Impedance Sensing of N2A and Astrocytes as Grounds for a Central Nervous
System Cancer Diagnostic Device

A Thesis

presented to

the Faculty of California Polytechnic State University,

San Luis Obispo

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biomedical Engineering

by

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June 2012

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ABSTRACT

Impedance Sensing of N2A and Astrocytes as Grounds for a Central Nervous System Cancer Diagnostic Device

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This thesis utilizes previously described manufacturing and design techniques for the creation of a PDMS-glass bonded microfluidic device, capable of electrochemical impedance spectroscopy (EIS). EIS has been used across various fields of research for different diagnostic needs. The major aim of this thesis was to capture cancerous and non-cancerous cells between micron sized electrodes within a microfluidic path, and to complete analysis on the measured impedances recorded from the two differing cell types. Two distinct ranges of impedance frequency were analyzed – the α dispersion range, which quantifies the impedance of the membranes of the cells of interest, and the β dispersion range, which quantifies the impedance of the cytosol of the cells of interest. This thesis is unique in the fact that it looks at the cellular impedances of two types of neural cells, which has not been documented previously in literature. The type of cancerous cells analyzed were Neuro-2-A cells, an immortalized line of murine glioma/neuroblastoma. The type of non-cancerous cells analyzed were murine primary astrocytes, a mortal line of neurological support cells found throughout the nervous system, and with great abundance in the brain.

By using a LabView program coded by a previous Cal Poly student, a sweep scan across a wide frequency range was completed on both cell types, and statistical analysis was completed on target frequencies of interest. A significant difference was found between the two cell lines' membrane impedances, however no difference was found between the cytoplasm impedances.

In total, this thesis aimed to fabricate a reusable microfluidic device capable of EIS for future Cal Poly students, create a protocol suitable for cell culturing and device operation, and to lay a foundation of knowledge for impedance comparisons regarding neural cancerous and non-cancerous cells.

Keywords: Electrochemical impedance spectroscopy, Cancer, Diagnostics, BioMEMS, Microfluidics, PDMS.

ACKNOWLEDGMENTS

First off, I would like to thank Dr. Robert Mahley of The J. David Gladstone Institutes at UCSF for his very generous donation of lab materials and reagents, as well as the cell lines which were used for this thesis. I would also like to give a very big thank you to Reeny Balestra of The J. David Gladstone Institutes at UCSF for teaching me all I know about cell culture, assisting me in preparing the cell lines for transfer to Cal Poly, and for the insight and answers she provided in response to my endless questions.

I would also like to thank The Eagles of Morro Bay, who provided funding for the creation of our microfluidic device creation, as well as for other lab materials.

I would like to thank Dr. Brian Thibeault of the University of California, Santa Barbara Nanofabrication Lab for his wealth of information regarding the manufacturing processes of glass-PDMS bonded microfluidics devices, and his selfless effort he put forth to help us build our microfluidic device.

I would like to thank Brian Wong for helping pave the way for the completion of this thesis, and for keeping me sane during our long hours in the lab.

I would like to thank Dr. Robert Szlavik and Dr. Dan Walsh for their assistance in the completion of my thesis as my committee members.

I am extremely grateful for the opportunity Dr. David Clague gave me to work in his lab, and for the opportunity to work on such an exciting and interesting project which fit into my interests. The guidance, insight, and support which you have provided for me have helped make this an enjoyable process.

I would finally like to thank my family and friends for their excessive support and encouragement during my college career, and for always being there to lend a helping hand.

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1 Introduction and Background Information

Specifically, the aims of this thesis are:

- 1) To manufacture a suitable platform for cell introduction, capture and electrochemical impedance analysis.
- 2) Develop a reproducible protocol for future cell analysis with the aforementioned manufactured platform.
- 3) Perform a sufficient amount of impedance measurements on cancerous and non-cancerous cells to be able to complete analysis with statistical validity.
- 4) Complete statistical analysis on the recorded information, used to determine if the membrane impedances and cytosol impedances between neural cancerous and non-cancerous cells are significant.

1.1 Clinical Problem

In the world today there are a myriad of disorders, diseases and infections which go undetected for too long, until the point where irreversible damage has been done. These occurrences are not limited to third world countries, but are found in all countries. With the advancements of technology and medicine, there is increased potential to successfully treat these ailments. The key factor governing treatment revolves around locating and identifying the disease in a timely fashion. The lack of foresight into disease identification has created a necessity for advanced screening methods, in order to utilize our modern day treatments.

A large culprit of eluding our diagnoses, which can quickly spiral out of control and mature into a lethal state, is cancer. Cancer is the uncontrolled growth of our own cells, as the cell's growth regulation systems are mutated and cannot inhibit drastic division [1]. Increased division of cells will result in the cancer growing, which can lead to metastasis of the tissue, the rampant spread of cancerous cells to various parts of the body. As the cancer spreads it can impede critical organ function and steal precious nutrients required for the rest of the body to function. As of 2008, there were around 12 million people in the United States alone with diagnosed forms of cancer, ranging from the central nervous system, to breast, to lung, essentially any part of the body [2]. Of these 12 million people, over half a million are expected to die each year, accounting for 1 in each 4 deaths that occur in the United States [2]. This traumatic disease not only takes loved ones from almost every family in the world, it also brings a catastrophic cost. It is so prevalent that approximately 50% of all men and 33% of all women in the United States are predicted to develop some form of cancer within their lifetime [2]. The National Institute of Health estimated that the overall cost of cancer in the United States in 2010 was on the range of \$260 billion, with \$140 billion of the total sum being directly related to the loss of productivity due to premature death of the patients [2].

Limiting the growth of cancer by diagnosing it before the tissue becomes malignant and begins to metastasize would help save millions of lives and billions of dollars worldwide.

1.2 The Mammalian Cell

The cell is commonly referred to as “the basic unit of life”, and is the primary tenant of the cell theory: all living organisms consist of one or more cells and their byproducts. As seen in Figure 1, all cells contain organelles within their cytosol that have particular responsibilities to keep the cell functioning, such as the nucleus which stores information and sends signals for protein production, the mitochondria which produces usable energy for the cell, and an array of other components [1]. Making up the remainder of the cytosol which is not occupied by organelles is fluid that harbors different ion, salts, and proteins which are readily used by cellular mechanisms.

Figure 1 – The principle features of the mammalian cell [1].

Surrounding the cytosol of the cell is the cellular membrane, a flexible bilayer of phospholipids, filled with proteins and cholesterol [1]. The proteins on the surface of the cell help drive intracellular reactions via ligand binding, control cell to cell interactions, help transport ions across the barrier, and facilitate various other functions [1]. The membrane itself is semipermeable to gases and other small molecules, with certain molecules exhibiting higher diffusion permeability. The inside and outside layers of the membrane are oppositely charged, with the exterior being positive and the interior being negative. It has been speculated that the cell surface may drive the affinity for cancerous cells and their interactions, including adhesiveness and new antigen production [3]. Cancerous cells have shown greater membrane permeability to ions, as well as significant pH differences [4, 5]. This is partially due to the alterations to the membrane which occur when it becomes malignant, the instability of proteins which congregate on their surface, and the malfunctioning of essential organelles including the mitochondria and endoplasmic reticulum [3, 5].

1.3 Neuroblastoma and Glioblastoma Introduction

Each region of the body is comprised primarily of unique cell types that are specialized for functionality. The central nervous system (CNS) is the combination of the brain and spinal cord, and this system is the epicenter of all information signaling for all vertebrates. It functions to control all aspects of motion, speech, thought, as well as various other responsibilities [6]. If any aspect of the central nervous system is damaged, it can lead to a vast array of

ailments including loss of motor control, loss of sensation in limbs, slurred speech, problems breathing - the list is extensive [6, 7, 8]. Both the brain and spinal cord are comprised of unique cell types, including neurons, glial cells and the meninges, and are surrounded by cerebrospinal fluid [6, 9, 10]. Glial cells are the primary support cells within the central nervous system, with three specific cell types composing this group: astrocytes, oligodendrocytes, and ependymal cells [9, 10].

Astrocytes specifically function to provide nutrients to neurons, and create a support system for these cells [6, 11]. This cell type can account for up to 90% of the tissue found in various regions of the brain [12]. If there is any neural damage which occurs, i.e. lesions in the brain or severed central nervous system axons, astrocytes will form a scar in this region to prevent further damage [12]. Another key aspect of the functionality of astrocytes is their ability to sequester various types of ions and molecules which have been released from neurons as transmission signals [6]. These transmissions signals range from ions such as potassium (K^+) and calcium (Ca^{2+}) to larger molecules such as the non-essential amino acid glutamate. Astrocytes' ability to uptake these signaling compounds is of great importance, as a buildup of neurotransmitters in the neural fluid will lead to excitotoxicity, a buildup of dysfunctional signaling cascades which eventually cause neural and glial cell death [13]. Some neurons within the brain fire up to 1000 times per second, so the astrocytes' ability to collect these molecule byproducts is critical to preventing excitotoxicity [1].

Glial cells are the preliminary location of most central nervous system tumors and cancers, and are in general called gliomas [9, 10]. Due to the fact that neurons do not undergo mitosis after the very early periods of life, they are very rarely the cause of a central nervous system cancer [6]. A cancer which arises from astrocytes is known as a glioblastoma or astrocytoma. These cancers account for over 13,000 adult deaths a year within the United States, and over 4,000 diagnoses in children and teens [9, 10]. The survival rate for children is relatively high, with $\frac{1}{4}$ of all diagnoses returning benign tissue masses, and a vast majority of patients surviving more than 5 years from initial diagnosis [9, 10]. Of all malignant central nervous system tumors, 8 in 10 are gliomas [9, 10]. These cancers can occur anywhere within the central nervous system, and are prone to spreading to other regions of the brain or spinal cord. Typical symptoms of glioblastomas include headache, numbness in extremities, loss of balance, and behavioral changes [8, 9, 10, 14]. Glioblastomas produce a complex local microenvironment, defined by edemic cells, a 'leaky neurovasculature,' and random protein congregation [8]. Surgery and radiotherapy are necessary for mature patients with these tumors, but the outlook is normally poor regardless of the treatment, as the average lifespan after diagnosis is 15 months [14]. Chemotherapy is typically ineffective in combating central nervous system malignancies, as the blood brain barrier is very selective towards chemicals crossing the lumen of the vasculature [6].

Neuroblastomas are another primary type of nervous system cancers. This type of cancer typically starts in very young nerve cells, and usually affects

children who are less than 10 years old. Neuroblastomas are the single most common extracranial solid tumor type in children [15, 16, 17]. There are serious symptoms of neuroblastomas, including the loss of limb control, loss of bladder function, and weakened extremities, as the cancer can invade the spinal cord and cause unwanted compression there [7, 16]. Neuroblastomas account for around 10% of the 8,000,000 childhood cancers (up to 15 years of age) diagnosed per year [18]. When diagnosed, neuroblastomas require complete removal of the tumorous mass, and radiation therapy [19]. However, there is little significant change in the long term prognosis of these patients, and surgery can lead to other problems such as scoliosis [7].

Once these tumors spread throughout the central nervous system, it is almost impossible to successfully treat them without some form of undesired damage to the patient. Unfortunately, over half of all children with positive initial screens for neuroblastoma do not have the disease, which can lead to unnecessary invasive surgeries added emotional stress [15]. MRI scans for cranial neuroblastomas can identify a foreign mass, but there are currently few methods to determine if the growth is a lethal malignant tumor, or an abscess or benign tissue. For both neuroblastomas and glioblastomas, there is the need for early detection, as well as lethal tumor confirmation.

