<th>LMCl/LMC</th>
<th>LMC/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN 5</td>
<td>25</td>
<td>4260</td>
<td>3264</td>
<td>3150</td>
<td>2907</td>
<td>76.6%</td>
<td>96.5%</td>
<td>92.3%</td>
<td>73.9%</td>
</tr>
<tr>
<td>MN 6</td>
<td>31</td>
<td>9175</td>
<td>6271</td>
<td>6168</td>
<td>3745</td>
<td>68.3%</td>
<td>98.4%</td>
<td>60.7%</td>
<td>67.2%</td>
</tr>
<tr>
<td>Overall</td>
<td>56</td>
<td>13435</td>
<td>9535</td>
<td>9318</td>
<td>6652</td>
<td>71.0%</td>
<td>97.7%</td>
<td>71.4%</td>
<td>69.4%</td>
</tr>
</tbody>
</table>
Very similar percentages are displayed by both analysis methods (Figure 19), demonstrating the robustness of the cell’s quantification and analysis (macros can be found in Appendix G). As seen in Table 6, the first method heavily undercounts the number of cells because the Z-Project function is applied instead of handling the whole stack, leading to the second analysis (Table 7).

Figure 23. A comparison of the two analyses for LMC percentage of devices with (coculture) and without (control) C2C12 in the muscle chamber.

The latter method was the one selected to discuss the significance of C2C12 present in the muscle chamber of the device. As seen in Table 7 and Figure 19, the percentage of LMC present in the MN chamber is high (>80%) for both the coculture (with C2C12 cells) and control (without C2C12 cells) experiments. After running a statistical analysis, it was found that there was no statistical difference between the control and coculture data using the
Wilcoxon rank sum test. These results are very surprising as they indicate that the presence of C2C12 cells is not required for MNs to differentiate into the specific subtype LMC. As mentioned earlier, the control devices were also stained for Lhx1 which stains specifically for lateral LMC MNs. It is very interesting to note that about 70% of the LMC MNs were part of the lateral subdivision of LMC (LMCₕ).

3.3. Discussion

In this study, we were able to differentiate mESCs into LMC MNs which was confirmed with high Foxp1 expression, a known marker for this subtype. Of the LMC MNs generated in the microdevice, about 70% belonged to the lateral LMC subtype (confirmed with Lhx1 expression), with the remaining 30% presumably belonging to the other LMC subtype (LMCₘ). The results obtained suggest that LMC (and LMCₕ) MNs naturally develop in vitro. The limb-innervating MN development might be induced by morphogens provided by neighboring neural cells spatially organized in the neural tube which could offer the essential environmental stimulus. These findings can be justified with those of other research efforts to create LMC MN subtypes, such as that of Patani et al., who found that RA (which is supplied in the ADFNK differentiation medium), synthesized by nearby MN populations in the spinal cord, contributes to a rostral (brachial level) identity, particularly of an LMC MN subtype and that when MNs were produced independently of RA, MNs of the MMC subtype were predominant.⁶¹–⁶³ Others have expressed that the specific differentiation medium (ADFNK) with RA/Shh conditions (here we use Purmorphamine, as an agonist) demonstrated that the emergence of lateral LMC through Lhx1 expression in MNs is greater when exposed to RA after five days of differentiation.⁴ Aside from the presence of specific morphogens, the timeline of MN differentiation could be crucial. As previously stated, preliminary research has shown
that LMC neurons were not found when recapitulating organized spinal tissue on-chip.\textsuperscript{13} The most significant difference between the preliminary results\textsuperscript{13} and the results generated in this work is the timeline of the differentiation experiments. Differentiating mESCs into MNs for seven days resulted in a majority of MMC neurons (expressing Lhx3), and it did not yield the LMC subtype\textsuperscript{13}, whereas carrying the differentiation for 14 to 15 days generated MNs of the LMC subtype. This can be explained by the fact that all developing MNs transiently express Lhx3 in the early stages of MN \textit{in vitro} development (~5 days), and that additional MN subtypes could emerge as Lhx3 disappears if the cells are cultured for extended periods of time.\textsuperscript{23,64,65}
CHAPTER 4: CONCLUSIONS AND FUTURE WORK

4.1. Conclusion

This work describes a microdevice that generates controlled, user-defined biochemical concentration profiles to provide unprecedented control over the cellular environment for the study of chemical signaling mechanisms occurring during development. As demonstrated throughout this thesis, the use of microfluidic technology can be a pertinent tool for investigating various aspects of the *in vivo* microenvironment in an *in vitro* system. This system is capable of 3D culturing two different cell types (MNs and C2C12) separately in chambers while still enabling intercellular communication through the interconnecting microchannels. The main hypothesis in this study was that the cues essential for the differentiation of motor neurons into limb-innervating neuron subtypes present in the limb motor column (LMC) are induced by growing adjacent skeletal muscle cells. Therefore, experiments examined the differentiation of motor neurons, specifically limb-innervating neurons (LMC and LMC\(_L\)) with and without the presence of skeletal muscle cells. The results of the experiments revealed that high yields of LMC MNs were generated whether the C2C12 cells were present or absent.

4.2. Future Work

To confirm these results, another experiment can be performed with a different type of cells in the muscle chamber. It could be mESCs with a distinct differentiation medium other than ADFNK or repeat the experiment with Geltrex only in the muscle chamber supplied with ADFNK to rule out the effect of C2C12 medium in sending signaling factors to the MNs to differentiate into LMC neurons. Lastly, to confirm the timeline supposition, some devices could be fixed on different days of differentiation such as day 5, day 7, day 10, and day 15. Other interesting avenues could be investigated such as identifying ~29% – 40.1% of other cell
types present in the MN chamber. Ultimately, the microdevice can be used to form neuromuscular junctions \textit{in vitro}. This can be accomplished in different ways; one way is to stain the muscle chamber for the postsynaptic (Acetylcholine) receptors of NMJ which could be done using $\alpha$-Bungarotoxin, another way would be to electrically stimulate the MN axons by inserting electrical wires into the medium tubes and applying a specific voltage, and then checking for any muscle contractions in live cells.
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68. Feeder-Dependent Culture and Passaging Mouse Embryonic Stem Cells (mESCs) in KnockOut™ Serum Replacement with Leukemia Inhibitory Factor (LIF) Publication Part Number MAN0007198. Published online 2012.


Microfluidics Overview

Microfluidics is a relatively new multidisciplinary field that combines physics, chemistry, fluid dynamics, microelectronics, biology, and material science principles. This new and unique engineering technology involves the precise control, distribution, and manipulation of fluids in channels that are geometrically limited to a small scale (typically tens of micrometers), where surface forces outweigh volumetric forces. In the microscale range, many materials can be manufactured into minuscule chips with channels and chambers using a wide range of

Figure A1. Development of in vitro models. Image Retrieved from Ref [9].
technologies for the desired size, shape, and geometry. Over the past decade, microfluidics and microinstruments have found increasing applications in a wide range of domains, including biology, chemistry, medicine, and physical sciences. Nanoparticle manufacturing, drug encapsulation, administration, and targeting, cell analysis, diagnostics, and cell culture are all examples of applications for microfluidic chips, whether employed alone or in combination with other devices. This thesis focuses on the cell culture applications of these innovative devices, which eliminates the requirement for cells to be contained in a Petri dish. As seen in Figure A1, the development of in vitro models and experiments has evolved from 2D cell culture to 3D cell culture in a Petri dish to the microfluidics technology. A battery of particular microtools, structures, and functions can now be added to the cell culture dish, allowing for more generic tissue motifs. The “organs-on-chip”, a fascinating concept, has recently arisen where multiple on-chip compartments provide diverse systemic organ tissues derived from organoids or dissected tissue. To replicate blood traveling between different organs, the compartments are connected by microfluidic channels. This method is considered to be quite effective in identifying drug metabolism mechanisms as well as the impact of drug byproducts or upstream secretions on numerous downstream organs. However, these technologies are not capable of manipulating the spatial chemical topography of cells, making them unsuitable for developmental studies, which are of interest to our research. As a result, we elaborate on this concept in this thesis to create an on-chip replica of the spatiotemporal neuromuscular chemical environment. This allows researchers to track how motor neurons (especially limb-innervating neurons) and skeletal muscle cells co-differentiate.
Fabrication of Microfluidic Devices

Materials:

In order to fabricate the microfluidic device, it is imperative to choose the suitable material as the properties of synthesized nanomaterials can be changed while working on a microscale surface.\textsuperscript{20} For instance, shorter retention times, laminar flows, large surface-to-volume ratios, and improved heat and mass transfer all contribute to unique phenomena in capillary.\textsuperscript{20,33} Moreover, the wetting and contact angles of an aqueous solution on the chip materials are of fundamental importance unlike for macroscale vessels. There are also many other essential properties that need to be considered when choosing the material for the microfluidic device. Some examples are the ease of fabrication, the durability, the transparency, the temperature and pressure needed, the biocompatibility, and the chemical compatibility with the implied reagents.\textsuperscript{20,30} There are many different types of materials used for manufacturing microfluidic devices that apply these properties such as silicon, glass, polymers, metals, and ceramics. Depending on its intended application, each material has advantages and disadvantages, and the diversity and quality of materials are unceasingly increasing.