1.4 Electrical Theory

Today, cellular investigation is used as a diagnostic tool in the realms of biotechnology, pharmaceuticals, biomedicine, food industries, and environmental

engineering [20]. While these different industries techniques for cell property validation may be drastically different, the key is that they all analyze cells and use this particular information to complete quantifiable analyses. Specifically for biomedical research, electrochemical impedance spectroscopy (EIS) has been utilized to detect viral infections, drug responses, DNA characterizations and living cell traits [20, 21]. This technology utilizes electrical circuit theory to quantifiably measure differences.

Electrical current flowing through a system is defined by the voltage of the current divided by the resistance to electron flow. If a system contains a capacitor, the system will be able to build-up and save a defined amount of charge, which is dependent on the electrical permittivity. In an alternative current system (AC), the resistance is called Impedance (Z) and is a complex function of the real and imaginary vector resistances. The impedance can be measured by the following equation [22]:

$$Z = R + (j * X)$$

1.4.1 Electrochemical Aspects of Mammalian Cells

By utilizing electric circuit theory, electrochemical impedance spectroscopy has been shown to be a capable method of differentiating between cell types, where the temperature, ion concentration, protein presence, and intracellular electrical fields all influence the membrane and cytosol impedances [23, 24]. It has also been documented that the components within cells (organelles, proteins, etc.) can resist flow or store charge like a capacitor, leading

to differing, unique impedance measurements [25]. When a cell is placed between electrodes of an electrical circuit and a voltage is passed through, an electrical field will be formed, which allows for measurement of the cell's impedance. Homologous cell types should provide standard and consistent impedance measurements, while an altered cell due to infection or genetic disruption will produce a modified impedance.

When a cell is placed between two electrodes, an electrical field will be created through the cell, which is used as a linkage for the current. The peripheral fluid's impedance will also be recorded and parlayed in the measurement. The impedance of the surrounding fluid can be calculated separately, and subtracted from the original value as a measure of background impedance.

1.4.2 Electrochemical Impedance Phenomena

When completing electrochemical impedance spectroscopy, a notable electrical phenomenon may occur called the electric double layer. This 'layer' is an electric field which is distributed uniformly around the electrode being used for analysis as well as on the surface of the analyzed item of interest and its proximal fluid [26, 27]. During this phenomenon, seen in Figure 2, a layer of immobile ions will congregate on the electrode and cell surfaces, producing the "Stern" layer. This layer is formed by oppositely charged ions being attracted to the surface, while like charged ions are repelled. This layer of ions in the "Stern" layer leads to a change in net charge along the surface. Outside of this

superficial layer, there is a segment of mobilized ions, which is called the “diffuse” layer. The further away from the electrode surface and the “Stern” layer, the net charge density will approach zero, signifying a balance of charge [27, 28]. Pure fluids such as water have a thicker “diffuse” layer, on the order of microns, while solutions with high media concentrations will only produce a layer in the nanometer range. These ions are mobilized due to principles of diffusion and other electrostatic forces [27, 29]. This electric double layer must be kept in mind when completing analysis as it produces an additional level of resistance, but it will be assumed that it is equivalent for all studied cells.

Figure 2 – The electric double layer, highlighting the Stern and diffuse layers [29].

Previous cell impedance sensing and electrical theory research has provided different target ranges to record when analyzing single cells or small tissue masses. A low frequency (10Hz-10 KHz) range, called the α dispersion range, will send the electrical current around the periphery of the cell, and will provide information on the cell membrane impedance [21, 22, 25, 30]. A higher frequency (2 KHz-1 MHz) range, called the β dispersion range, will allow for measurement of the cell's cytosol and internal compartments, as the frequency of the current decreases the cell's electrical shielding ability [21, 22, 25, 30]. In this frequency range, the membrane becomes polarized, allowing current to freely pass through it and into the cytoplasm [26].

1.5 Electrochemical Impedance Spectroscopy Technology

Using electrochemical impedance spectroscopy has been proposed as a future medical diagnostic technique, with the benefit of higher accuracy than currently employed practices. There are various types of current techniques being used to screen for cancers, including X-ray technology, magnetic resonance imaging (MRI), and visual skin inspection [2]. The use of all of these methods are widespread, but typically are only used for preliminary analysis. Further invasive tissue biopsy is typically needed for a definitive verdict on the presence of malignant tissue. Electrochemical impedance sensing may be a suitable replacement for modern methods.

There are currently various methods of electrochemical impedance sensing being researched today, with the primary goal of disease status

identification. Arresting the current disease state, also known as secondary prophylaxis, is of the utmost importance, so damage to the host can be limited as early as possible [31]. Using biological micro-electro-mechanical systems (BioMEMS), single cells or clusters of tissue can be captured and analyzed, leading to near instantaneous results. As diseased cell types may have unknown local agents (either self-produced or infiltrated) or altered physiological functions, various aspects of the cell may be changed, including pH, temperature, and osmolarity. These altered states all effect electrical activity within the cell, and are the ticket for differentiation between cell types while using impedance sensing technology [24].

There are a wide array of electrochemical impedance sensing devices, including optical tweezers, physical cell capturing, non-invasive probes, patch clamps, magnetic tweezers, and dielectrophoresis (DEP) devices [32, 33, 34, 35]. All of these technologies employ a system to measure the impedance across a single cell, or a small amount of tissue. Therefore, a sample of the tissue must be sampled via a biopsy. EIS has been used to differentiate normal and malignant excised prostate tissue, and has been utilized to separate malignant skin tumors by providing recordings of higher conductivity and permittivity due to increased salt and water content [32, 36]. A non-invasive method of measuring impedance is electrical impedance tomography (EIT), which involves placing a device on the exterior of the skin and measuring through a mass of tissue [22]. Non-invasive probes, EIT, optical tweezers and other EIS methods have shown

potential to improve cancer diagnoses, but these methods still have limitations and necessary improvements.

1.5.1 Electrochemical Impedance Sensing Case Studies

One specific use of impedance sensing to identify cancerous tissue was completed by Halter *et al.* (2008) with a hand held coaxial impedance sensing probe [36]. The EIS device constructed for their research consisted of an outer conductor with external electrodes, and can be seen in Figure 3. The use of this device requires an excised segment of tissue from a biopsy. The tissue was cut into a cylindrical volume, and then placed under the probe for analysis. Various samples of pancreatic tissue were sampled, including those which were suspected of being cancerous. The recording and analyses of the different tissues, paired with histological examinations found that there were statistically significant differences between cancerous tissue and various types of benign tissue including glandular and connective. Although there were positive aspects of the use of the device for a diagnostic tool, there were some downsides to the use of the device, which are explained in the following section.

Figure 3 – Coaxial impedance sensing probe developed by Halter *et al.* for prostate cancer sensing [36].

Aberg *et al.* (2004) also studied the use of EIS for cancer detection, specifically to identify malignant skin lesions from benign tissue [32]. In this study, the group created another version of a hand held probe, which had four gold electrodes patterned in a concentric manner on a ceramic plate. This device was placed directly over both malignant and benign areas of skin and scanned the region by applying a voltage. The output recorded an impedance value for each sample they measured. As seen in Figure 4, their results showed success in employing EIS technology to differentiate between the two types of tissue. The device produced 75% specificity during their testing, meaning that it was 75% accurate when identifying between malignant or benign tissue. Although their results are promising, there were some limitations of the device, which are documented in the following section.

Figure 4 – Impedance magnitude and phase graphs produced by Aberg *et al.* showing the differences between benign (dotted lines) and malignant (solid lines) skin tissues [32].

1.5.2 Current Limitations of Electrical Impedance Sensing

During diagnosis of cancer, it is of the utmost importance to not miss a rare event. Some currently used cancer diagnosis methods do not provide the utmost accuracy. For example, the primary method for breast cancer screening, X-ray mammography, produces a significant amount of false positives, which must later be confirmed via a visualized tissue biopsy [22]. Another example is skin cancer screening, where a mass of tissue is typically analyzed visually by trained personnel, which is completely dependent on the skill of the observer, increasing the chance of a missed diagnosis and therefore escalating risk [32]. These analysis techniques suggest that there is a large amount of room for improvement when it comes to cancer diagnosis.

Electrochemical impedance spectroscopy has been proposed as a next generation diagnosis method, with a higher affinity for accurate sensing. As opposed to MRI scans and visual inspections, electrochemical impedance spectroscopy can help take out the guess work. This method may reduce the

complete dependence on the skill of the radiologist. However, there are some user born errors which may occur with EIS. Large cell mass impedance sensing diagnostics are one possibility, which can be completed non-invasively, or with a tissue biopsy. The exterior impedance sensing method has been studied, but there are various problems with analysis using this technique. Exterior impedance sensing does not always provide a concise readout due to poor contact with the skin, the unique shape of an object being read, among other challenges of use [22]. This can lead to a misdiagnosis of cancer. Also, in a cancerous mass, only some of the cells will show 'malfunction symptoms,' leading to off measurements [21, 31, 37]. When analyzing a tissue biopsy sample, other problems may arise with the impedance sensing. If the tissue is not well separated and a cluster of cells forms onto one electrode, a loss of signal can occur which leads to poor data read outs [38].

Single cell sensing may lend a more precise method of impedance spectroscopy, but it also has limitations. It has also been noted that it is important to have the individual cells positioned uniformly between the electrodes from run to run. Also the cells must be nicely adhered to the electrode in use to fully optimize the sensing, especially during low frequency recordings [26, 37]. There are various aspects that can affect cellular analysis, such as the cell size and membrane thickness. As cell size increases, it has been documented that a large 'insulator volume' is produced where the current passes around the periphery of the target cell, and can lead to impedance error [26]. Patch clamp single cell analysis is another single cell sensing alternative, but using this class

of device requires a high level of skill, while optical tweezers require expensive and complex optics systems [34, 39]. Using micropipette single cell analysis systems can yield accurate data, but are prone to cause cellular damage [35].

In the study completed by Halter *et al.*, there were various documented limitations of their hand help impedance sensing device [36]. It was noted that any variation in the shape of the excised tissue can lead to improperly balanced contact, and therefore a poor readout. Furthermore, there is some operator induced variability with the device contact which can further accentuate this problem. Also, all tissue sampled will have variable cell concentrations, which can drastically alter the electrical properties, therefore leading to inconstant data [36]. The study further describes the hand held device's limitations by noting that the polarization at the electrode to tissue interface would produce an additional capacitance layer and lead to inaccuracies within the alpha dispersion range, where the membrane properties of cells would be recorded.

In the study completed by Arberg *et al.*, the group documented that this skin sensing method showed 75% specificity, which is very good but far from perfect [32]. It was also noted that the device could not scan with any significant depth into the tissue, so if a malignant growth is burrowed beneath the epidermis, it would not be identified. Also of note was the physical size of the electrodes, which were significantly larger than the area of interest being scanned. The difference in size of the electrodes can lead to the inclusion of impedance measurements of the tissue surrounding the target. This could potentially alter the final results, and therefore the diagnosis.

Overall, it is very important to separate cancerous cells from noncancerous cells to be able to analyze them specifically. With advances in electrochemical impedance spectroscopy and MEMS technology, this can be realistically achieved. By utilizing principles from these two areas of research, a microfluidic channel with miniaturized electrodes can be manufactured to isolate and localize single cells for impedance sensing.

1.6 Microfluidic Device Design and Manufacturing

To complete single cell impedance analysis, a microscopic scale device must be manufactured. This device must include a cell and media flow passageway, as well as a set of miniature electrodes to measure the impedance. Being able to isolate and analyze a single cell is beneficial over analyzing a mass of tissue or a small cluster of cells, as it allows for a very specific individual characterization which may be missed elsewhere. Advancements in engineering have made single cell measurement a possibility, specifically using MEMS technology and fabrication methods. MEMS technology utilizes small scale machining and manufacturing, for pieces that are on the scale of 10^{-6} meters, or a micron.

1.6.1 MEMS Technology Applications

The field of MEMS technology began in with microelectronics, where manufacturing processes were used to produce electrical circuit boards for computer processors and radio frequency capacitors [40, 41]. Advancement in

the field lead to production of micro scaled sensors, valves, pumps and a variety of other devices [42, 43, 44, 45]. A key use of MEMS systems related to biological research is the production of microfluidics devices, which can control fluid flow with great precision. This technology is currently being employed in the design of LAB-on-A-chip devices, biomarker sensing, low cost assays, and other devices used in the fields of biotechnology, chemical energy and biomedical sciences [27, 39, 46, 47, 48, 49].

Another application of microfluidic systems is microflow cell cytometry, which is a cheap and simple method to count various types of cells including red blood cells [50]. Microfluidic systems behave uniquely depending on the geometry and size of the channel, as well as the viscosity of the utilized fluid. One benefit of the small channels is the ability to control the flow through the channel, and avoid turbulence [51]. In addition to utilizing flow through such a small channel, micron scale electrodes can be incorporated for analysis, which may help advance the field of biological diagnostics [26, 35]. A viable method of manufacturing these minute electrodes is by photolithography.

Biological micro-electro-mechanics systems (BioMEMS) include the previously mentioned Lab-on-a-chip and low cost assay systems, and have been a target of research for diagnostic uses. The direct benefit of these systems is the small amount of fluid or tissue required to complete analysis, and a smaller volume of used reagents. This will lead to more accurate and specialized results, and savings in cost. These devices may then be used for definitive medical diagnostics in hospitals and laboratories across the world.

1.6.2 Materials Selection

The design and manufacturing of an ideal in house device for single cell diagnostic use requires low cost and biocompatible materials as well as simple but efficient manufacturing techniques. To create such a device there is an array of materials which can be chosen, including using glass for a device base, gold and titanium for impedance sensing electrodes, and a polydimethylsiloxane (PDMS) molded chamber. These materials have all been previously used for microfluidic devices, as they all exhibit biocompatibility, and do not affect cell proliferation or differentiation [39, 49, 52]. Gold is commonly used in the world of microfabrication for biologics, both in the form of electrodes and surface modifications. It is a very biocompatible element, and can reduce biofouling [53, 54, 55]. PDMS is an ideal material for the molded chamber, as it can be easily fabricated with a high level of precision, is biocompatibility, has low cost, and is physically transparent for cell viewing [35, 45, 49]. To produce a fully functional microfluidic device in a short period of time, PDMS is ideal due to its extremely short manufacturing time [49].

A PDMS-glass bonded microfluidic device allows for easy passage of cells, a large area for single cell capture, and easy removal and replacement of cells on the electrodes with its flow through capability. These devices are operated under the control of a set of syringe pumps, which can be connected to inflow ports and outflow ports [56]. This set up allows for a single media and cell mixture introduction, as well as the addition of a single compound for quantifiable

real time reactions. This set up of syringes allows for complete flow control, and an array of testing possibilities.

1.6.3 Manufacturing Methodology

To produce a suitable PDMS-glass microfluidic device, photolithography is essential in the manufacturing process. Photolithography is used to pattern the electrodes onto the glass base. Prior to applying the photoresist, HMDS is applied to the glass substrate surface, which acts as an adhesion promoter by making the surface more hydrophobic. HMDS functions by reacting with the surface hydroxyl groups and producing siloxane groups (silicone, oxygen and alkaline) [57, 58, 59]. After HMDS is placed onto the substrate, a positive photoresist is spun across the surface of the glass to produce a micron scale layer. The thickness of this photoresist layer can be calculated by the following equation [60]:

$$T = \frac{KC^{\beta}\eta^{\gamma}}{\omega^{\alpha}}$$

Where: T = thickness of the produced layer, K = Calibration constant, C = Polymer concentration (g/100 mL), η = Intrinsic viscosity, and ω = Rotations per minute (RPM). The photoresist is then baked at 100 degrees Celsius to confirm the removal of all defects and improve its adherence [61].

After the photoresist is firmly attached to the glass base, it must be exposed to UV light, with the positive mask protecting the area where the electrodes will be formed. Positive photoresist masks can be created on glass wafers, which are then held above the glass base, and allows UV light to shine

outside the region of interest. This UV light disrupts the positive photoresist bonds to make the undesired features capable of being etched away [35, 44]. The intensity of the UV light is dependent on the photoresist's sensitivity. The mask and glass base are put into a vacuum induced contact by a contact aligner, then the UV light is exposed. After UV exposure, the glass base must be processed in a developing solution, which removes all unwanted photoresist material.

Metal deposition is a manufacturing method ideal to pattern electrodes onto completed onto the remaining photoresist area. This is completed either by electron beam deposition or metal sputtering [35]. To form a robust electrode on the glass, a thin (~150 angstrom) layer of titanium must be deposited first, followed by a ~1000 angstrom layer of gold. The metals are deposited onto the glass surface, and fully cover the device. After the metal layers are deposited onto the surface of the glass base, the excess metal which does not cover the positive photoresist is etched away to reveal the specifically designed electrodes.

To finish the device manufacturing, the PDMS and electrode laid glass base must be sufficiently adhered to prevent any form of fluid leakage. PDMS is naturally hydrophobic however, and must have surface alterations completed to promote bonding [62]. A commonly used method to bond PDMS and glass is by exposing both surfaces to O₂ plasma, then pressing the two components together, followed by a period of heat treating. The application of O₂ plasma produces a hydrophilic surface and drastically increases the surface energy, shown by the decrease in water contact angle [63]. O₂ plasma is commonly

used to bond PDMS to glass, silicone and PDMS itself [64]. Plasma bonding has also been shown to be a successful method of adhering other materials including metals, polymers and silicone [47].

However, O_2 plasma deposition does have some limitations. The process has been shown to fail due to hydrophobic recovery of the surface, where the oligomers within the PDMS migrate to the surface, leading to the material losing its hydrophilic capacity [64]. As seen in Figure 5, Bodas *et al.* showed that hydrophilicity decreases at an exponential rate after initial application, and can lose most of its function within a few hours [63].

Figure 5 – Graph produced by Bodas *et al.* highlighting the drastic increase in water contact angle when PDMS is treated with O_2 plasma [63].

Fortunately, there is a technique to reduce the chance of hydrophobic recovery, and can improve the success of bonding PDMS to glass. This method is ultraviolet ozone treatment, which has been shown to be less problematic than using O_2 plasma to make some surfaces hydrophilic, including PDMS [65]. This

method uses short wave UV radiation to excite the surface molecules, and generate atomic oxygen to produce a hydrophilic surface. As compared to O₂ plasma deposition, UV ozone deposition significantly increases the surface oxygen levels and decreases the carbons levels [66]. As seen in Figure 6, this leads to more silicone oxygen bonding, and improved long term hydrophilicity. Even after 3 months from the time of UV deposition, surfaces exhibit 65% of the instantaneous hydrophilicity, where O₂ plasma treated surfaces would have lost all of its hydrophilic properties [66]. Therefore, the most efficient bonding of PDMS and glass will be completed by exposing O₂ plasma to the glass element, and exposing the PDMS to UV generated ozone. After both components have sufficient exposure, they are to be aligned and bonded with a contact aligner under manually applied pressure. To finalize a device of this nature, it must be heat cured, and is then ready for use.

Figure 6 – Graph produced by Wu *et al.* showing improved low water contact angle retention after UV plasma deposition [66].

1.7 Purpose and Objectives

Each year, millions of people across the world are diagnosed with cancer, and millions more die due to the myriad types of this body ravaging disease. The overall cost of cancer per year is in the order of hundreds of billions of dollars, and there is no concise cure in sight. Without early diagnoses, the chance of cancerous tissue metastasizing and overwhelming the body is greatly increased. Even with our current technologies, there are widespread occurrences of missed diagnoses, whether false positives or negatives. These errors can lead to unnecessary mental and physical pains associated with subsequent testing. To reduce the number of deaths and to lessen the economic burdens of cancer, new acute diagnostics methods are needed.

The purpose of this thesis is to provide a proof of concept for a microfluidic based BioMEMS device which can produce fast and highly accurate diagnoses of single cells, which were removed from a supposedly cancerous tissue mass. This device will improve on currently researched electrochemical impedance sensing cancer diagnostic devices, as it attempts to remove human based error, and requires a smaller tissue sample for analysis. The design and manufacturing of the device will be based on the simplest and lowest cost methodology, so that similarly produced future devices can be accessible throughout all countries across the globe. Specifically, this thesis will look into testing for neural based cancers, which are categorized as glioblastomas and neuroblastomas. These

diseases can affect people of all ages, and are associated with various ailments such as loss of motor control, nausea, problems breathing, and of course death.

In section 2, Methods and Materials, the steps of fabricating a low cost microfluidics BioMEMS device out of glass, PDMS, titanium and gold, which is capable of analyzing cells using electrochemical impedance spectroscopy will be covered. Also in this section, the methodology of cell culturing will be explained, followed by the protocol used to introduce the cells into the chamber, and over the electrodes for scanning. Next in section 3, Results, experimental analysis will be explained, including statistical methods. Finally in section 4, Discussion and section 5, Conclusion, an overview of the success of the research, interpretation of the results, as well as future applications of the device in a medical scenario will be conferred.

2 Methods and Materials

This section is intended to describe the manufacturing processes and equipment required to create the PDMS-Glass bonded microfluidic device used for this thesis project, the methodology to successfully harvest murine primary astrocytes, the methodology of culturing the Neuro-2-A and primary astrocyte cell lines for use, and the methodology for recording the impedance of these cell lines in the microfluidic device. The techniques described primarily were derived from protocols from previous Cal Poly Biofluidics group researchers, researchers at The Gladstone Institutes at UCSF, as well as others found within literature. As this project's aim was to create a proof of concept device for diagnostic sensing, the methods and materials utilized for device creation focused on both low cost and functionality. Therefore the device was not created for purpose of superiority to existing devices, and there is much room for improvement if this device were to be used in the field today.

2.1 Device Fabrication

As previously mentioned, when designing an ideal microfluidic device that is low in cost and biocompatible for cell use, the material selection process is of utmost importance. Previous research has shown that PDMS is an ideal material for a microfluidic devices to be used with biologics, as it can be formed to a wide array of shapes, is low cost, and is biocompatible. As previously shown in Josh Fadriquela and Stephanie Hernandez's theses (both Cal Poly 2009), a device can be manufactured with segments created in house at Cal Poly and other

components nearby at UCSB that consists of a PDMS channel segment, bonded to a glass base. Their device exhibited functionality in its recording capability; however the inflow and outflow system the device utilized caused a congregation of cells in the vicinity of the electrodes. This is not ideal, as capturing a group of cells can lead to impedance recording inaccuracies. Therefore a simpler device was developed, that consisted of an inflow region, and outflow region, and a microfluidic channel which passes over a set of electrodes for recording.