Silicon has been a prominent material for microfluidic systems for decades due to its 3D micromachining capability, highly reproducible manufacture, micron size feature capability, design flexibility, semiconducting properties, and surface modification capabilities. Some applications of silicon microfluidic devices can be found in medical diagnostics and organ-on-chip devices for drug toxicity screening.\textsuperscript{30,31,33} Despite the numerous advantages that silicon has to offer, several disadvantages must be considered when incorporating this material into practical applications. Silicon highly absorbs visible light (opaque), making optical detection in the visible and ultraviolet ranges more difficult than with transparent materials. If in-situ
imaging is required, at least a portion of the device must be non-silicon to allow for optical detection.\textsuperscript{30,31} Furthermore, because silicon is quite fragile and has a high elastic modulus, the attachment of external components such as a pump increases the risk of fragmentation. Lastly, using silicon as a material for microfluidic devices is relatively expensive.\textsuperscript{30}

Another common material used in microfluidic devices is glass which allows easy surface functionalization, it is chemically inert, electrically insulating, thermostable, and importantly, biologically compatible. Because of these properties, glass can be used in microfluidic devices to carry out chemical reactions that require extreme conditions such as high temperatures and pressures, as well as aggressive solvents.\textsuperscript{30,31} Glass is also advantageous due to its excellent optical transparency, which can be used for optical detection, as well as its low cost and the ability to integrate active components. Moreover, performing experiments with this material allows for the effective cleaning of the device done by heating or washing it with chemicals because of the thermal and chemical stability of glass (similar to silicon).\textsuperscript{30,31} Also, in laboratories, biologists and chemists usually chose glass as a traditional material because glass is compatible with biological samples and makes it useful for biochemical analyses. It's no surprise that glass is a popular material for microfluidics devices given all of these advantages. However, using glass introduces some microfabrication challenges because glass is expensive to manufacture into chips with submillimeter features which requires time-consuming and costly procedures, e.g., laser micromachining.\textsuperscript{30}

Metals have numerous benefits that make them ideal for chip microfabrication. They are inexpensive, widely available, and can withstand high heat loads, high pressure, and most toxic chemicals, making them ideal for cleaning processes. Some examples of popular metals include aluminum, iron, and copper, but they are frequently alloyed with other metals in order
to improve their chemical resistance.\textsuperscript{30} An aluminum oxide-based material, also known as low
temperature co-fired ceramic (LTCC), is a substrate technology that supports active and
passive components while providing reliable, high density electrical interconnection. This
material has a distinct surface chemistry and is resistant to corrosion, high temperatures, and
mechanical stresses.\textsuperscript{30,31} The microfabrication of intricate fluidic channels is made possible by
the multilayer fabrication method.\textsuperscript{31} However, ceramics have several drawbacks in terms of
dimensional stability, porosity, and fragility, making it challenging to incorporate this type of
material into a comprehensive microsystem.\textsuperscript{30}

Silicon, glass, and metals are all inorganic materials used for microfluidics, but they are
not the best choice for prototyping experiments. Nevertheless, they have useful applications
where some of their qualities can be used. For instance, inorganic materials are preferred when
high temperatures or pressures are required. Glass is the best material for applications requiring
sensitive optical detection or high voltage, and silicon materials are frequently used when
semiconductor features or devices are required.\textsuperscript{31}

Finally, polymer is an organic material with numerous advantages and practicalities,
making it a viable alternative to silicon and glass chips in microfluidic device fabrication. The
majority of polymers are organic and cheap, unlike the other materials utilized in
microfabrication. Polymers are inexpensive due of their low cost and simple manufacturing
procedures.\textsuperscript{30,31} Polymer microfluidic devices can be used for everything from nanoparticle
manufacturing to fluid manipulation. Any microfluidic system made of polymers are suitable
for applications at either room temperature or higher temperatures up to 200ºC.\textsuperscript{30} They can
also be either transparent or semitransparent, allowing for optical access to follow the progress
of a reaction, which is especially important when nano-crystallizations are performed.
Polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), fluoropolymers, cyclo-olefin polymers and copolymers (COPs/COCs), and Thiol-ene polymers are among the most commonly used polymers in the creation of microfluidic devices (TEs). PDMS, an elastomer with outstanding microchip production capabilities, is one of the most well-known materials in this category. A PDMS microfluidic device is made by pouring uncured PDMS into micro-structured molds, curing, and releasing them, which results in the PDMS replicating tiny geometries. To produce microfluidic channels, the molded portion was then bonded to a surface (usually glass). Due to the soft, elastomeric character of PDMS, this method of manufacturing is known as soft lithography. This material is less expensive, easy to mold and reusable, and ideal for prototyping. It also has optical transparency, gas permeability, biocompatibility, low auto-fluorescence, natural hydrophobicity, and high elasticity. All of these characteristics make PDMS an excellent choice for a wide range of microfluidic device developments and applications in biomedical research, particularly cell culture, cell screening, and biochemical tests. Bacteria, as well as their proteins and DNA, can be detected using PDMS micro-devices, which is extremely valuable for disease detection. However, some difficulties arise in organic synthesis and cell biology. Because of its porosity and its hydrophobic nature (contact angle ~110), PDMS is an absorbing polymer that allows numerous small molecules like proteins and signaling molecules to diffuse into it, which causes a problem when being used for drug discovery, cell culture and molecular biology. Organic solvents are incompatible with the material because their molecules can be absorbed into channel walls and cause the platform to inflate. Another issue can be water evaporation via channel walls, which causes a change in solution concentration. Depending on the needed features and
eventual usage, various polymeric materials are currently being explored for microfluidic production to overcome these difficulties.

Recently, the combination of silicon and glass-based microfluidic devices has emerged as the materials of choice as it has none of the aforementioned limitations. For the purpose of this project, a silicon-based material was used in the fabrication of the microdevice.

**Microfabrication Techniques**

Due to numerous technological developments from various domains which have drawn research attention, microfluidics technology has advanced significantly in a very short period of time. As previously mentioned, a range of materials with unique properties can be used to create microfluidic devices, which leads them to behave differently during processing. Because of the distinctive properties of the material in question and the limitations of the final result, fabrication techniques must be adjusted. Additionally, when choosing the fabrication method, the cost must be taken into account. This is crucial for microfluidic platforms because they are frequently discarded and difficult to reuse, maintain and clean. For single-use chips, the method must be financially viable.\(^\text{30}\)

Fortunately, several fabrication methods have been reported and are still in use today. A classification of these techniques has been created by Waldbaur et al. based on whether the microfluidic structure is generated by removing material, known as "removing techniques," or by depositing material, known as "depositing techniques" (see details of the classification at reference [30]). Fabrication techniques can be further divided into different categories according to the type of processes used, such as mechanical, chemical, laser-based, and other processes.\(^\text{30}\) In this project, chemical processes used for material removing techniques are wet
and/or dry etching of silicon, using a patterned mask of either silicon dioxide or photoresist. The device fabrication process follows the same procedure as previously published by C.J. Demers\textsuperscript{13} and P. Chennampally.\textsuperscript{53}
APPENDIX B

Cell Culture Basics

One of the most important tools in cellular and molecular biology is cell culture, which offers superior model systems for studying the typical physiology and biochemistry of cells (such as metabolic studies, and aging), the effects of drugs and toxic substances on the cells, and mutagenesis and carcinogenesis. Additionally, it is utilized for biological compound manufacture on a big scale as well as drug research and screening (e.g., vaccines, therapeutic proteins). The main benefit of employing clonal cells in cell culture for any of these applications is the consistency and repeatability of the results that may be obtained.67

Cell culture involves taking cells from an animal or plant and letting them grow in a friendly artificial environment. The cells may be extracted directly from the tissue and split before cultivation using mechanical or enzymatic procedures, or they may come from a cell line or strain that has already been created. Typically, the cells are isolated from the tissue and multiplied under the right conditions until confluence. The following stage is called the "primary culture" stage, where the cells must be sub-cultured, or in other words, passaged before they are transferred to a new dish containing fresh growth medium to allow for greater area for further growth. The primary culture becomes a cell line after the first subculture.67 The lifespan of cell lines derived from primary cultures is constrained and as they are passed through successive generations, the cells with the greatest capacity for growth predominate, causing a certain level of genotypic and phenotypic uniformity in the population. Finally, a cell line becomes a cell strain when a portion of it is successfully isolated from the culture by cloning or another technique. Following the beginning of the parent line, a cell strain frequently develops additional genetic alterations.67
Every cell type has different culture requirements, but every artificial environment for growing cells always includes a suitable vessel with a substrate or medium that provides the necessary nutrients, growth factors, hormones, and gases (O2, CO2), as well as controls the physiochemical environment. While certain cells can be grown floating in the culture media, suspension culture, the majority of cells are anchorage-dependent and must be cultured while attached to a solid or semisolid substrate (adherent or monolayer culture). Every cell type has varied culture requirements; hence the culture conditions are highly crucial. A suitable vessel containing a substrate or medium that offers the required nutrients, such as amino acids, carbohydrates, vitamins, and minerals, is always included in every artificial environment for growing cells. The medium also requires the necessary hormones, growth factors, gases like oxygen and carbon dioxide (O2, CO2), as well as a good level of control over the physiochemical environment, which includes pH, osmotic pressure, temperature, and other parameters. While certain cells can be grown floating in the culture medium, known as suspension culture, the majority of cells are anchorage-dependent and must be cultured while attached to a solid or semisolid substrate, and this is known as adherent or monolayer culture which is the method used in this research.

Subculturing, also known as passaging, is the removal of the medium and the transfer of cells from an earlier culture into new growth medium. This method allows the cell line or cell strain to continue to proliferate. Following seeding, the cells expand in culture from the lag phase to the log phase, when they multiply exponentially. Cell proliferation is significantly decreased or stops altogether when the cells in adherent cultures occupy all the available substrate and have no room for development, or when the cells in suspension cultures exceed the medium's capacity to sustain further growth. The culture must be divided, and new medium must be added, in order
to maintain the culture at the ideal density for ongoing cell growth and to encourage additional proliferation.\textsuperscript{67}
APPENDIX C

Cell Culture Procedures

Subculture Procedure:

Subculture of cells is the most used process in this thesis, and it involves retrieving and thawing a vial of cells from the liquid nitrogen storage tank. Before anything is done, a 60-mm Petri dish is coated with an attachment factor and placed in the incubator during the next few steps. Then, to separate the cells from the medium, the mixture of thawed cells and medium was mixed with 4-5mL of fresh media specific to the type of cells used in a 15mL tube and then centrifuged for 5 minutes. After centrifugation, the cells stuck to the bottom of the tube with the medium on top of the cell pellet. To protect the bottom cell pellet, the medium was cautiously sucked without disturbing it. Next, the cell pellet needs to be broken by gentle mechanical agitation to facilitate the dispersion of the cells in the medium. This was done by softly dragging the centrifuged tube across the hood grill. Fresh medium (~3-4mL, depending on the density of cells and Petri dish used) was then added to the separated cells and mixed a few times before being placed in a dish coated with an attachment factor for proliferation.

Trypsinization Procedure:

Trypsinization consists of removing the adherent cells from the cell culture dish and is the most frequently used procedure after subculturing but is done prior to the start of the experiment or another subculture. PBS without calcium (Ca^{2+}) and magnesium (Mg^{2+}) is used in the dissociation process before trypsin solution to wash out or remove any medium or serum components from the dish, and resuspend cells as opposed to DPBS with the presence of calcium and magnesium which may inhibit trypsin activity. After the washing steps, about 1–3 milliliters
of trypsin solution; depending on the size of the dish and the density of cells present, are added to the dish and was placed in the incubator for 4 minutes. Following this, the cells should be floating around in the dish and the mixture of cells and trypsin solution is transferred to a 15 mL tube, after which the trypsin is neutralized by adding 4–8 milliliters of new cell culture medium, depending on the size of the dish. Centrifugation of the mixture of cells/trypsin and fresh medium is performed at 1000 rpm for 5 minutes. The isolated cells were employed in the appropriate manner after centrifugation. After centrifugation, the cells stuck to the bottom of the tube with medium/trypsin on top of the cell pellet. Here, the mixture of medium and trypsin is decanted without disturbing the cell pellet at the bottom. Then, the cell pellet was loosened, and the isolated cells were employed in the appropriate manner based on the need and type of cells used. Most of these operations were completed in a cell culture hood and the cell culture dish or devices were kept in an incubator set at 37°C, 5 % CO₂ and 60% relative humidity (RH) to promote cell growth and differentiation. Unless otherwise specified, the aforementioned criteria apply to all research involving cell culture.

**Plating Protocol:**

*Mouse Embryonic Fibroblast Plating Procedure:*

1. Before starting, ensure enough MEF medium (*Table 1*) is made and warm it up in the water bath for 30 minutes.

2. Cover the whole surface of a 60-mm culture dish with about 2 mL of Attachment Factor (AF) solution and place the Petri dish in the incubator at 37°C for 15–30 minutes.

3. Remove the vial of mitomycin C-treated (MitC) MEFs from the liquid nitrogen storage tank using metal forceps.
4. Remove the frost by rolling the vial between your gloved hands. This should take about 10–15 seconds.
5. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
6. Remove the vial from the water bath when only an ice crystal remains inside of it.
7. Before placing the vial in the hood, spray the outside of the vial with 70% ethanol.
8. Gently pipet the thawed cells into a sterile 15-mL conical tube using a 5-mL sterile pipette.
9. Slowly pour 4 mL of pre-warmed MEF medium into the 15-mL conical tube with the cells inside. Gently move the tube back and forth while adding the medium to mix the cells. This lessens the risks of an osmotic shock to the cells.
10. Rinse the vial with 1 mL of pre-warmed MEF medium and add it to the 15-mL conical tube with cells. This step is to ensure most of the cells from the thawed vial are transferred to the conical tube.
11. Centrifuge the cells in the conical tube at 1100 rpm for 5 minutes.
12. Aspirate the supernatant. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.
13. Resuspend the cell pellet by adding 3.5mL of MEF medium and mix by gently pipetting the cells up and down in the tube a few times.
14. Remove the AF-coated Petri dish from the incubator and aspirate the AF solution.
15. Add the 3.5mL of cells mixed in MEF medium to the pre-coated Petri dish.
16. To spread the cells evenly throughout the surface of the Petri dish, move the culture dish in figure-eight and circular motions.
17. Label the dish as MitC MEFs with the passage number from the vial, the date, and the user initials.
18. Check the cells under the microscope.

19. Place the MEFs Petri dish into a 37°C, 5% CO₂, and 60% relative humidity (RH) incubator.

20. Change the medium 24 hours after plating for optimal proliferation.

This procedure was adapted from the original source.⁶⁸

*Mouse Embryonic Stem Cell Plating Procedure:*

This procedure is done once the MEFs reach about 80% confluency (~24–48 hours after plating) as seen in Figure 11.

**Steps:**

1. Before starting, ensure enough mESC medium (*Table 2*) is made and warmed up in the water bath for 30 minutes.

2. Remove the vial of mESCs (or named KM4 wild cells) from the liquid nitrogen storage tank using metal forceps.

   **Note:** If the vial is kept outside for more than 15 seconds before being moved and thawed, place it into a container containing a small amount of liquid nitrogen.

3. Remove the frost by rolling the vial between your gloved hands. This should take about 10–15 seconds.

4. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.

5. Remove the vial from the water bath when only an ice crystal remains inside of it.

6. Before placing the vial in the hood, spray the outside of the vial with 70% ethanol.

7. Gently pipet the thawed cells into a sterile 15-mL conical tube using a 5-mL sterile pipette.

8. Slowly pour 4 mL of pre-warmed mESC culture medium dropwise into the 15-mL conical tube with the cells inside. Gently move the tube back and forth while adding the medium to mix the mESCs. This lessens the risks of an osmotic shock to the cells.
9. Rinse the vial with 1 mL of pre-warmed mESC culture medium and add to the 15-mL conical tube with cells. This step is to make sure most of the cells from the thawed vial are transferred to the conical tube.

10. Centrifuge the cells in the conical tube at 1100 rpm for 5 minutes.

11. Aspirate the supernatant. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

12. Resuspend the cell pellet by adding 3.5mL of mESC medium and mix by gently pipetting the cells up and down in the tube a few times.

13. Take the Petri dish with MEFs (at 80% confluency) out of the incubator and aspirate the MEF medium

14. Slowly add the 3.5mL mixture of mESCs and medium onto the dish

15. To spread the cells evenly throughout the surface of the Petri dish, move the culture dish in figure-eight and circular motions.

16. This dish should already be labeled as MitC MEFs. So, write under it mESCs with the passage number from the vial, the date, and the user initials.

17. Check the cells under the microscope.

18. Gently place the Petri dish into a 37°C, 5% CO₂, and 60% RH incubator.

19. Incubate the cells overnight.

20. Change the medium 24 hours after plating for optimal proliferation

Notes:

- The mESCs will be smaller and rounder than the feeder cells, and more uniform in shape as seen in Figure 12.
- Frozen vials of mESC may also contain MEFs if the mESCs were cultured on MEF feeder layers before cryopreservation.

This procedure was adapted from the original source.68

**Skeletal Muscles (C2C12) Plating Procedure:**

1. Before starting, ensure enough MEF medium (*Table 1*) is made and warm it up in the water bath for 30 minutes.
2. Cover the whole surface of a 60-mm culture dish with about 2 mL of Attachment Factor (AF) solution and place the Petri dish in the incubator at 37°C for 15–30 minutes.
3. Remove the vial of C2C12 from the liquid nitrogen storage tank using metal forceps.
4. Remove the frost by rolling the vial between your gloved hands. This should take about 10–15 seconds.
5. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
6. Remove the vial from the water bath when only an ice crystal remains inside of it.
7. Before placing the vial in the hood, spray the outside of the vial with 70% ethanol.
8. Gently pipet the thawed cells into a sterile 15-mL conical tube using a 5-mL sterile pipette.
9. Slowly pour 4 mL of pre-warmed MEF medium into the 15-mL conical tube with the cells inside. Gently move the tube back and forth while adding the medium to mix the cells. This lessens the risks of an osmotic shock to the cells.
10. Rinse the vial with 1 mL of pre-warmed MEF medium and add it to the 15-mL conical tube with cells. This step is to ensure most of the C2C12 cells from the thawed vial are transferred to the conical tube.
11. Centrifuge the cells in the conical tube at 1100 rpm for 5 minutes.
12. Aspirate the supernatant. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

13. Resuspend the cell pellet by adding 3.5mL of MEF medium and mix by gently pipetting the cells up and down in the tube a few times.

14. Remove the AF-coated Petri dish from the incubator and aspirate the AF solution.

15. Add the 3.5mL of C2C12 cells mixed in MEF medium to the pre-coated Petri dish.

16. To spread the cells evenly throughout the surface of the Petri dish, move the culture dish in figure-eight and circular motions.

17. Label the dish as C2C12 with the passage number from the vial, the date, and the user initials.

18. Check the cells under the microscope.

19. Place the C2C12 Petri dish into a 37°C, 5% CO₂, and 60% RH incubator.

20. Change the medium 24 hours after plating for optimal proliferation.

This procedure was adapted from the original source.
APPENDIX D

Expansion and Cryopreservation Protocol:

Mouse Embryonic Fibroblast Expansion Example Procedure:

In stem cell culture, feeder cells (MEFs) play two crucial roles: they produce a number of vital growth factors into the medium, which aids in maintaining pluripotency, and they give ES cells a cellular matrix on which to grow. The cells used were PMEF, Strain CF1, Not Treated, and Passage 3 – all of which were purchased from Sigma. The density of fibroblasts per vial was 5-6×10^6. As a result of this high density, one vial was plated onto two 100-mm dishes, as seen in Table 5.

Table D1. Recommended seeding cells densities per dish size.

<table>
<thead>
<tr>
<th>Dish Size</th>
<th>Volume (mL) / Dish</th>
<th>Growth Area (cm^2)</th>
<th>No. of Fibroblasts / Dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>60-mm dish</td>
<td>5</td>
<td>21</td>
<td>1.0×10^6</td>
</tr>
<tr>
<td>100-mm dish</td>
<td>10</td>
<td>56</td>
<td>2.8×10^6</td>
</tr>
</tbody>
</table>

Steps for plating the MEF cells:

1. Before starting, ensure enough MEF medium (Table 1) is made and warm it up in the water bath for 30 minutes.
2. Cover the whole surface of two 100-mm culture dishes with 4 mL of Attachment Factor (AF) solution and place the Petri dishes in the incubator at 37°C for 15–30 minutes.
3. Remove one vial of PMEFs, Strain CF1, Not Treated, and Passage 3 from the liquid nitrogen storage using metal forceps.
4. Remove the frost by rolling the vial between your gloved hands. This should take about 10–15 seconds.
5. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
6. Remove the vial from the water bath once only an ice crystal remains inside of it.

7. Before placing the vial in the hood, spray the outside of the vial with 70% ethanol.

8. Gently pipet the thawed cells into a sterile 15-mL conical tube using a 5-mL sterile pipette.

9. Slowly pour 4 mL of pre-warmed MEF medium into the 15-mL conical tube with the cells inside. Gently move the tube back and forth while adding the medium to mix the MEFs. This lessens the risks of an osmotic shock to the cells.