2.1.1 Silicon Wafer Master Mold Fabrication

Prior to the production of the PDMS section of the microfluidic device, Brian Wong (Cal Poly 2012) designed and fabricated a master mold for the PDMS to be poured over which would create the designed channel geometries. In short, Mr. Wong designed the microfluidic channel in AutoCAD, and submitted the design to the photo-plotting service company CAD/Art Services Inc. for production of a negative transparency mask. After receiving the mask, 100 mm diameter silicon wafers were cleaned by soaking with a Piranha solution (98% concentration Sulfuric Acid and 30% concentration Hydrogen Peroxide mixed to a 9:1 ratio) for 15 minutes, followed by a de-ionized water rinse. The wafers were then rinsed with buffered oxide etchant (BOE) solution (HF acid/H₂O, Transene) for a period of 5 minutes, followed by a de-ionized water rinse. Next the wafers were dried with nitrogen gas and baked at 205° Celsius for 10 minutes then allowed to cool to room temperature. After cooling, the wafers were spin-coated with the epoxy based polymer SU-8 (MicroChem SU-8 2000), then baked

on a hot plate at 85° Celsius for 3 minutes, and again allowed to cool to room temperature. After coating with the SU-8, the wafer was placed on the photolithography mask aligner with the negative transparency mask above it. UV light was then exposed to the wafer for 100 seconds at 125 mJ/cm² to de-cross link the portion of the SU-8 that will not be used for the PDMS mold. To remove this SU-8, the wafer was then baked on a hot plate at 85° Celsius for 5 minutes, allowed to cool to room temperature, then placed into a developing solution of propylene glycol monomethyl ether acetate (Microchem SU-8 Developer) for 3 minutes. After rinsing the wafer with de-ionized water and allowing it to dry, the finished master mold for PDMS pouring was created. See the thesis of Josh Fadrique (Cal Poly 2009) for a more detailed methodology, as well as Appendix A for a step by step process.

2.1.2 PDMS Pouring

To prepare the PDMS for pouring, the PDMS base and curing agent (Dow Corning Sylgard 182A and 182B respectively) were mixed in a 1:10 volume ratio. To mix the two components, they were decanted in a pouring cup, and then stirred with a metal stirring rod. After stirring the mixture thoroughly (enough to see a significant amount of bubbles), the PDMS mixture was degassed in a vacuum chamber that was pressurized to -22 inches Hg for approximately 25 minutes to remove all bubbles. After it was evident that the bubbles were removed, the SU-8 master mold was placed ridge (mold) side up in a 140 mm diameter petri dish with forceps. Next the PDMS mixture was poured slowly over

the entirety of the mold. After pouring the PDMS, any air bubbles produced by the pouring process were popped or drag away from the features of interest via a needle. A curing oven was heated to 65° Celsius, and after obtaining this temperature, the entire petri dish containing the master mold and the freshly poured PDMS was placed into the oven to bake cure for 4 hours. After the heat curing was completed, the PDMS was peeled off of the mold with a scalpel. The microfluidic channel features were then covered with clear tape for storing until device fabrication, as to prohibit debris from entering the channel. A step by step process of the PDMS pouring can be found in Appendix B.

2.1.3 Electrode Patterning

Standard 2 x 3" laboratory glass slides were prepared for electrode patterning by cleaning the surface with acetone, followed by isopropanol, and finally a nitrogen gas dry. After the initial cleaning period, a Technics PEII Plasma Etcher (seen in figure 7) was used to deposit oxygen plasma onto the surface, which completely eliminates any organic material. The device was run at 300 mT O₂ and 100 Watts power for a duration of 60 seconds. To produce siloxane groups from greater hydrophobicity, HMDS (hexamethyldisilazane) (Transene HMDS – X10) was placed onto the surface of the glass slip with a dropper and allowed to rest for 30 seconds. The glass slide was then placed onto a spin coater, and spun at 4000 RPM for 20 seconds to remove excess liquid. After spinning on the HDMS, AZ nLOF 2020 (Capitol Scientific #AZNLOF2020-1Q) negative tone lift-off photoresist was added to the surface via a dropper, and then

spun at 3000 RPM for 30 seconds to create an ideal layer across the glass. The glass slide was then placed onto a hot plate which was heated to 110° Celsius for 2 minutes, to dry the photoresist onto the surface.

Figure 7 – The Technics PEII Plasma Etcher device, which is used to remove organic material from desired surfaces and also deposits oxygen plasma for device bonding.

As the photoresist is present across the entire surface, a vast majority of the photoresist must be removed to leave a pattern for the electrodes. The glass slide was placed onto the stage of a Karl-Suss MA6 contact mask aligner, and the transparency with the pattern for the electrodes was placed over the top of the slide. The 350 Watt UV lamp then ran for 5 seconds to dissociate the bonds of all regions not protected by the transparency. After the UV exposure was

completed, the slide was returned to the 110° Celsius hot plate for 90 seconds. Following the baking period, the slide was developed in AZ 300 MIF developing solution (Clariant AZ 300 MIF Developer) for 1 minute. This step removes the surrounding photoresist from the glass surface, and allows the remaining photoresist to bond the electrodes during further processing. After the slide was developed, it was rinsed with de-ionized water for 30 seconds, and finally dried with nitrogen gas.

Figure 8 – The glass portion of the device, resting in a developing solution to strip away any excess material not attached to the deposited photoresist.

Next, the electrodes were patterned onto the glass slide with a CHA Model SEC600 electron beam evaporator. First, the chamber was pumped to 2.0×10^{-6}

Torr pressure, and then a 15 nanometer layer of titanium was deposited onto the glass slide at a rate of 0.15 nm/second, followed by a 100 nanometer layer of gold deposited at 0.3 nm/second. The titanium layer is deposited onto the glass prior to gold deposition, as titanium will maintain a solid bond to the glass, and gold will flake off easily if patterned directly onto the glass. After the two layers of metal were deposited onto the glass surface, the metal which is not in direct contact with the photoresist was etched away by soaking the slide in Shipley 1165 photoresist stripper (NMP) (Shipley Microposit Remover 1165) for 1 hour at 80° Celsius. After being removed from the photoresist stripping solution, the slide was rinsed off with de-ionized water for 2 minutes, and then dried with nitrogen gas. At this stage, the electrodes were successfully patterned onto the glass surface, and can be seen in figure 9.

Figure 9 – Finished electrodes bonded to the glass surface after excess metal etching.

2.1.4 Device Bonding

After completing the PDMS molding and patterning the electrodes onto the glass slide, the two components were bonded together to complete the device. The PDMS section of the device was placed into a bath of acetone under ultrasonic agitation for a period of 5 minutes. After the acetone bath, it was rinsed with isopropanol for 1 minute, and finally dried with nitrogen gas. The PDMS was then placed into a UVP, Inc. PR-100 UV-Ozone Photoreactor to deposit UV generated ozone for 5 minutes. At the same time, the glass slide was returned to the Technics PEII Plasma Etcher, and had oxygen plasma deposited across its surface at 300 mT O₂ and 100 Watts power for 90 seconds. At this stage, both halves of the device were sufficiently coated with ozone plasma for bonding. Both sections of the device were brought to a Karl Suss MJB-3 contact aligner which was set to soft-contact mode. The two components were visually analyzed to confirm proper bonding location of the electrodes inside of the microfluidic channel, and then were manually pressed together to bond. During the manual bonding process, it was difficult to align the electrodes in an exact location of the microfluidic channel, as there was some minor slipping which occurred between the glass and PDMS. The minute geometry of the channel accentuated this difficulty, as the width is on the micron scale. The device was then removed from the contact aligner, and the entire surface of the device was pressed together to assure no bubbles were present between the two materials. After any bubbles were removed, the device was placed onto the hot plate at 110° Celsius for 10

minutes of heat curing. The device at the end of the fabrication can be seen in figure 10.

Figure 10 – The microfluidic device upon completion of its fabrication at the UCSB nanofabrication lab, shown with test tubing inserted into the three ports.

2.2 Cell Harvesting and Culturing

As Neuro-2-A cells are an immortalized cancerous cell line, they can be ordered online from various cell culturing facilities, therefore their harvesting for culturing is not discussed. The primary astrocytes are a mortal cell line, and do not survive long term in culture. They must be harvested fresh from mice (or corresponding animals) into passage “zero” and then can be split in culture for a limited time. The harvesting of these cells is explained in detail here.

2.2.1 Murine Primary Astrocyte Harvesting

On the day of the primary astrocyte harvesting, ice cold 90% Dissociation Media/ 10% Kynurenic Acid (DM/KY) mixture was poured into five separate tissue culturing dishes, and placed onto ice. Dissociation Media (DM) was prepared to a volume of 1000 mL by mixing then sterile filtering the following ingredients (all components listed at final concentration): Na₂SO₄ at 81.8 mM (Sigma-Aldrich S9627), K₂SO₄ at 30 mM (Sigma-Aldrich P0772), MgCl₂ at 5.8 mM (Sigma-Aldrich M8266), CaCl₂ at 0.25 mM (Sigma-Aldrich 53704), HEPES buffer at 1 mM (Sigma-Aldrich 83264), Glucose at 20 mM (Sigma-Aldrich D9434), Phenol Red at 0.001% (Sigma-Aldrich P0290), and NaOH at 0.16 mM (Sigma-Aldrich 43617 FLUKA). The Kynurenic Acid (KA) mixture was prepared to a volume of 1000 mL by mixing then sterile filtering the following ingredients (all components listed at final concentration): Kynurenic Acid at 10 mM (Sigma-Aldrich K3375), Phenol Red at 0.0025%, HEPES buffer at 5 mM, and MgCl₂ at 100 mM. One 100 mm culture dish was used for the pups while in the uterine sac, one 60 mm dish was used for the heads of the pups, one 60 mm dish was used for the intact brains, one 60 mm dish was used for used under the dissecting microscope, and finally one 60 mm dish was used for the dissected segments of the brain. Next, the papain solution was prepared, by mixing 6 mL papain (Sigma-Aldrich 76216) per 5 cortices to be harvested into a 15 mL conical tube, which was then diluted with DM/KY. The papain was then activated with the addition of a few L-cysteine crystals (Sigma-Aldrich C7880), and finally adjusted to a neutral pH via the addition of 0.1 N NaOH. This solution was then

sterile filtered with a 0.22 μ M syringe filter less than 30 minutes before use. Next the trypsin inhibitor solution was created, by mixing 2 mL solution per cortex to be harvested. This was added to a 50 mL conical tube, and diluted with DM/KY, and finally adjusted to a neutral pH by adding 0.1 N NaOH. This solution was then sterile filtered with a 0.22 μ M syringe filter less than 30 minutes before use.

To acquire the pups for brain harvesting, the mother was sacrificed by cervical dislocation. The abdominal area of the mother was then sprayed with 70% EtOH, and then opened with scissors. The uterine sac (with pups) was then removed and placed into the 100 mm culture dish. The mother was then discarded in a biological waste bag. Next, the pups were removed from the uterus, and then the heads of the pups were removed with surgical scissors and placed into the first 60 mm culture dish. The bodies of the pups were discarded in the biological waste bag. After the heads were isolated, the brains were removed by cutting the skull in the middle region of the head and creating a transverse cut behind the eyes then finally peeling the skull away. The brains were then scooped out gently with a brain spatula, and placed into the next 60 mm culture dish, as seen in figure 11. After the brains were isolated, the olfactory bulbs were removed with surgical scissors, and the cortexes were removed from the remainder of the brain with fine forceps. The meninges were then removed from the cortical lobes, and the cortical lobes were placed into the final 60 mm culture dish.