10. Rinse the vial with 1 mL of pre-warmed MEF medium and add it to the 15-mL conical tube with cells. This step is to make sure most of the cells from the thawed vial are transferred to the conical tube.

11. Centrifuge the cells (in the conical tube) at 1100 rpm for 5 minutes.

12. Aspirate the supernatant. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

13. Resuspend the cell pellet by adding 2 mL of MEF medium and mix by gently pipetting the cells up and down in the tube a few times.

14. Remove the two AF-coated Petri dishes from the incubator and aspirate the AF solution.

15. Add 7–9 mL of MEF medium to each dish.

16. Add 1 mL of the mixture of cells and medium from the tube into each dish. Each dish should now have 8–10 mL of medium and fibroblasts.

17. To spread the cells evenly throughout the surface of the Petri dishes, move the culture dishes in figure-eight and circular motions.

18. Label both dishes as untreated MEFs with the passage number from the vial (3 in this case of this expansion), the date, and the user initials.

19. Check the cells under the microscope.
20. Gently place the Petri dishes into a 37°C, 5% CO$_2$, and 60% relative humidity (RH) incubator.

21. Incubate the cells overnight.

About 16 hours later, the media was changed and about 24 hours after the cells were plated (~80% confluency), each 100-mm dish was expanded into three 100-mm dishes for a total of six 100-mm dishes. The two techniques used here are trypsinization and subculturing or passaging.

Steps for expanding the MEFs cells:

1. Before starting, ensure enough MEF medium, at least 100 mL (*Table 1*), is made and warm it up, as well as the trypsin in the water bath for 30 minutes.

2. Take six 100-mm culture dishes and cover each of their surfaces with 4 mL of AF solution and then place the Petri dishes in the incubator for 15–30 minutes at 37°C.

3. Remove the two dishes with MEFs from the incubator and aspirate the old medium.

4. Wash the cells with Phosphate-Buffered Saline (PBS) without calcium (Ca$^{2+}$) and magnesium (Mg$^{2+}$) at least three times at 3–5 minutes intervals. This is the dissociation process to wash out or remove any medium or serum components from the dish before trypsin solution is added.

5. Add 3–4 mL of trypsin to each of the two dishes and leave them in the incubator for 4 minutes (until the adherent cells dissociate from the dish). The proteins that allow the cells to adhere to the dish are broken down by trypsin.

6. Gently pipet the trypsinized cells from each dish into a sterile 15-mL conical tube using a 5-mL sterile pipette.
7. Slowly pour 2 mL of pre-warmed MEF medium into each of the two 15-mL conical tubes with the trypsinized cells inside. To mix the trypsinized cells with the medium, gently move the tube back and forth while adding the medium.

8. Rinse each dish with 3 mL of pre-warmed MEF medium and add it to the 15-mL conical tube with cells. This step is to make sure most of the cells from the dishes are transferred to the conical tubes.

9. Centrifuge the cells (in the conical tube) at 1100 rpm for 5 minutes.

10. During the wait, remove the six AF-coated dishes from the incubator and aspirate the AF solution, then add 7–9mL of the pre-warmed MEF medium in each of the six dishes.

11. After centrifugation, aspirate the supernatant of the two tubes. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

12. Resuspend the cell pellet by adding 3 mL of MEF medium in each of the two tubes and mix thoroughly by gently pipetting the cells up and down in the tube a few times.

13. Add 1 mL of the mixture of cells and medium from one tube in each of three dishes. Repeat this with the second tube. There should be a total of six dishes with 8–10mL of medium and fibroblasts.

14. To spread the cells evenly throughout the surface of the Petri dishes, quickly move the culture dishes in figure-eight and circular motions.

15. Label the six dishes as untreated MEFs with the passage number from the vial (4 in this case), the date, and the user initials.

16. Gently place the Petri dishes into a 37°C, 5% CO₂, and 60% RH incubator.

17. Incubate the cells overnight.
About 24 hours later, the media was changed in all six dishes. Then when the untreated MEFs reach ~80% confluency, the same steps for expanding the cells were used to expand the six dishes into twelve dishes. The medium was appropriately changed every 24 hours until the cells reached ~80% confluency, and at this threshold, they were subsequently treated with Mitomycin C (MitC) and then frozen.

**Steps for Mitomycin C Treatment and Cryopreservation:**

1. Prepare 10 µL of MitC for every 1 mL of MEF medium. In this case, because we have six dishes and we want 7mL per dish, 42 mL of MEF medium with the addition of 420 µL of MitC is made and warmed up in the water bath for 30 minutes.
2. Remove six out of the twelve dishes with MEFs from the incubator and aspirate the old medium.
3. Add 7 mL of the MitC–MEF medium into each of the six dishes.
4. Incubate the six dishes for two hours.
5. After the two hours have elapsed, remove the six dishes from the incubator and aspirate the MitC–MEF medium.
6. Repeat steps 3–5 once.
7. Wash the cells with PBS without calcium (Ca^{2+}) and magnesium (Mg^{2+}) at least three times at a 3–5 minutes interval. Once again, this is the dissociation process to wash out or remove any medium or serum components from the dish before trypsin solution is added.
8. Add 3–4 mL of trypsin in each of the six dishes and leave them in the incubator for 4 minutes (until the adherent cells dissociate from the dish). Again, the proteins that allow the cells to adhere to the dish are broken down by the trypsin.

9. Gently pipet the trypsinized cells from each dish into a sterile 15-mL conical tube using a sterile 5-mL pipette.

10. Slowly pour 2 mL of pre-warmed MEF medium (Table 2) into each of the six 15-mL conical tubes with the trypsinized cells inside. To mix the trypsinized cells with the MEF medium, gently move the tube back and forth while adding the medium.

11. Rinse each dish with 3 mL of pre-warmed MEF medium and add to the 15-mL conical tube with cells. Again, this step is to make sure most of the cells from the dishes are transferred to the conical tubes.

12. Centrifuge the cells (in the conical tube) at 1100 rpm for 5 minutes.

13. During the centrifugation time, prepare a solution of 10% dimethyl sulfoxide (DMSO), a cryoprotective agent, in the MEF culture medium (the solution is also referred to as freezing medium). Each of the six conical tubes should receive 2 mL of the freezing medium. Therefore, in this case, 12 mL (6 × 2 mL) of freezing medium was prepared in a 15-mL conical tube; 1.2 mL of DMSO was added to 10.8 mL of MEF medium.

14. After centrifugation, aspirate the supernatant of the six tubes. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

15. Resuspend the cell pellet by adding 2 mL of DMSO–MEF medium (or freezing medium) in each of the six tubes and mix thoroughly by gently pipetting the cells up and down in the tube a few times.
16. Take twelve sterilized cryovials under the hood and label them as MitC treated MEFs with the passage number from the vial (5 at this point), the date, and the user initials.

17. Add 1 mL of the mixture of cells and DMSO–MEF medium from the tubes into each of the vials. Repeat this with all six tubes for a total of twelve cryovials with 1 mL of cells mixed in DMSO–MEF medium in each.

18. Cells should be frozen slowly at a rate of 1°C/min. This can be achieved by placing the twelve cryovials in an insulated box placed in a −80°C freezer for about 24 hours, then transferring them to a liquid nitrogen storage tank.

19. In this case, repeat steps 1–18 for the other six dishes left in the incubator. There will then be a total of twenty-four cryovials in the liquid nitrogen.

Notes:

- Every step should use proper aseptic techniques.

- To prevent any contaminations or complications, six dishes will be frozen at a time.

- Some vials can be left untreated and frozen to be used for future expansions. This procedure can be changed based on each expansion's needs.
Expansion of MEFs example diagram – total of 16 vials (1 mL) of MitC treated MEF and 2 vials of untreated MEF shown in Figure 2.

Figure D1. Diagram representing the expansion procedure for MEF cells.
Mouse Embryonic Stem Cells Expansion Example Procedure:

For this procedure, a 60-mm Petri dish was used and MitC MEFs were plated onto it. Then, once the MEFs reach about 80% confluency (~24–48 hours after plating), the Mouse Embryonic Stem Cells Plating Procedure was followed. We allowed the mESCs to proliferate for 3–5 days or until they reach about 80% confluency before we transferred them onto a 100-mm dish coated with MitC MEFs (feeder layer), then expand them.

Steps for expanding the mESCs:

1. MEF cells were plated onto two 100-mm Petri dishes. Once the cells reach about 80% confluency, which is about 24 to 48 hours after plating them, the expansion of mESCs can start as these cells need to grow on top of the feeder layer, MEFs.

2. Before starting, ensure enough mESC medium, at least 100 mL (Table 2), is made and warm it up (as well as trypsin) in the water bath for 30 minutes.

3. Remove the 100-mm dish with mESC from the incubator and aspirate the old medium.

4. Wash the cells with PBS without calcium (Ca$^{2+}$) and magnesium (Mg$^{2+}$) at least three times at 3–5 minutes intervals.

5. Add 3–4 mL of pre-warmed trypsin to the dish and leave it in the incubator for 4 minutes (until the adherent cells dissociate from the dish). The proteins that allow the cells to adhere to the dish are broken down by trypsin.

6. Gently pipet the trypsinized cells from the dish into a sterile 15-mL conical tube using a 5-mL sterile pipette.

7. Slowly pour 2 mL of the pre-warmed mESC medium into the 15-mL conical tube with the trypsinized cells inside. To mix the trypsinized cells with the medium, gently move the tube back and forth while adding the medium.
8. Rinse the dish with 3 mL of pre-warmed mESC medium and add it to the 15-mL conical tube with cells. This step is to make sure most of the cells from the dish are transferred to the conical tubes.

9. Centrifuge the cells (in the conical tube) at 1100 rpm for 5 minutes.

10. During the wait, remove the two 100-mm culture dishes with MEFs coated on them from the incubator and aspirate the media, then add 7–9mL of the pre-warmed mESC medium in each of the two dishes.

11. After centrifugation, aspirate the supernatant of the tube. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

12. Resuspend the cell pellet by adding 2 mL of mESC medium into the tube and mix thoroughly by gently pipetting the cells up and down in the tube a few times.

13. Add 1 mL of the mixture of cells and medium from the tube in each of the two dishes (MEFs) with already 7–9mL of the pre-warmed mESC medium.

14. To spread the cells evenly throughout the surface of the Petri dishes, move the culture dishes in figure-eight and circular motions.