Figure 11 – The harvested murine pup brains on ice in culturing dishes filled with DM/KY solution.

The cortices were then brought to the biological safety cabinet, and minced into $\sim 1 \text{ mm}^3$ segments with a razor blade that had been sprayed with 70% isopropanol. Next, the brains and their fluid were transferred to a 15 mL conical tube and allowed to settle to the bottom. Next, the DM/KY solution was removed slowly with a 5 mL pipette, and the filtered papain solution was added (6 mL/5 brains). The conical tube was then inverted slowly every 2 minutes for a period of 15 minutes in the solution. The tissue was then allowed to settle to the bottom, and the papain solution was removed with a new 5 mL pipette, and the trypsin inhibitor solution was then added (2mL/1 brain). The tube was then inverted slowly every minute for a period of 5 minutes, and then the tissue was allowed to settle to the bottom. The trypsin inhibitor solution was then removed with a new 5 mL pipette. Next, the tissue pellet was washed with 1 mL per brain

of Opti-MEM/Glucose solution (4 mL of 2.5 M Glucose per 500 mL of Opti-MEM, Opti-MEM: Invitrogen 31985), and the tube was gently inverted several times. The tissue was then allowed to settle, and the Opti-MEM/Glucose solution was removed with a new 5 mL pipette. 5 mL of fresh Opti-MEM/Glucose solution was then added with a fresh pipette, and the tissue mass was gently ground with the pipette tip until the solution became cloudy throughout. Once there was no visible sign of tissue remaining, the cell solution was added to a 50 mL conical tube, and the solution was diluted to 50 mL total volume.

The diluted cell suspension was then briefly mixed, and 10 μ L of cell solution was added to 10 μ L of DM/KY solution in an Eppendorf tube. 10 μ L of Trypan Blue (Sigma-Aldrich T8154) was then added to this mixture, and 10 μ L of the mixture was then pipetted onto a hemocytometer. The number of blue cells (viable) was counted in 16 different squares from two separate regions of the hemocytometer. This total number of cells from the two regions was then averaged, and this number was multiplied by 30,000 to identify the number of cells present per mL of the diluted cell suspension. This number comes from the 1:10,000 dilution of removing 10 μ L from 10 mL of the cell mixture, and the 1:3 dilution completed by adding more media and the Trypan Blue solution. For this step, it is important to recount two new regions of the hemocytometer if the number of viable cells differs by more than 10% between the two regions. The two regions which were counted amassed to 47 and 51 viable cells per 16 squares, which was within the 10% distribution range. The initial concentration was therefore $(49 \text{ cells/region}) \times (30,000 \text{ dilution factor}) = 1.47 \times 10^6 \text{ cells/mL}$.

This diluted cell suspension was then further diluted with Opti-MEM/Glucose so that the final concentration of 1×10^6 cells per mL was achieved, then the entirety of the solution was placed into a T175 flask. After the cell solution rested for two hours in the flask, the Opti-MEM/Glucose solution was removed with an aspiration pipette by placing the flask screw top side up, and aspirating directly off the base which was resting on the working space. The flask was then filled with 50 mL of the astrocyte media which consists of DMEM + GlutaMAX (GIBCO 10569), 10% Fetal Bovine Serum (Hyclone ASJ 30474), and 1% Penicillin-Streptomycin (Sigma-Aldrich P4458). The flask was then placed into a 37° Celsius and 5% CO₂ incubator for 24 hours. After the 24 hour period, the flask was slapped firmly on the side to dislodge any loose cells which are not astrocytes. The media was then aspirated as previously described, and replaced with fresh media. A step by step process can be seen in Appendix C. This flask was then split into new flasks, as described in the next section.

2.2.2 Cell Culture Splitting for Passaging and Device Use

Prior to handling the cells for culturing, the appropriate media for the specific cell line is removed from the refrigerator, and placed into a heated water bath to bring it to 37° Celsius. The media for the Neuro-2-A cells is a mixture of MEM + GlutaMAX (GIBCO 41090) with 10% Fetal Bovine Serum, 5% Non-essential Amino Acids (GIBCO 11360), 5% Sodium Pyruvate (GIBCO 11140), and 1% Penicillin-Streptomycin. The media for the primary astrocyte cells is a mixture of DMEM + GlutaMAX, 10% Fetal Bovine Serum, and 1% Penicillin-

Streptomycin. After warming the media, the surface within the biologic safety cabinet is sprayed with 70% isopropanol then wiped with a paper towel. Next the media bottle, bottle of trypsin-EDTA (Invitrogen 25300), DPBS (Invitrogen 14190), and the new T25 flasks (Thermo Scientific 136196) to be used were wiped with a paper towel saturated with the 70% isopropanol, then placed within the biologic safety cabinet. The flasks of interest were then removed from the 37° Celsius and 5% CO₂ incubator. The base of the flasks and the stage of the inverted microscope were then wiped down with a 70% saturated isopropanol paper towel. The flasks were inspected via the microscope to confirm that a confluent layer of cells had grown across the base of the specific flask. If the flask does not have a confluent monolayer of cells, such as in figure 12, it is not ready to be split and should be returned to the incubator until a confluent layer is visible.

Figure 12 – A 200x image captured with an inverted microscope of a monolayer of N2A cells which has not reached confluency.

After confirmation of a confluent monolayer of cells, the flasks to be split were brought to the biologic safety cabinet. Using a 2 mL aspiration pipet (VWR 414004-265) attached to a vacuum pump and waste collection chamber, the media present in the flask was completely aspirated by resting the flask cap side up as seen in figure 13, and aspirating directly off of the base of the flask which is resting on the work space. After the flask is emptied, 5 mL of DPBS was dispensed into the flask via a 5 mL pipette (VWR 89130-896), directly onto the base still resting on the work space. After the flask was filled with DPBS, the flask was tilted side to side gently to rinse off any residual media, then aspirated away as described previously for the removal of media. Next, 2.5 mL of trypsin-EDTA was added to the flask via a 5 mL pipette, and the flask was again gently rocked side to side for approximately 5 seconds so that the trypsin covers the side of the flask where the cells are attached to the surface. The trypsin was then aspirated as described above, and the flask was set aside to rest for 2 minutes so the bonds between the cells and the flask wall are dissociated.

Figure 13 – The proper method for aspirating fluid from a cell culturing flask, with media resting on the base of the flask away from the monolayer of cells to avoid disruption.

After allowing the flask to rest, the sides of the flask perpendicular to the growth field were slapped vigorously to dislodge the cells from the wall. It is best to confirm that the cells are floating by analyzing the flask with the inverted microscope, but it is also possible to see that they are dissociated from the flask by holding the flask up towards a light and looking for a hazy layer that is slowly free floating – this is the layer of cells. As seen in figure 14, one problem which occurred during the splitting procedure was that some cells would not fully dissociate from each other, and would form small clusters. If this was evident via visualization with the inverted microscope, it was important to allow the trypsin

solution to remain in the flask a bit longer, and to repeat the process of slapping the sides of the flask. With a longer trypsinization time, or with more slaps to the flask (or a combination of both), the cells would no longer form clusters and then become individually isolated.

Figure 14 – A 400x image of astrocytes post splitting, exhibiting a clustering phenotype due to insufficient time in the trypsin solution or a lack of slaps to the side of their flask.

After the cells were confirmed to be dislodged from the surface of the flask, a pipette was filled with the cell's respective growth media, and dispensed over the growth field where the cells are. Either 10 mL or 3.33 mL of growth media was dispensed into the flask, depending on the required cell concentration for testing in the impedance sensing device (discussed later). This media with cells was then aspirated slowly with the same pipette and dispensed over all walls of the flask multiple times, and repeated until all sides of the flask had been washed off. This will ensure that the maximal amount of cells from the flask have

been washed off and collected. Then the flask (with all media and cells) was slowly shaken side to side to mix the cells evenly in the growth media. Next, the entire volume of media (10 mL or 3.33 mL) with the cells was aspirated into the pipette slowly.

After the media with the cells has been aspirated, the mixture may be split into flasks for future use, or into a conical tube for device testing. To split the cells, a new T25 flask was brought into the biologic safety cabinet after being wiped down with a 70% isopropanol soaked paper towel. When using 10 mL of growth media (the standard amount of media used to split a T25 flask), two fifths of the cell and media mixture (4mL volume) was then dispensed into the new flask for a 1:2.5 passaging ratio. When using 3.33 mL of growth media, two fifths of the cell and media mixture (1.33 mL volume) was then dispensed into a new flask, also to accommodate a 1:2.5 passaging ratio. The remainder of the cell media mixture may be split into new flasks if desired. However if it is not required to passage more cells, the remaining volume of the mixture was dispensed into a conical tube, and placed into the incubator temporarily. The use of this tube will be discussed in the next section. The flask with the new passage of cells was then labeled with the day it was split as well as its new passage number. Next, enough fresh media was added to this flask so the total volume within it is 5 mL, and then it was placed into the incubator. A flask which has been split 1:2.5 (two fifths of the previous cell and media mixture) will grow to into a confluent layer in approximately 7-10 days. At day 4 post splitting, the flask had its media removed

via a 2 mL aspiration pipette as previously described, and 5 mL of fresh warmed media added via a 5 mL pipette as previously described.

2.3 Cell Impedance Sensing

To utilize the conical tube that has the remainder of the cells and media, the solution may be diluted further, or analyzed at its current concentration of cells. In an attempt to cover a wide array of testing conditions with the device, three different scenarios were analyzed. The testing conditions were as follows: 1) a 1:10 diluted version of the typical 10 mL (1:2.5) split ratio, 2) a 1x stock concentration of the typical 10 mL (1:2.5) split ratio, and 3) a 3x concentrated version of the typical 10 mL split ratio, as the flask was washed down with 3.33 mL of cell media. The following protocol is identical for both astrocytes and N2A cells, with the only difference being the growth media used for the specific type of cells.

2.3.1 1:10 Diluted Cell Concentration Preparation

For a 1:10 diluted cell concentration, a T25 flask was rinsed with 10 mL of growth media to pick up all cells present in the flask. The leftover media with cells not utilized for passaging was then placed into a conical tube. A 1 mL aliquot of the remaining cell and media mixture was removed with a pipette, and added to a new conical tube. Then 9 mL of DPBS with Calcium and Magnesium (Invitrogen 14040) was added to the tube to dilute it, and 0.08 mL of glucose at 2.5 M was added for a final concentration of 4 mL glucose/ 500 mL DPBS. The

DPBS with the Calcium and Magnesium is added to the new solution (as opposed to plain DPBS) to help retain cell membrane functionality when in a low concentration setting of growth media. The glucose was supplemented to provide some energy for the cells as they are out of the incubator without their growth media for a sufficient amount of time. After the diluted cell mixture was prepared, it was brought to the device for use. The original conical tube with the media and cells was placed in the incubator until testing was completed. When the first aliquot of cells is exhausted, a new one was prepared as previously described, then used for scanning.

2.3.2 1x Stock Cell Concentration Preparation

For a '1x' stock cell concentration, a T25 flask was rinsed with 10 mL of growth media, as previously described for the 1:10 diluted cell concentration. The leftover media with cells not utilized for passaging was then placed into a conical tube. An aliquot of the remaining cell and media mixture (volume depending on the number of samples required for recording, ranging from 1-3 mL) was added to a new conical tube, and brought to the device for testing. The original conical tube with the media and cells was placed in the incubator until testing was completed.

2.3.3 3x Stock Cell Concentration Preparation

For a '3x' stock cell concentration, a T25 flask was rinsed with 3.33 mL of growth media to pick up the cells from the flask. This was completed to

concentrate the cells in the media by three times the normal amount. The leftover media with cells not utilized for passaging was then placed into a conical tube. An aliquot of the remaining cell and media mixture (volume depending on the number of samples required for recording, ranging from 0.5-2 mL) was added to a new conical tube, and brought to the device for recording. The original conical tube with the media and cells was placed in the incubator until testing was completed.

2.3.4 Computer and Microscope Setup

When inside of the ATL, the two Biofluidics computers which power the NVIDIA controllers and Microscope #1 were turned on. Microscope #1 was then turned on. On the NVIDIA controller computer, the 'Single Cell Impedance Files (SH)' folder was opened, and 'Impedance Generator Final' was opened. This opens a LabView8 program written by Stephanie Hernandez (Cal Poly 2009) which powers the impedance generator. The layout and displays present in the program are seen in figure 15. Inside of the LabView program, the Function Generator Resource Name was set to 'FGEN', the amplitude of the waveform was set to 0.2, and the Source Resource Name was set to 'SCOPE'. The Minimum Sampling Rate was left unchanged at 5,000,000. On the computer which powers Microscope #1, the program uSCOPE was opened. The video was set to 'Live Feed' unless the display automatically showed the view of the microscope.

Figure 15 – An image of the impedance sensing program home screen, written by S. Hernandez, which is used to record impedance values.

After the computers were set up, two Harvard Apparatus 11 Plus syringe pumps were plugged in, and set for use. The rate of the pumps was set to 0.055 ml/min for filling the device, the volume was set to the total volume of the media and cell mixture to be used (ranging from 0.5 to 3 mL), the configuration was set to 'Units ml/min', and the diameter was set to 8.66 mm to accommodate for the 3 mL BD syringes (BD 309570) which were used. A piece of tygon tubing with a syringe tip for the 3 mL BD syringes was placed into both the inflow and outflow ports, and then attached to a syringe. As the depth of the PDMS layer which was produced for device was relatively thin, the holes which were designed to accept the tygon tubing were somewhat difficult to work with. Inserting the tubing into the holes was difficult, and in multiple cases, the tubing would fall out, causing a

leakage of fluid over the device. To alleviate this problem, the tubing was taped to the microscope or to a conical tubing rack as to minimize the possible motion and dislodging of the tubing. Another method which was explored to improve the connection between the tubing and the device was cutting the end of the tube which was to be inserted at different angles, but this did not seem to play a large factor into the success of attachment. As the secondary inflow port was not utilized for this experimentation, it needed to be sealed so that the fluid would not be diverted towards it as it provided the path of least resistance. Two methods were employed to stop leakage of fluid through this port. One method was inserting a third piece of tygon tubing with a clipped/sealed end, but this proved difficult much like the previously described inflow and outflow tubing insertion. A second method which was used was to cover the hole with a piece of sticky poster-wall tape. This provided a decent seal on the device when low flow rates on the syringes were used (< 0.050 mL/min), but with higher flow rates, some leakage was visible. The microscope was then moved to center on the electrodes inside of the device, and brought into focus. Then wire leads were attached to the cables that are connected to the NVIDIA controllers. The cable labeled with orange tape is to be attached to the left side of the device and the phasor resistor. The cable labeled with red tape is to be attached to the right side of the device, and is the function generator. Finally, miniaturized alligator clip-wiring sections were attached from the wire leads to the corresponding wire on the device. The wires protruding straight from the wire lead setup were attached to the electrodes leading into the device, and the wires protruding perpendicularly

were attached to the grounded segment of the device. The device setup can be seen in figure 16.

Figure 16 – A close up view of the device during a recording session, with the outflow tube on the left, the plugged tube in the middle blocking the unused port, and the inflow tube on the right.

2.3.5 Device Preparation, Testing and Cleanup

When the entire setup was ready, the inflow syringe was filled with 1mL of trypsin, and then flushed through the entire system manually and allowed to rest for 5 minutes. This was completed as debris was typically found left over in the device from the previous recording session, and as a precautionary step to fully clean the inside of the channel. This process cleans the device of any protein bonded material (i.e. cells, growth media) by cleaving protein bonds. Next the

device was manually evacuated of all trypsin and poured into a flask. The inflow syringe was then filled with distilled water, and the device was manually flushed with the water. This liquid was then manually evacuated, and discarded into the same flask. The syringe was then filled with 1 mL of the media specific to the cell type being tested, and the device was manually filled as seen in figure 17. When the media had settled, its impedance was recorded by running the impedance generator program. After the recording was completed (1 minutes 15 seconds per complete scan), the files for the media were recorded in the following order: 1) Voltage Out, 2) Phase, 3) Magnitude, 4) Frequency. The media was recorded multiple times to assure normalization of data.

Figure 17 – Screen shot of media within the microfluidic device during impedance recording.

After the growth media impedance values were recorded, the inflow syringe was emptied, and filled with the media and cell mixture. The syringes

were then attached to the syringe pumps, and both were run at 0.055 ml/min until the device was filled with the media and cell mixture. The syringe pumps were then shut off to allow for the fluid to stop flowing inside of the device. When the target cell or cells were seen between the electrodes, the impedance scan was completed, and then the subsequent files were saved as described above. The syringe pumps were then set to 0.005 ml/min to move along the fluid to focus a new cell or field of cells over the electrodes. After one sample had been recorded, the syringe pumps were activated until the new sample arrived for scanning, where they were then shut off. To assure an equal concentration of cells in the media, the syringe was turned over in the syringe pump, and new samples of media with cells were brought into the syringe at evenly spaced intervals.

There were various difficulties regarding the recording of cell impedance values. A primary concern which was noted early on in the process of recording was that it was difficult to isolate a single cell between the electrodes for various reasons. One reason for the difficulty was the fact that the device was not completely sealed, and even through precise manipulation of the syringe pumps, the fluid within the device would continue to slowly move even with both syringe pumps shut off. The flow would eventually stop, but it could take up to a minute (depending on the previously set speed of the syringe), and there was no guarantee that a single cell would settle between the electrodes. To accommodate for this difficulty in isolating and recording a single cell, the testing of cells in media at the 1x and 3x concentrations was completed. These tests did

not isolate a single cell, but made the process of isolating a section of the media with an evenly dispersed amount of cells much easier. Another reoccurring difficulty in the recording process was the formation of an air bubble just upstream of the electrodes. Due to the curving geometry of the channel, when the syringe was run at certain speeds, any air bubbles which were introduced into the device through the tygon tubing would accumulate. As seen in figure 18, when the syringes were turned back on to dispense the cell mixture, the air bubble or a portion of the air bubble would flow over the electrodes and disrupt the ability to scan. To fix this problem, the syringe pumps would be set to a high dispensing speed (> 0.100 mL/min) and run until the air bubble was fully dislodged and evacuated into the outflow syringe.

Figure 18 – Screen shot during a recording session of an air bubble flowing from right to left over the recording electrodes.

After recording was completed, the device was manually evacuated of all fluid into one of the syringes. This fluid, along with any unused media with cells, was added to the previously mentioned waste flask. The device was then manually filled with trypsin as previously described and allowed to rest for 5 minutes. Next, the trypsin was manually evacuated and added to the flask. The device was then flushed with distilled water two complete times to remove any remaining debris. This fluid was then added to the flask. An equivalent volume of bleach was then added to the fluid in the flask, and let to rest for 10 minutes. This process kills any viable cells within the fluid. This mixture was then poured down the drain, and all utilized glassware was washed with 70% EtOH or isopropanol and dried. All syringes and tubing which were used were flushed with water and allowed to air dry, along with the device.

2.3.6 LabView File Access

As previously mentioned, after the device completes its recording cycle, the files were saved in the order of 1) Voltage Out, 2) Phase, 3) Magnitude, and 4) Frequency. These files all appear as a 'blank' file type, and can only be opened with OpenOffice.org. To view the content of the files, the OpenOffice.Org Writer program was opened, and the specific file to be analyzed was selected. It was a somewhat common occurrence for the impedance data file to be saved as in one of the other files (i.e. a file saved as 'Phase' would contain the impedance data for one scan). When this occurred the data would

be swapped between the two files. Why this was occurring was never determined. After opening the file of interest with the OpenOffice.Org Writer program, the data was displayed in a Word type document that is tab separated. The data was then copied and pasted into the OpenOffice.Org Calc program (Excel style), as 'Paste Special' was chosen, followed by 'Unformatted Text'. After this was completed, the data was separated into individual cells which are capable for further manipulation.

3 Results

3.1 Fabrication Results

After completing the fabrication of the device at the UCSB Nanofabrication facility, the device was returned to Cal Poly for preliminary testing. Although the design for the device had envisioned the electrodes in the lower area of the microfluidic channel, during the manual process of pressing together the PDMS and the glass, the recording region of the electrodes ended up directly in the middle of the channel, which can be seen in figure 19. This ended up being beneficial, as the passage of cells through the channel was more consistent in the middle of the channel, as opposed to the boundaries. Prior to any impedance measurements being recorded, the device was tested for water tightness, as well as the ability to handle flow of fluid without disrupting the PDMS-glass bond. The device was flushed with water, and there were no evident disruptions between the two material layers, and it did not leak any fluid. The device accepted water from the syringe pumps up to 1 mL/minute with no visible damage. There were a few very small chunks of the PDMS which could be seen flowing through the device at random times, but these pieces were likely from the edges that had been in contact with the mold, and were slightly torn when the PDMS was removed for application to the glass base. These pieces of PDMS were either flushed from the system, or congregated in the corners of the device, where they did not affect flow or recording.

Figure 19 – Screenshot captured during recording of cells, which highlights the recording position of electrodes in the middle region of the microfluidic pathway.

3.2 Cell Culturing and Transportation

The murine N2A cells were brought up successfully from a -80° C freezer, and were plated out onto fresh flasks. These cells were in good condition, and grew up successfully to confluency within the time period of 7-10 days. These cells were passaged over a period of 5 weeks without any adverse effects.

These cells are immortalized, and therefore would continually split and grown unless there was a lack of media. The fresh murine primary astrocytes were successfully harvested, and attached well to the flasks on which they were plated onto. These cells also grew to confluency within the stipulated time period of 7-10 days, without any adverse events. These cells were passaged over a period

of 5 weeks up to a passage level of 4, at which there was evidence of the cells becoming insufficient for further splitting. The astrocytes at a 7 days post splitting into passage 4 can be seen in figure 20, where the cells are noticeably losing their ability to grow to confluency.

Figure 20 – A 400x image of astrocytes 7 days post splitting into passage 4 captured with the inverted microscope. The lack of confluency highlights the effects of long term passing of the mortal cell line.

All cells were initially brought up or harvested at The Gladstone Institutes at UCSF in San Francisco, and then had fresh media added to their flasks for the trip down to San Luis Obispo. The flask caps were covered with paraffin plastic to prevent gas exchange, and the flasks were placed into a foam box to retain some heat. Upon arrival to Cal Poly, the cells had their excess media removed so that 5 mL remained per T25 flask, and were placed into the 37° C and 5% CO₂ incubator. Each cell line survived the 3 and a half hour drive, and continued

to grow normally once on campus. By 7-10 days post splitting (on the day of transportation) the cells grew up to a confluent layer, suggesting their viability.