15. Label the two dishes as MitC–MEF–ESC with the passage number from the vial, the date, and the user initials.

16. Gently place the Petri dishes into a 37°C, 5% CO₂, and 60% RH incubator.

17. Incubate the cells overnight.

The medium was appropriately changed every 24 hours until the cells reached ~80% confluency in both dishes. Then, the cells were cryopreserved.
Steps for Cryopreservation of mESCs:

1. Add trypsin and mESC medium to the water bath approximately 30 minutes before starting.
2. Remove the two dishes with MEF–mESCs from the incubator and aspirate the old mESC medium.
3. Wash the cells with PBS without calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)) at least three times at 3–5 minutes intervals.
4. Add 3–4 mL of pre-warmed trypsin in each of the two dishes and leave them in the incubator for 4 minutes (until the adherent cells dissociate from the dish).
5. Gently pipet the trypsinized cells from each dish into a sterile 15-mL conical tube using a sterile 5-mL pipette.
6. Slowly pour 2 mL of pre-warmed mESCs medium (*Table 2*) into each of the two 15-mL conical tubes with the trypsinized cells inside. To mix the trypsinized cells with the medium, gently move the tube back and forth while adding the medium.
7. Rinse each dish with 3 mL of pre-warmed mESC medium and add to the 15-mL conical tubes with cells.
8. Centrifuge the cells (in the conical tube) at 1100 rpm for 5 minutes.
9. During the centrifugation time, prepare a solution of 10% dimethyl sulfoxide (DMSO), a cryoprotective agent, in the mESC culture medium (the solution is also referred to as freezing medium). Each of the two conical tubes should receive 2 mL of the freezing medium. Therefore, in this case, 4 mL (2 × 2 mL) of freezing medium was prepared in a 15-mL conical tube; 0.4 mL of DMSO was added to 3.6 mL of mESC medium.
10. After centrifugation, aspirate the supernatant of the two tubes. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

11. Resuspend the cell pellet by adding 2 mL of DMSO–mESC medium (or freezing medium) in each of the two tubes and mix thoroughly by gently pipetting the cells up and down in the tube a few times.

12. Take four sterilized cryovials under the hood and label them as mESCs with the passage number from the vial, the date, and the user initials.

13. Add 1 mL of the mixture of cells and DMSO–mESC medium from the tubes into each of the vials. Each of the four cryovials should have 1 mL of cells mixed in DMSO–mESC medium.

14. Cells should be frozen slowly at a rate of 1°C/min. This can be achieved by placing the four cryovials in an insulated box placed in a –80°C freezer for about 24 hours, then transferring them to a liquid nitrogen storage tank.

Notes:

- Every step should use proper aseptic techniques.
- It is recommended to use a low passage number for mESCs. The lowest the better, but the cells can be expanded until passage 20.
Expansion of mESCs example diagram – total of 4 vials (1 mL) of mESCs with MEFs shown in Figure 2.

Figure D2. Diagram representing the expansion procedure for mESC cells.
Skeletal Muscles (C2C12) Expansion Example Procedure:

1. Start by plating C2C12 in a 60-mm dish using the plating procedure for C2C12.
2. Change the medium after 24 hours
3. Once the cells reached 80% confluency, transfer them onto a 100-mm dish.
4. Change the media 24 hours after the transfer
5. When the 100-mm dish reached 80% confluency, it is time to expand the dish into three 100-mm dishes.

Steps for expanding the C2C12 cells:

1. Before starting, ensure enough MEF medium, at least 100 mL (Table 1), is made and warm it up in the water bath for 30 minutes.
2. Take three 100-mm culture dishes and cover each of their surfaces with 4 mL of AF solution and then place the Petri dishes in the incubator at 37°C.
3. Remove the 100-mm dish with C2C12 from the incubator and aspirate the old medium.
4. Wash the cells with Phosphate-Buffered Saline (PBS) without calcium (Ca^{2+}) and magnesium (Mg^{2+}) at least three times at 3–5 minutes intervals.
5. Add 3–4 mL of trypsin to the dish and leave it in the incubator for 4 minutes (until the adherent cells dissociate from the dish).
6. Gently pipet the trypsinized cells from the dish into a sterile 15-mL conical tube using a 5-mL sterile pipette.
7. Slowly pour 2 mL of pre-warmed MEF medium into the 15-mL conical tube with the trypsinized cells inside. To mix the trypsinized cells with the medium, gently move the tube back and forth while adding the medium.
8. Rinse the dish with 3 mL of pre-warmed MEF medium and add it to the 15-mL conical tube with cells. This step is to make sure most of the cells from the dish are transferred to the conical tubes.

9. Centrifuge the cells (in the conical tube) at 1100 rpm for 5 minutes.

10. During the wait, remove the three AF-coated dishes from the incubator and aspirate the AF solution, then add 7–9mL of the pre-warmed MEF medium in each of the three dishes.

11. After centrifugation, aspirate the supernatant of the tube. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

12. Resuspend the cell pellet by adding 3 mL of MEF medium in the tube and mix thoroughly by gently pipetting the cells up and down in the tube a few times.

13. Add 1 mL of the mixture of cells and medium from the conical tube in each of the three dishes. The three dishes should now have 8–10mL of MEF medium and cells.

14. To spread the cells evenly throughout the surface of the Petri dishes, move the culture dishes in figure-eight and circular motions.

15. Label the six dishes as C2C12 with the date, and the user initials.

16. Gently place the Petri dishes into a 37°C, 5% CO₂, and 60% RH incubator.

17. Incubate the cells overnight.

About 24 hours later, the media was changed in all three dishes. Then when the C2C12 reached ~80% confluency, the same steps for expanding the cells were used to expand the three dishes into nine dishes. In the nine dishes, the media was properly replaced every 48 hours up until the cells reached 80% confluency, at which point they were cryopreserved.
Steps for Cryopreservation of C2C12:

1. Place trypsin and MEF medium into the water bath approximately 30 minutes before starting.
2. Remove the nine dishes with C2C12 from the incubator and aspirate the old MEF medium.
3. Wash the cells with PBS without calcium (Ca$^{2+}$) and magnesium (Mg$^{2+}$) at least three times at 3–5 minutes intervals.
4. Add 3–4 mL of pre-warmed trypsin in each of the nine dishes and leave them in the incubator for 4 minutes (until the adherent cells dissociate from the dish).
5. Gently pipet the trypsinized cells from each dish into a sterile 15-mL conical tube using a sterile 5-mL pipette.
6. Slowly pour 2 mL of pre-warmed MEF medium (Table 2) into each of the nine 15-mL conical tubes with the trypsinized C2C12 cells inside. To mix the trypsinized cells with the medium, gently move the tube back and forth while adding the medium.
7. Rinse each dish with 3 mL of pre-warmed MEF medium and add to the 15-mL conical tubes with cells.
8. Centrifuge the cells (in the conical tube) at 1100 rpm for 5 minutes.
9. During the centrifugation time, prepare the freezing medium; a solution of 10% dimethyl sulfoxide (DMSO), a cryoprotective agent, in the MEF culture medium. Each of the nine conical tubes should receive 2 mL of the freezing medium. Therefore, in this case, 18 mL (9 × 2 mL) of freezing medium was prepared in a 50-mL conical tube; 1.8 mL of DMSO was added to 16.2 mL of MEF medium.
10. After centrifugation, aspirate the supernatant of the nine tubes. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

11. Resuspend the cell pellet by adding 2 mL of freezing medium (DMSO–MEF) in each of the nine tubes and mix thoroughly by gently pipetting the cells up and down in the tube a few times.

12. Take eighteen sterilized cryovials under the hood and label them as C2C12 with the date, and the user initials.

13. Add 1 mL of the mixture of cells and freezing medium from the nine tubes into each of the eighteen vials. Each tube has 2mL of cells in a freezing medium and is used for two cryovials (1mL each).

14. Cells should be frozen slowly at a rate of 1°C/min. This can be achieved by placing the eighteen cryovials in an insulated box placed in a –80°C freezer for about 24 hours, then transferring them to a liquid nitrogen storage tank.
Expansion of C2C12 example diagram – total of 18 vials (1 mL) of C2C12 cells as shown in Figure 2.

Figure D3. Diagram representing the expansion procedure for C2C12 cells.
APPENDIX E

Experiment Day Protocol

Steps for sterilizing the microdevice and coverslips:

1. First, a tiny amount of uncured PDMS was put 500 \( \mu \text{m} \) from each side of the chambers before proceeding with sterilization. This small amount of PDMS functions as a greasy barrier between the coverslip and the device, facilitating a greater exchange of gases, as well as keeping the coverslips attached to the chambers and device in general.

2. All the parts of the coculture device that will come into contact with culture media or cells must be adequately sterilized before cell culture can begin to prevent infection. This was done by rinsing and drying the microdevices with 70% ethanol for 30 minutes under the hood. It is important to spray 70% ethanol everywhere in the device and to let the top and bottom sides of the device be exposed to UV light for at least 30 minutes. Any ethanol residue was then rinsed away several times using autoclaved and filtered (0.45 \( \text{m} \)) deionized (DI) water.

3. Spray the bag with sterilized 5-mL cryotubes with 70% ethanol and place it under the hood. Then cut the bottom part of the tube to make an opening. Each device needs four tubes as each device has four inlets.

4. Before the experiment began, the channels were vacuumed to remove any lingering water droplets in order to keep everything dry.

5. Using a dicing cutter, many coverslips (160 \( \mu \text{m} \) thick) that can cover the chambers (2), the interconnecting microchannels (1), and the entirety of the chambers and interconnecting
microchannels (1) were sized appropriately for a total of four coverslips per device. They were then disinfected by soaking them in a 60-mm dish with 70% ethanol for 10 minutes under UV light, followed by 10 minutes in DI water with UV treatment under the hood. For further sterilization, this can be repeated two to three times.

6. The sterilized coverslips were arranged in a certain pattern around the device so that they were manageable to reach and handle with tweezers. This was done to facilitate experimentation and improved time management while loading the cells onto the chambers of the microdevice.