For both the astrocyte and N2A cell lines, the base of the T25 flasks would be covered with a monolayer of cells at approximately 7-10 days post splitting, as long as the flasks were fed once at 3-5 days post splitting. As seen in figure 21 C, the N2A cells grow into a 'cobble-stone' formation, with the originally free floating circular cells stretching out and attaching to the base of the flask. The cells were confirmed to be contact inhibited, so at the point when the cells divided enough to fill the flask, they would stop their growth period. This is the time when the cells were split for passaging, and were also isolated for recording. Utilizing the cells at this period most likely limits any natural variability in impedance, as the cells are no longer undergoing cell division, so all the cells present in the flask are releasing minimal growth factors. Also seen in figure 21 A, the astrocytes grow in a different manor than the N2A cells, as they elongate as they attach to the flask base, and produce slight projections. These cells have a bulb shaped appearance at the center of the cell, and when trypsinized for splitting they regain their circular shape.

Figure 21 – Inverted microscope images of N2As & astrocytes prior to and post splitting while in T25 flasks. (A) A confluent layer of astrocytes prior to splitting with visible projection attachments. (B) Isolated astrocytes at the 1x concentration post splitting in their balled up form. (C) A semi-confluent layer of N2A cells prior to splitting exhibiting 'cobblestone' attachment to the flask base. (D) Isolated N2A cells at the 1x concentration post splitting in their balled up form. All images captured at 200x.

3.3 Cell Impedance Sensing

As previously described in the methods, when each type of cells was split for passaging a portion of the cells in media mixture was saved and placed into a conical tube for testing. The initial protocol which was tested was to take the 'stock' cells in media mixture (one confluent T25 flask rinsed with 10 mL of media after the monolayer of cells was trypsinized), then dilute this mixture 1:10 with DPBS (plus Magnesium and Calcium). One mL of 'stock' cell mixture was diluted

with 9 mL of the DPBS, and supplemented with 80 μ L of glucose. This 1:10 diluted cell mixture, as well as a separate flask of 1:10 diluted cell specific media was then taken to the device for recording. Prior to the recording of the cells in media, the device was filled with the specific diluted media. This fluid had its media recorded at 0.2 program amplitude from a frequency of 1096 Hz up to 42.77 MHz (75 frequency steps), and it produced an exponential curve which was previously found during the LabView program creation testing by S. Hernandez. The media was recorded 5 separate times at the same voltage, and it was determined that this voltage was acceptable for recording as the impedance values followed the same pattern of exponentially decreasing and the standard errors produced were relatively small. The recorded impedance values of the 1:10 diluted N2A media ranged from 34 K Ω at 1096 Hz to 7 K Ω at 42.77 MHz. The recorded impedance values for the 1:10 diluted astrocyte media ranged from 34 K Ω at 1096 Hz to 3.6 K Ω at 42.77 MHz.

After each set of diluted media was scanned, an air bubble was introduced into the tube leading from the syringe which was filling the system to identify where the transition from media to cells and media would occur. The syringe was then filled with the 1:10 diluted cells in media mixture, and successfully introduced into the device. Under visual inspection by the inverted microscope in the laboratory, it was evident when cells had entered the channel. Although the N2A and astrocytes are approximately the same size, it was more difficult to see the astrocytes. This was also noted when analyzing the flasks for confluency with the standard inverted microscope, as the N2A cells were a slightly darker

shade and the astrocytes were more transparent. During the introduction of the cells in media, it was difficult to immediately cease the flow of cells in the media as the entrances to the device via the syringe tubing were not ideally sealed. Therefore the fluid was pushed slowly through the device at a rate of 0.005 ml/min to gently move along the cells over the electrodes. It was fairly difficult to isolate single cells in this scenario however, so when recording occurred, there were instances when a single cell was not the only one being recorded between the electrodes.

During the time period of impedance recording, 50 samples of each cell type at a 1:10 dilution was completed. As expected the LabView graphical output of frequency versus impedance values seemed to signify that the diluted media which contained cells appeared to be higher on average than diluted media on its own. After the 50 samples of data were collected for both N2A and astrocytes, the actual impedance recordings were averaged. The recorded impedance values of the 1:10 diluted N2A cells in media ranged from 32 K Ω at 1096 Hz to 6.6 K Ω at 42.7 MHz. The recorded impedance values of the 1:10 diluted astrocytes in media ranged from 42 K Ω at 1096 Hz to 3.6 K Ω at 42.77 MHz. The values followed the same exponentially decreasing pattern as described earlier for both cell types. Although the impedance values produced at the initial (1096 Hz) and final (42.77 MHz) values are not indicative of any of the intermediate recording frequency values, the fact that they were so close to the impedance values of the diluted media coupled with the fact that single cells were difficult to isolate, further testing was required.

Following the splitting protocol previously described in the methods, the two cell lines were split and washed from their T25 flasks at the standard 10 mL volume, as well as with a 3.33 mL volume of media. The split of each cell lines with the 10 mL volume was designated the '1x stock' solution of cells in media, and this was tested in the device without any dilution with DPBS. Prior to these tests, 5 samples of stock media for the respective cell type were recorded. The split of each cell lines with the 3.33 mL volume was designated as the '3x stock' solution of cells in media, and these were also tested (also versus the stock media for the respective cell type). When these mixtures of cells in media were introduced to the device, it was visually evident that there were much more cells, as would be expected. As it was difficult to isolate a single cell for recording with the 1:10 dilutions, these concentrations helped to move an equally concentrated volume of fluid in between the electrodes for recording numerous times. This testing method is similar to the procedure completed by S. Hernandez and J. Fadriquela, where they isolated a dense concentration of cells in between electrodes. The '3x' cells in media produced greater differences between media and cells in media than the '1x', for example as the '3x' N2A media impedance values ranged from an average of 21 K Ω at 1096 Hz to 2.6 K Ω at 42.77 MHz and the '3x' N2A cells in media impedance values ranged from 25 K Ω at 1096 Hz to 3.4 K Ω at 42.77 MHz.

Although there were evident differences in impedance between the media and the cells in media for the various concentrations at the initial and final frequencies, the most important thing analyze was if there were differences

across the wide frequency range. When viewing the data, it was evident that there was the possibility of statistical significance between the media and cells in media mixture at certain frequencies, but not at all frequencies. Also, identifying specific frequencies within the α dispersion range (~ 10 Hz to 10 KHz) and the β dispersion range (~2 KHz to 1 MHz) to identify the membrane and cytosol impedances of neuronal cells was of great importance. To complete this, statistical analysis was completed on the recorded data.

3.4 Statistical Analysis

As previously mentioned, there were some potential differences between the cells in media mixtures and their respective media at various frequencies. It was also evident that at certain frequencies, there were no differences between the two groups. After organizing all of the impedance data from the 1:10, 1x, and 3x concentrations of astrocytes and N2A cells along with their respective media, the data was analyzed. The experimental units for the study are the cells in media mixture for each respective concentration. The measurement units are the impedance values which were recorded from the cells in media mixture at the various frequencies. Due to the nature of the device and the visualization capabilities of the microscope, the study could not be completed as a blinded study. There were 5 recorded impedance spectrum replicates for each media control, and 50 recorded impedance spectrum replicates for each cell in media mixture at each of the three concentrations. There were no recording errors

which occurred during the experimentation process, therefore no data points were excluded as outliers.

For each data set, a two tailed T-Test with equal variances was completed comparing the cells in media value and the respective media value for each recorded frequency, compared at a predetermined P-value of 0.1. Each frequency which produced statistical significance between the cells in media and its respective media was noted for further analysis. The frequencies which recorded significant differences between the cells in media mixture and its respective media can be seen in Table 1.

Table 1 – Frequencies from scans at various concentrations which demonstrated significance between the cell mixture and its respective media are marked with an X ($P < 0.1$). Further explored data sets contain their respective P values.

	N2A Cell Mixtures			Astrocyte Cell Mixtures		
Frequency (Hz)	1:10x Dilution	1x Conc.	3x Conc.	1:10x Dilution	1x Conc.	3x Conc.
1096.633				x		
1459.303	x					
1683.401	x					
1941.912		x				
2240.122	x					
2584.127			x			
2980.958			x			
3966.797			x	x		
7024.384			x	x		
8103.084		x	x			
9347.434			x			
10782.873	x		x	0.26		0.29
12438.744			x			
19094.256			x			
25408.96			x			
29310.887			x			
33812.013	x		x			
39004.354	x					
44994.058			x			
59874.142			x			
69068.714			x			
141088.53			x			

288205.36	x					
332463.62	x					
903729.81	x	x		0.27		
1042511.1	x					
2456592.3	x					
8886110.5						x
10250706						x

3.4.1 10782 Hz Frequency Comparison

After completing T-Tests between the two groups for the three separate concentrations, it was evident that the frequency of 10782 Hz was a good candidate for late range α dispersion or early range β dispersion comparison between the two cell type's membranes or cytosol, respectively. At this frequency, the P value between the 1:10 N2A cell mixture and its respective diluted media was 0.0074, and at the P value between the 3x N2A cell mixture and its respective media was 0.0857. However, at this frequency the P value between the 1x N2A cell mixture and its respective media was 0.5434. Due to the two cases of significance which were found for N2A samples, the T-Tests of the astrocytes for 1:10 dilution and 3x concentration were further scrutinized for further testing at this frequency. At the frequency of 10782 Hz, the P value between the 1:10 astrocyte cell mixture and its respective diluted media was 0.2629, and the P value between the 3x astrocyte cell mixture and its respective media was 0.2991. Although these two values were not within the predetermined significance level of 0.1, they exhibited a noticeable trend that the cell and media mixture produced a larger impedance than the respective media, which can be seen in figure 22. This circumstance, plus the fact that the P value produced between the astrocytes in media mixture and its respective media was greater

than 0.45 for most other frequency samples lead to its continued analysis. Therefore the data produced related to these samples was further compared to the N2A data.

Figure 22 – LabView generated impedance spectra of 1:10 diluted astrocyte mixture & 1:10 diluted media. Select LabView raw data points at various frequencies comparing astrocytes in media at a 1:10 dilution (red) and astrocyte media at a 1:10 dilution (black). * Significantly different from 1:10 diluted astrocyte media value ($P < 0.05$). Data represent means $\pm$ S.E.M. Cell solution sample sizes $n = 50$.

After the T-Tests between the cell mixtures and their respective medias were completed, two different analyses were completed to compare the cell types at the 10782 Hz frequency. First the media impedance values for the respective groups (Astrocytes 1:10 & N2A 1:10; Astrocytes 3x & N2A 3x) were averaged to produce background impedance values. The correlating

background value was then subtracted from each of the 50 samples for each cell type and specific concentration group (1:10 or 3x). The average impedance value with background subtracted of astrocytes at 1:10 dilution was 4342 Ω , and the average impedance value with background subtracted of N2A cells at 1:10 dilution was 10685 Ω . The average impedance value with background subtracted of astrocytes at a 3x concentration was 1260 Ω , and the average impedance value with background subtracted of N2A cells at a 3x concentration was 4937 Ω . A two tailed T-Test with equal variances was then completed comparing these impedance values for both cell types at a 1:10 dilution, as well as at a 3x concentration. The P value between the cell types at a 1:10 dilution was 0.000163, suggesting that there are differences between the cell types at 10782 Hz. The P value between the cell types at a 3x concentration was 0.000978; also suggesting that there are differences between the cell types at this same frequency, where the N2A cells having higher impedances at both concentrations. Although both sets of data suggest the N2A cells have a higher impedance value than astrocytes at these specific concentrations, as seen in figure 23, the impedance values recorded seemed counterintuitive, for example as the average impedance for N2A cells at 1:10 dilution was vastly greater than that of N2A cells at a 3x concentration. A two tailed T-Test with equal variances confirmed the statistical differences between each cell type's two analyzed concentrations, as the P value comparing the two different concentrations of N2A cells was 0.000345, and the P value comparing the astrocytes was 0.010.