**Stem Cells to Motor Neuron Differentiation Procedure:**

This procedure is done after following the “Plating Protocol” for MEFs and mESCs and once the mESCs have reached 80% confluency in a 60-mm (this can take 4 to 6 days). This is considered the start of an experiment, where there are two gravity separation procedures for separating the MEFs (feeder layer cells) to the mESCs. Before anything is done, place the Geltrex in the refrigerator the night before the experiment to ensure that it gets to 4°C by the time the experiment gets started.

**First Gravity Separation Steps:**

1. Place trypsin and mESC medium in the water bath for 30 minutes, then change the medium in the MEF–mESC dish about an hour before starting the experiment.

2. Take a new sterile 60-mm culture dish and cover its surface with 2 mL of AF solution, and then place it in the incubator at 37°C.

3. Remove the 60-mm dish with mESC from the incubator (an hour after the medium got changed), move the dish around, and aspirate the old medium.
4. Wash the cells with PBS without calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)) at least three times at 3–5 minutes intervals.

5. Add 2.5 mL of pre-warmed trypsin to the dish and leave it in the incubator for 4 minutes (until the adherent cells dissociate from the dish).

6. Gently pipet the trypsinized cells from the dish into a sterile 15-mL conical tube using a 5-mL sterile pipette.

7. Slowly pour 2 mL of the pre-warmed mESC medium into the 15-mL conical tube with the trypsinized cells inside. To mix the trypsinized cells with the medium, gently move the tube back and forth while adding the medium.

8. Rinse the dish with an additional 3 mL of pre-warmed MEF medium and add it to the 15-mL conical tube with cells. This step is to make sure most of the cells from the dish are transferred to the conical tubes.

9. Centrifuge the trypsinized cells (in the conical tube) at 1100 rpm for 5 minutes.

10. During the wait, remove the AF-coated 60-mm culture dish from the incubator and aspirate the solution.

11. After centrifugation, aspirate the supernatant of the tube. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

12. Resuspend the cell pellet by adding 3–4 mL of mESC medium into the tube and mix thoroughly by gently pipetting the cells up and down in the tube a few times.

13. Once the cells are fully mixed in the fresh medium, transfer the whole 3–4 mL of it to the AF-coated dish.

14. To spread the cells evenly throughout the surface of the Petri dish, move the culture dishes in figure-eight and circular motions.
15. Label the Petri dish as “Gravity Separation 1” with the date, and user’s initials
16. Gently place the Petri dish into a 37°C, 5% CO₂, and 60% RH incubator.
17. Incubate the cells for 60 minutes

Second Gravity Separation Steps:
1. About 15 minutes before starting, take a new sterile 60-mm culture dish and cover its surface with 2 mL of AF solution, and then place it in the incubator at 37°C.
2. Once the hour for the first gravity separation has elapsed, remove the new AF-coated dish from the incubator (put in 15 minutes prior), and aspirate the AF solution.
3. Remove the dish labeled as “Gravity Separation 1” and transfer it to the new AF-coated dish using a pipette.
4. To spread the cells evenly throughout the surface of the Petri dish, move the culture dishes in figure-eight and circular motions.
5. Label the Petri dish as “Gravity Separation 2” with the date, and user’s initials
6. Gently place the Petri dish into a 37°C, 5% CO₂, and 60% RH incubator.
7. Incubate the cells for another 60 minutes

Steps for getting the mESCs ready for loading onto the microdevice:
1. Prepare the ADFNK medium (Table 3) in a 50-mL tube and warm it up for at least 30 minutes.
2. After the hour for the second gravity separation has elapsed, remove the dish labeled “Gravity Separation 2” from the incubator.
3. Transfer the whole 3–4 mL from the dish to a 15-mL conical tube.
4. Centrifuge the cells at 1100 rpm for 5 minutes.

5. After centrifugation, aspirate the supernatant of the tube. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

6. Add 1–1.5 mL (depending on the cells’ density) of pre-warmed mESC medium to the conical tube to resuspend the cell pellet and mix by gently pipetting the cells up and down in the tube a few times.

7. Once the cells are mixed in the medium, transfer 1 mL into an Eppendorf tube (2 mL).

8. Centrifuge the cells (in the Eppendorf tube) at 1100 rpm for 5 minutes.

9. After centrifugation, aspirate the supernatant of the Eppendorf tube. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

10. Add 100 µL of Geltrex in the Eppendorf tube

11. Leave the Eppendorf tube in the fridge with the vial of Geltrex

Steps for getting the C2C12 ready for loading onto the microdevice:

C2C12 cells will be plated about 24–48 hours prior to the experiment day.

1. Place trypsin and C2C12 medium (Table 4) in the water bath for 30 minutes.

2. Remove the 60-mm dish with C2C12 from the incubator, move the dish around, and aspirate the old medium.

3. Wash the cells with PBS without calcium (Ca$^{2+}$) and magnesium (Mg$^{2+}$) at least three times at 3–5 minutes intervals.

4. Add 2.5 mL of pre-warmed trypsin to the dish and leave it in the incubator for 4 minutes (until the adherent cells dissociate from the dish).
5. Gently pipet the trypsinized cells from the dish into a sterile 15-mL conical tube using a 5-mL sterile pipette.

6. Slowly pour 2 mL of the pre-warmed C2C12 medium into the 15-mL conical tube with the trypsinized cells inside. To mix the trypsinized cells with the medium, gently move the tube back and forth while adding the medium.

7. Rinse the dish with an additional 3 mL of pre-warmed C2C12 medium and add it to the 15-mL conical tube with cells. This step is to make sure the maximum amount of the cells from the dish is transferred to the conical tubes.

8. Centrifuge the trypsinized cells (in the conical tube) at 1100 rpm for 5 minutes.

9. After centrifugation, aspirate the supernatant of the tube. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.

10. Resuspend the cell pellet by adding 1 mL of pre-warmed C2C12 medium into the tube and mix thoroughly by gently pipetting the cells up and down in the tube a few times.

11. Once the cells are mixed in the medium, transfer 0.5–1 mL of it into an Eppendorf tube (2 mL).

12. Centrifuge the cells (in the Eppendorf tube) at 1100 rpm for 5 minutes.

13. After centrifugation, aspirate the supernatant of the Eppendorf tube. This leaves the cell pellet at the bottom of the tube which can be broken by scraping the tube on the grid inside the hood.


15. Leave the Eppendorf tube in the fridge with the vial of Geltrex.
Notes:

- Both types of cells should be handled simultaneously in order for them to be ready in Eppendorf tubes and placed in the fridge around the same time to avoid getting any cells dried.

Steps for loading the cells onto the microdevice:

After preparing the gel mixtures with the proper concentration of mESCs and C2C12 in Eppendorf tubes as previously mentioned, these steps are carried out.

1. About 0.2 μL of Geltrex is pipetted onto the middle coverslip (covering the interconnecting microchannels), and after a 30-second pause, the coverslip is flipped over and inserted over the interconnecting microchannels using tweezers, filling that area with Geltrex.

2. Immediately, blob 0.8 μL of the gel mixture (mESCs and Geltrex) onto a coverslip for the MN chamber, wait 30 seconds and flip it over before placing it onto the MN chamber.

3. Repeat step 2 for C2C12 chambers, so blob (0.8 μL) the gel mixture (C2C12 cells and Geltrex) onto a coverslip for the C2C12 chamber, wait 30 seconds and flip it over before placing it onto the C2C12 chamber.

4. Blob 0.8 μL of Geltrex on the big coverslip, and after a 30-second pause, the coverslip is flipped over and gently placed on top of all three coverslips to hold them together and to prevent uneven drying or the production of bubbles.

5. Place the device in a 37°C, 5% CO2, and 60% RH incubator for four minutes in order for the Geltrex to solidify and form the desired 3D matrix for the cells.

6. When the time is up, remove the device from the incubator.

7. Take the four already cut cryotubes and twist them onto the inlets of the device.
8. Pipet about 4 mL of C2C12 medium in the two C2C12 tubes, supplying the medium to the C2C12 chamber, and 4 mL of ADFNK medium in the other two tubes, supplying the medium to the ES cells (which will differentiate them into MNs).

9. Place a cap on top of the tube to seal.

10. Wait for the media (C2C12 and ADFNK) to flow out of the two outlets, then place the device in the incubator.

11. Repeat steps 1-12 for all the devices.

Notes:

- Try to avoid air bubbles when blobbing and placing the coverslips on the respective chambers.

- When placing the caps, they cannot touch the medium. If they did, a new cap needs to be used.

- The caps are autoclaved in a box before experiment day.

- A picture of the layout of the coverslips after flipping them to load the cells can be found in Figure ?

- A slight vacuum was supplied to the outlet to kick-start the media flow if it didn't begin on its own.
APPENDIX F

Immunocytochemistry

Fixation:

1. Prepare a 4% paraformaldehyde solution (PFA). The lab has a 16% PFA flask that is diluted in DPBS (with Ca\(^{2+}\)/Mg\(^{2+}\)) to get to a 4% PFA.

2. Aspirate the medium from the tubes of the devices.

3. Unscrew the tubes and aspirate any of the excess medium remaining.

4. In a 100-mm Petri dish with 10 mL of medium (any type), place up to 3-4 devices to soak as it makes it easier to gently remove the coverslips without disturbing the cells and Geltrex in the chambers.

5. Once the coverslips are removed, aspirate the medium from the 100-mm Petri dish and add 10 mL of DPBS (with Ca\(^{2+}\)/Mg\(^{2+}\)).

6. Wash twice with DPBS – aspirate and add 10 mL of DPBS (with Ca\(^{2+}\)/Mg\(^{2+}\))

7. Aspirate the DPBS, add 10 mL of the 4% PFA solution, and let that sit for 10 minutes at room temperature.

8. Aspirate the PFA solution and wash twice with DPBS (with Ca\(^{2+}\)/Mg\(^{2+}\)) at a 5-minute interval.

9. Prepare the antigen retrieval buffer (10 mL of autoclaved DI water, 10 µL of sodium citrate (1M), 5 µL Tween 5%) and add 10 mL to the dish after aspirating the DPBS from the washes.

10. Place the dish(es) with devices in the oven at 50°C overnight.
11. The next day, remove the dish(es) from the oven and let it cool down for 30 minutes at room temperature. Switch off the oven. Remove the DPBS (with Ca\(^{2+}/\) Mg\(^{2+}\)) from the refrigerator and let it warm up to room temperature for 30 minutes as well.

12. Wash twice with DPBS at a 5-minute interval – aspirate and add 10 mL of DPBS (with Ca\(^{2+}/\) Mg\(^{2+}\)).

13. Prepare the wash buffer (1% goat serum, 0.3% Triton x100) in a 50 mL conical tube.
   
   a. 44.5 mL of DPBS (with Ca\(^{2+}/\) Mg\(^{2+}\))
   
   b. Add 5 mL of Triton x100 detergent (0.3%) to dilute in 44.5 mL in order to get 0.3% of Triton x100.
   
   c. 0.5 mL of goat serum

14. Permeabilization step: add 12 mL of wash buffer to the dish and let it sit under the hood for 30 minutes. Intermittently move the dish to swirl.

15. Wash twice with DPBS at a 5-minute interval – aspirate and add 10 mL of DPBS (with Ca\(^{2+}/\) Mg\(^{2+}\)).

16. Prepare the block buffer in a 50 mL conical tube.
   
   a. 47.5 mL of DPBS (with Ca\(^{2+}/\) Mg\(^{2+}\))
   
   b. 2.5 mL of goat serum

17. Blocking: add 12 mL of block buffer to the dish and let it sit under the hood for 60 minutes.

18. Once the hour has elapsed, wrap the dish(es) with parafilm and transfer the dish(es) to the refrigerator and let it sit there overnight.
Notes:

- Some cells can be damaged by the abrupt change between the culture media's osmolarity and the fixation solution's osmolarity.

- One 100-mm dish can have up to 3 devices, therefore, if more devices are being fixed, repeat these steps in another 100-mm Petri dish.

- The antigen retrieval can be prepared for a volume of 50 mL and can be reused for later cell fixation.

- Steps 9 and 10 are done because some antibodies work best when cells are heated in an antigen retrieval buffer. Consult the package information for recommendations for each primary antibody being used.

- The wash and blocking buffers can be stored in a 4°C refrigerator.

**Immunostaining**

Immunostaining is a scientific procedure that makes use of antibodies to identify certain proteins, antigens, or markers in a sample of cells. Based on whether they directly bind to antigens or target another antibody, there are two types of antibodies. The primary antibody locates the marker, gene, or protein in the cell sample; in contrast, the secondary antibody is made to have a fluorophore affixed to it for visibility. In order to see the particular gene of interest from the primary antibody, secondary antibodies attach to primary antibodies and give them a fluorescent color. Secondary antibody will only bind to the primary antibody of the same species. If more than one primary antibody from the same species needs to be stained, the process must be carried out one antibody at a time in order to successfully have a secondary antibody bind to it.
Primary Antibody Staining Steps:

1. Remove the 100-mm dishes with devices and the block buffer out of the refrigerator let them warm up for 30 minutes or until it reaches room temperature.

2. Aspirate the blocking buffer from the dishes and around the devices while avoiding the chambers.

3. Take non-sterile 60-mm dishes, one for each device.

4. Cut a square size parafilm wrapping film and place it on the inside of the bottom part of the 60-mm dish. This is to make the dish less hydrophilic.

5. Make the correct dilution in an Eppendorf tube for the primary stain in the block buffer. The volume made must account for the number of devices needed to be stained.

6. Blob 160 μL on the parafilm on the dish and place one device (chambers facing down) on the blob.

7. Check if the chambers (cells) are covered.

8. Repeat steps 4 and 5 for as many devices as needed.

9. Wrap the dishes with parafilm and put them in a sealed box. Place the box in the refrigerator overnight.

10. Repeat steps 2-9 for each primary antibody after the devices are stained with secondary antibody.

Notes:

- Staining is still done under the hood.

- Antibody stains used are anti-GFP, Foxp1, and Lhx1 and are all from the same species, therefore, they need to be stained separately. In other words, if we start by staining for anti-
GFP, we need to do the entire process including secondary staining before staining for another primary antibody such as Foxp1 or Lhx1.

**Table F1.** Important characteristics of the primary antibodies used in the experiments.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host Species</th>
<th>Storage</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GFP</td>
<td>Rabbit</td>
<td>4°C refrigerator</td>
<td>1:200</td>
</tr>
<tr>
<td>Foxp1</td>
<td>Rabbit</td>
<td>-20°C freezer</td>
<td>1:100</td>
</tr>
<tr>
<td>Lhx1</td>
<td>Rabbit</td>
<td>-20°C freezer</td>
<td>1:100</td>
</tr>
</tbody>
</table>

- Unlike the secondary antibodies, the primary antibodies have different dilution ratios.
  - Example (anti-GFP): if we have ten devices needed to be stained, and we need to blob 160 µL for each device (total 1600 µL), a total volume of 2000 µL (block buffer and primary antibody) will be prepared. Always make sure to prepare more than is necessary, just in case. And because the dilution is 1:200, we will add 10 µL of primary antibody (anti-GFP) in 1990 µL of block buffer.
  - Example (Foxp1/Lhx1): if we have ten devices needed to be stained, and we need to blob 160 µL for each device (total 1600 µL), a total volume of 2000 µL (block buffer and primary antibody) will be prepared. Always make sure to prepare more than is necessary, just in case. And because the dilution is 1:100, we will add 20 µL of primary antibody (anti-GFP) in 1980 µL of block buffer.
**Washing steps:**

1. The next morning, remove the box with the devices, the DPBS (with Ca\(^{2+}\) / Mg\(^{2+}\)) bottle, the block buffer, and wash buffer out of the refrigerator let them warm up for 30 minutes or until it reaches room temperature.

2. Aspirate the excess of the primary stain off each device and transfer the devices from each 60-mm dish back to a 100-mm dish (3 devices per big dish).

3. Pipet 12 mL of wash buffer in each 100-mm dish.

4. Aspirate the wash buffer.

5. Repeat steps 3-4 three times at a 10 minutes interval. Intermittently move the dish in circular motions.

6. Aspirate the wash buffer in each dish and add 10 mL of DPBS (with Ca\(^{2+}\) / Mg\(^{2+}\)).

7. Rest for 15 minutes, then aspirate the DPBS (with Ca\(^{2+}\) / Mg\(^{2+}\)). Intermittently move the dish in circular motions.

8. Pipet 12 mL of block buffer in each 100-mm dish.

9. Rest for 60 minutes, then wrap the dishes with parafilm and place them in the refrigerator overnight.

**Notes:**

- To make the process a bit quicker, the devices can sit in block buffer (step) for two hours instead of overnight.

- The washing steps have to be done to wash out the primary antibody before staining with the secondary antibody.
Secondary Antibody Staining Steps:

1. Remove the 100-mm dishes with devices and the block buffer out of the refrigerator and let them warm up for 30 minutes or until it reaches room temperature.
2. Aspirate the blocking buffer from the dishes and around the devices while avoiding the chambers.
3. Take non-sterile 60-mm dishes, one for each device.
4. Cut a square size parafilm wrapping film and place it on the inside of the bottom part of the 60-mm dish. This is to make the dish less hydrophilic.
5. Make the correct dilution in an Eppendorf tube for the secondary stain (1:500) in the block buffer. The volume made must account for the number of devices needed to be stained.
6. Blob 160 μL on the parafilm on the dish and place the device (chambers facing down) on the blob.
7. Check if the chambers (cells) are covered.
8. Wrap the dishes and put them in a sealed box. Place the box in a dark room for two hours.

Notes:

- For secondary staining, the dilution ratio is 1:500.
  o Example: if we have ten devices needed to be stained, and we need to blob 160 μL for each device (total 1600 μL), a total volume of 2000 μL (block buffer and secondary antibody) will be prepared. Always make sure to prepare more than is
necessary, just in case. Because the dilution is 1:500 we will add 4 μL of secondary antibody in 1996 μL of block buffer.

- The primary antibody stains are all of the rabbit species; therefore, the secondary antibodies also need to match.

**Table F2.** Important characteristics of the secondary antibodies used in the experiments.

<table>
<thead>
<tr>
<th>Secondary Antibody Name (Host Species)</th>
<th>Storage</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-Rabbit IgG, Alexa Fluor 488 (green)</td>
<td>4°C refrigerator</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG, Alexa Fluor 555 (red)</td>
<td>4°C refrigerator</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG, Alexa Fluor 647 (magenta)</td>
<td>4°C refrigerator</td>
<td>1:500</td>
</tr>
</tbody>
</table>

**Washing steps:**

1. Remove the box with the devices and the DPBS (with Ca²⁺/Mg²⁺) bottle, the block buffer, and wash buffer out of the refrigerator let them warm up for 30 minutes or until it reaches room temperature.

2. When the two hours are up, place the box under the hood and aspirate the excess of the secondary stain off each device.

3. Transfer the devices from each 60-mm dish back to a 100-mm dish (3 devices per big dish).
4. Pipet 12 mL of wash buffer in each 100-mm dish.
5. Aspirate the wash buffer.
6. Repeat steps 3-4 three times at a 10-minute interval.
7. Aspirate the wash buffer in each dish.
8. Pipet 10 mL of DPBS (with Ca\(^{2+}\)/Mg\(^{2+}\)) in each dish and rest for 5 minutes, then aspirate the DPBS (with Ca\(^{2+}\)/Mg\(^{2+}\)).
9. Repeat step 8 two more times. And intermittently move the dish in circular motions.
10. Pipet 12 mL of DPBS (with Ca\(^{2+}\)/Mg\(^{2+}\)) in each 100-mm dish.
11. Wrap the dishes with parafilm and place them in the refrigerator overnight.

Notes:
- This is a good time to image the devices, either right after the secondary washing steps are done or the next day.
- Again, because the primary antibodies are of the same species, the whole process needs to be repeated for each marker. After all the staining steps are done for each marker, the devices need to be imaged under the microscope again using fluorescence imaging and multi-channel imaging (including z-stack) techniques.

*Hoechst-33342 Staining Steps:*

Hoechst-33342 is an organic compound used as a fluorescent stain for specifically staining the nuclei of living or fixed cells.
1. Remove the 100-mm dishes with devices and the DPBS out of the refrigerator and let them warm up for 30 minutes or until it reaches room temperature.
2. Aspirate the PBS out of the 100-mm dishes.

3. Pipet 12 mL of fresh PBS into each one of the dishes (depending on how many devices need to be stained).

4. Prepare the Hoechst dye stock solution by dissolving 24 μL of Hoechst stain in the dish with 12 mL of PBS and move the dish around in circular motions to ensure that the stain is properly dissolved.

5. Let the dish(es) under the hood for 45 minutes.

6. After 45 minutes, aspirate the stain solution and wash the devices three times with either PBS or wash buffer at a 10-minute interval.

Notes:

- The dilution for Hoechst-33342 is 1:500.
- The devices can be immediately imaged.
APPENDIX G

**Image Analysis Code using Masks**

```plaintext
path = "C:/Users/MISL/Desktop/Kailey and Ines/";
image_list = getFileList(path);

for (i = 0; i < 6; i++) {
    image = image_list[i];
    print(image);
    open(path + image);

    selectWindow(path + image + " - C=2"); // C2 is GFP
    run("Enhance Contrast", "saturated=0.35");
    run("Auto Threshold", "method=IsoData white stack");

    selectWindow(path + image + " - C=0"); // C0 is Foxp1
    run("Enhance Contrast", "saturated=0.35");
    run("Auto Threshold", "method=IsoData white stack");

    selectWindow(path + image + " - C=1"); // C1 is Hoechst
    run("Enhance Contrast", "saturated=0.35");
    run("Subtract Background...", "rolling=10 stack");
    run("Enhance Contrast", "saturated=0.35");
    run("Auto Threshold", "method=IsoData white stack");
    run("Fill Holes", "stack");
```
run("Watershed", "stack");
setOption("BlackBackground", true);
run("Erode", "stack");
run("Dilate", "stack");
run("Analyze Particles...", "size=100-300 pixel circularity=0.50-1.00 show=Masks display exclude clear stack");
totcellcount = nResults;
print("Image " + i+1 + " TCC = " + totcellcount);
run("Divide...", "value=255.000 stack");
run("Enhance Contrast", "saturated=0.35");
imageCalculator("AND create stack", path + image + " - C=2", "Mask of " + path + image + " - C=1"); // Mask of Hoechst
selectWindow("Result of " + path + image + " - C=2");
run("Enhance Contrast", "saturated=0.35");
run("Auto Threshold", "method=Otsu white stack");
run("Fill Holes", "stack");
run("Watershed", "stack");
run("Analyze Particles...", "size=100-300 pixel circularity=0.50-1.00 show=Nothing display exclude clear stack");
MNcellcount = nResults;
print("Image " + i+1 + " MNCC = " + MNcellcount);

imageCalculator("AND create stack", path + image + " - C=0","Result of " + path + image + " - C=2");
selectWindow("Result of " + path + image + " - C=0");
run("Auto Threshold", "method=Otsu white stack");
run("Fill Holes", "stack");
run("Watershed", "stack");
run("Analyze Particles...", "size=100-300 pixel circularity=0.50-1.00 show=Nothing display exclude clear stack");
LMCcellcount = nResults;
print("Image " + i+1+ " LMCCC = " + LMCcellcount);

if (i > 3) {
    selectWindow(path + image + " - C=3"); // C3 is Lhx1
    run("Enhance Contrast", "saturated=0.35");
    run("Auto Threshold", "method=IsoData white stack");

    imageCalculator("AND create stack", path + image + " - C=3","Result of " + path + image + " - C=0");
    selectWindow("Result of " + path + image + " - C=3");
    run("Auto Threshold", "method=Otsu white stack");
    run("Fill Holes", "stack");}
run("Watershed", "stack");
run("Analyze Particles...", "size=100-300 pixel
circularity=0.50-1.00 show=Nothing display exclude
clear stack");
LatLMCcellcount = nResults;
print("Image " + i+1+ " LatLMCCC = " + LatLMCcellcount);

selectWindow("Result of " + path + image + " - C=3");
run("Z Project...", "projection=[Max Intensity]"");
}

selectWindow("Result of " + path + image + " - C=0");
run("Z Project...", "projection=[Max Intensity]"");

selectWindow("Result of " + path + image + " - C=2");
run("Z Project...", "projection=[Max Intensity]"");

selectWindow("Mask of " + path + image + " - C=1");
run("Z Project...", "projection=[Max Intensity]"");
run("Multiply...", "value=255.000");

*Z Project was done for visualization purposes only to be able to represent every z-stack slice.*

waitForUser;
run("Close All");
}
Image Analysis code (Binary GFP_Hoechst and GFP_FoxP1)

path = "C:/Users/MISL/Desktop/Kailey and Ines/";
image_list = getFileList(path);

for (i = 0; i < 6; i++) {
    image = image_list[i];
    print(image);
    open(path + image);
    selectWindow(path + image + " - C=2");
    run("Subtract Background...", "rolling=10 stack");
    run("Enhance Contrast", "saturated=0.35");
    run("Z Project...", "projection=[Max Intensity]" edx);
    selectWindow("MAX_" + path + image + " - C=2");
    rename("Max Projection GFP" + i);
    run("Auto Threshold", "method=IsoData white");
    setOption("BlackBackground", true);
    run("Erode");
    run("Dilate");
    run("Fill Holes");
    run("Watershed");

    selectWindow(path + image + " - C=0");
}
run("Subtract Background...", "rolling=10 stack");
run("Enhance Contrast", "saturated=0.35");
run("Z Project...", "projection=[Max Intensity]" gravity="auto""");
selectWindow("MAX_" + path + image + " - C=0");
rename("Max Projection FoxP1" + i);
run("Auto Threshold", "method=IsoData white");
setOption("BlackBackground", true);
run("Erode");
run("Dilate");
run("Fill Holes");
run("Watershed");

selectWindow(path + image + " - C=1"); // C1 is Hoechst
run("Subtract Background...", "rolling=10 stack");
run("Enhance Contrast", "saturated=0.35");
run("Z Project...", "projection=[Max Intensity]" gravity="auto""");
selectWindow("MAX_" + path + image + " - C=1");
rename("Max Projection Hoechst" + i);
run("Auto Threshold", "method=IsoData white");
setOption("BlackBackground", true);
run("Erode");
run("Dilate");
run("Fill Holes");
run("Watershed");
run("Set Measurements...", "area bounding redirect=None
decimal=3");

selectImage("Max Projection Hoechst" + i);

run("Analyze Particles...", "size=100-300 pixel
circularity=0.5-1.00 display exclude clear");

GFP_MN = 0;

FoxP1_LMC = 0;

storeresults = nResults;

for (j = 0; j < storeresults; j++) {
    area = getResult("Area", j);
    xval = getResult("BX", j);
    BX = xval/0.645;  //Unit conversion from microns to
    pixels (image > properties for conversion)
    yval = getResult("BY", j);
    BY = yval/0.645; //Unit conversion from microns to
    pixels (image > properties for conversion)
    pixwidth = getResult("Width", j);
    pixheight = getResult("Height", j);
    width = pixwidth/0.645;
    height = pixheight/0.645;
    print(area + " " + BX + " " + BY + " " + width + " " +
    height);
    selectImage("Max Projection Hoechst" + i);
    makeRectangle(BX, BY, width, height);
}
run("Copy");
run("Internal Clipboard");
run("Keep Largest Region");
close("Clipboard");
selectWindow("Clipboard-largest");
rename("Hoechst_box" + j);

selectWindow("Max Projection GFP" + i);
makeRectangle(BX, BY, width, height);
run("Copy");
run("Internal Clipboard");
rename("GFP_box" + j);

imageCalculator("AND create", "Hoechst_box" + j, "GFP_box" + j);
selectWindow("Result of Hoechst_box" + j);
rename("Hoechst_GFP_Combo" + j);
selectWindow("Hoechst_GFP_Combo" + j);
getStatistics(area, mean); // for GFP-Hoechst
    if (mean > 0) {
        GFP_MN ++;
        selectWindow("Max Projection FoxP1" + i);
        makeRectangle(BX, BY, width, height);
run("Copy");
run("Internal Clipboard");
rename("FoxP1_box" + j);
imageCalculator("AND create", "Hoechst_box" + j,"FoxP1_box" + j);
selectWindow("Result of Hoechst_box" + j);
rename("Hoechst_'GFP'_FoxP1_Combo" + j);
selectWindow("Hoechst_'GFP'_FoxP1_Combo" + j);
getStatistics(area2, mean2); // for GFP-Foxp1
if (mean2 > 0) {
    FoxP1_LMC ++;
}

Table.create("MN/LMC Percentage" + i);
Table.set("MN Count", 0, GFP_MN);
Table.set("LMC Count", 0, FoxP1_LMC);
Table.set("Total Cell Count", 0, nResults);
Table.update();
close("GFP_box" + j);
close("Hoechst_box" + j);
close("Hoechst_GFP_Combo" + j);
close("FoxP1_box" + j);
close("Hoechst_'GFP'_FoxP1_Combo" + j);

resultspath = "C:/Users/MISL/Desktop/Ines Thesis Analysis Results/Binary_FoxP1_GFP_Hoechst_Results/";
Table.save(resultspath + image + "_Hoechst_GFP_FoxP1_Results.csv");
waitForUser;
run("Close All");
APPENDIX H

Raw Images Examples

MN3

MN5
Masks generated from Images analysis for Hoechst, GFP, Foxp1, Lhx1
Figure I. Images of the microfluidic devices.

a) Top view of the chip with the four inlets and fluidic channels where the media flows in.
b) Bottom view of the chip with two outlets and the two adjacent chambers connected by microchannels.
BIography of the Author

Inès Khiyara was born in Brussels, Belgium on May 20th, 1998. She was raised in Crisnée, Belgium, and graduated from Saint-Servais Secondary School in 2016. She enjoyed swimming and competitively swam throughout secondary school, representing her two countries, Belgium and Morocco. She then decided to use her passion for swimming to swim abroad in the United States while continuing her education. She graduated in May 2020 with a bachelor’s degree in Biomedical Engineering and a Minor in Environmental Engineering. Throughout her three years at the University of Maine, she enjoyed swimming for the varsity team for which she has broken several records, as well as her classes in the biomedical engineering program. She then entered the Biomedical Engineering graduate program in the Summer of 2020. After receiving her degree, Inès looks forward to gaining some work experience in the Biotechnology Industry as well as continuing to enjoy swimming. Inès Khiyara is a candidate for the Master of Science degree in Biomedical Engineering from the University of Maine in December 2022.