Figure 23 – LabView generated data of N2As & astrocytes impedance accounting for background impedance. (A) LabView raw data recording of a cell solution impedance spectra. (B) 0.9 MHz average impedance values of N2A and astrocyte cells at a 1:10 dilution with respective average background impedance subtracted out. (C) 10.8 KHz average impedance values of N2A and astrocyte cells at a 1:10 dilution with respective average background impedance subtracted out. (D) 10.8 KHz average impedance values of N2A and astrocyte cells at a 3x concentration with respective average background impedance subtracted out. * Significantly different from N2A value ($P < 0.05$). Data represent means $\pm$ S.E.M. Cell solution sample sizes $n = 50$.

Although the outcome was consistent regarding the larger impedance value of N2A cells at 10782 Hz, the relatively large difference between average impedance values with background subtracted out warranted further analysis. For this second set of analyses, the percentage increase of cells in media impedance over the media impedance values was completed. Each cells in media sample impedance value was subtracted by the respective background impedance average value, and then this number was divided by the respective

background impedance average value. This yielded the percentage increase in impedance over the media, as completed by the following example equation:

$$\frac{(3x \text{ N2A Impedance} - \text{N2A Media Impedance})}{(\text{N2A Media Impedance})} = \% \text{ Impedance Above Background}$$

The average percentage increase of impedance over background for astrocytes at 1:10 dilution was 11.12%, and the average percentage increase of impedance over background for N2A cells at 1:10 dilution was 49.45%. The average percentage increase of impedance over background for astrocytes at a 3x concentration was 7.69 %, and the average percentage increase of impedance over background for N2A cells at 3x concentration was 45.37%. A two tailed T-Test with equal variances was then completed comparing these impedance values for opposing cell types at a 1:10 dilution, as well as at a 3x concentration. The P value between the opposing cell types at a 1:10 dilution was 9.914×10^{-9} (~0.0), suggesting that there are differences in impedance between the two cell types at 10782 Hz. The P value between the cell types at a 3x concentration was 0.000166; also suggesting that there are differences in impedance between the two cell types at this same frequency. The tests suggest that the N2A cells have a greater percentage increase over background impedance at both concentrations. After completing the T-Test comparing the different cell types percentage increase in impedance over background at the same concentration, a two tailed T-Test was completed between the percent increase over background values between the N2A cells at 1:10 dilution and 3x concentrations, as well as between the astrocytes at these concentrations. The P value comparing the

astrocytes at differing concentrations was 0.3395, and the P value comparing the N2A cells was 0.7084. As seen in figure 24 D this data suggests that there is no difference in percentage increase of impedance over background for the cell types at differing concentrations, validating this comparison method (as opposed to comparing straight impedance values with media impedance values subtracted out).

Figure 24 – LabView generated data of N2As & astrocytes percentage increase of impedance above background. (A) 10.8 KHz average percentage increase over background impedance values of N2A and astrocyte cells at a 1:10 dilution. (B) 10.8 KHz average percentage increase over background impedance values of N2A and astrocyte cells at a 3x concentration. (C) 0.9 MHz average percentage increase over background impedance values of N2A and astrocyte cells at a 1:10 dilution. (D) 10.8 KHz average percentage increase over background impedance values comparing like cell types between 1:10 dilution and 3x concentration. * Significantly different from N2A value (P < 0.05). Data represent means $\pm$ S.E.M. Cell solution sample sizes n = 50.

3.4.2 0.9 MHz Frequency Comparison

After completing the T-Tests between the two groups for the three separate concentrations, it was evident that the frequency of 903729 Hz (0.9 MHz) was also a candidate for a comparison between the two cell types. This frequency would fall into the β dispersion range, and measure the frequency of the cell's cytosol and internal components. At this frequency, the P value between the 1:10 N2A cell mixture and its respective diluted media was 0.0284, and at the P value between the 1x N2A cell mixture and its respective media was 0.0211. However, at this frequency the P value between the 3x N2A cell mixture and its respective media was 0.6974. Due to the two cases of significance which were found, the T-Tests of the astrocytes at this frequency were further scrutinized for further testing at this frequency. At the frequency of 0.9 MHz, the P value between the 1:10 astrocyte cell mixture and its respective diluted media was 0.2742. Although this value was not within the predetermined significance level of 0.1, the data exhibited a noticeable trend that the cell and media mixture produced larger impedance than the respective media. This circumstance, in accordance with the fact that the P value produced between the astrocytes in media mixture and its respective media was greater than 0.40 for most other samples in the β dispersion range of frequency samples lead to its continued analysis. Therefore the data produced related to these samples was further compared to the N2A data.

After the T-Tests between the cell mixtures and their respective medias were completed, two different analyses were completed much like the samples

recorded at the frequency of 10782 Hz. First the media impedance values for the respective groups (Astrocytes 1:10 & N2A 1:10) were averaged to produce background impedance values. The correlating background value was then subtracted from each of the 50 cells in media samples for each cell type and concentration group. At the frequency of 0.9 MHz, the average impedance value with background subtracted of astrocytes at 1:10 dilution was 2660 Ω , and the average impedance value with background subtracted of N2A cells at 1:10 dilution was 3500 Ω . A two tailed T-Test with equal variances was then completed comparing these impedance values for both cell types at a 1:10 dilution. The P value between the cell types at a 1:10 dilution was 0.339, suggesting that there is no difference between the cell types at 0.9 MHz.

Although the T-Test comparing the cell impedance values with the background subtracted out did not yield statistically significant data, the previous findings at 10782 Hz regarding percentage increase in impedance over background warranted further analysis. For this second analysis, the percentage increase of impedance over the media impedance values was completed. Each media sample impedance value was subtracted by the respective background impedance average value, and then this number was divided by the respective background impedance average value, as described in the previous section. This yielded the percentage increase in impedance over the media (background). The average percentage increase of impedance over background for astrocytes at 1:10 dilution was 19.81%, and the average percentage increase of impedance over background for N2A cells at 1:10 dilution was 30.58%. A two tailed T-Test

with equal variances was then completed comparing these impedance values for both cell types at a 1:10 dilution. The P value between the cell types at a 1:10 dilution was 0.120 suggesting that there is no difference between the cell types at 0.9 MHz, which can be seen in figure 24. Although significance was found at the 10782 Hz frequency level, there was no significance found at the 0.9 MHz level.

4 Discussion

4.1 Device Creation, Use, and Associated Difficulties

As a principle component of the completion of this thesis, manufacturing a functional microfluidic device for cell introduction and electrochemical impedance analysis was of the utmost importance. Lightly based upon the devised design and using the instated manufacturing methods of an earlier generation EIS device completed by S. Hernandez and J. Fadriquela for Dr. David Clague, the microfluidic device was successfully created with few fabrication challenges. Aside from the somewhat difficult orientation of the electrodes during device bonding, the device was produced overall with great success – utilizing low cost materials and methods, and a short production time.

To show the capability of the device and justify its (or like devices) use for future cancerous diagnostic testing, two cell lines were grown in tissue culture for analysis. The two cell lines assessed were glio/neuroblastomas (N2A) which are a type of neural cancer cell, and astrocytes which are a type of primary support cell for the entire central nervous system. Both of these cell lines were grown *in vitro* in plastic flasks, and were identified to have relatively similar sizes and growth periods. These cell types provided a good comparison between cancerous and non-cancerous cells for this thesis as both lines are readily accessible, and they also provided a new field of analysis for EIS as central nervous system cancers had not previously been analyzed via this method. During the preparation of the cell lines for scanning, the largest reoccurring problem which occurred was clustering of cells. If the cells were not allowed to

sit in the trypsin solution long enough (to dissociate their protein bonds), or if the sides of the flask were not hit firmly, the cells would dissociate from the base of the flask – but remain connected in small groups. This problem was easily identified and solved however, as each flask which was being prepared for splitting was analyzed with an inverted microscope, and if necessary more trypsin solution could be added, or the flask could be hit again to shear the cells apart.

Although the device fabrication process was completed with little problems and limited required troubleshooting, the operation of the device provided more difficult. The first main problem associated with the device setup was the connection of the tubing from the syringes to the PDMS. In the initial process of pouring the PDMS to create the microfluidic channel, an insufficient amount of liquid PDMS was poured over the mold prior to bake curing to create a thick enough layer for stable tubing insertion. The thickness of the PDMS ended up being around 1 mm, whereas a PDMS layer of approximately 3-5 mm would have provided greater support for the tubing to prohibit it from falling out of the device. Although the tubing would fit snugly into the holes in the PDMS, there were times during set up or experimentation when the tubes would fall out and leak fluid across the device. To help support the tubing and reduce the stress on the PDMS layer of the device, the tubing was taped onto the microscope surface or onto a centrifuge tube rack. This helped to alleviate the problem, but it was not always a fail proof method of connecting the device to the syringe pumps. Another problem which arose during device setup was the method to block off

the third unused port. Two methods were used to attempt to seal the device, the first by placing a sealed tygon tube into the hole, and the second option being placing a piece of wall-poster tape (sticky pad) over the hole. As previously mentioned, placing the tubing into any of the holes and stopping them from falling out was difficult enough, so using the sticky pad was explored as a second option. Although this method provided a decent seal of the device when the fluid was being pumped through at relatively low speeds (< 0.050 mL/min), if the flow rate was increased it was susceptible to leaks. The fact that the sticky pad could not provide a perfect seal over the hole meant that it was difficult to completely control the flow through the device. There were various occasions during scanning when fluid would revert its direction to flow out the path of least resistance (if a leak had occurred through the sticky pad), and that the flow would not stop immediately when the syringe pumps were shut off. Also during recording of the cells, at various points particles would stick to the glass near or between the electrodes. Air bubbles were another somewhat consistent problem, as they would form and stop over the recording region. To alleviate both of these problems, it was most efficient to turn up the flow rate on the syringe pumps to a relatively high speed (> 0.100 mL/min) to pass them across the recording region and out the outflow tube.

A final noted difficulty associated with the device was the ability to isolate single cells between the electrodes for recording. At the 1:10 dilution from the 'stock' solution of cells the ability to halt one cell between the electrodes without the presence of another few cells was extremely difficult with the limited control

of the device. This was not due to the cells clustering, but the fact that cells were found interspersed throughout the mixture in a relatively consistent manner. Therefore, the dilemma to further dilute the cell mixture or to 'concentrate' the amount of cells was reached. As it was somewhat difficult to identify the presence of the cells with the microscope in the lab, it was deduced that if further dilutions were completed, there would be a significantly increased risk of recording the impedance of the media alone – as opposed to single cells. Thus, to provide diversity in the testing methodology and to provide more results to compare, two sets of samples at the '1x stock' concentration and a '3x' concentration were scanned. At these concentrations of cells, there is relatively little area within the fluid which is not populated with cells. Therefore, when these concentrations were scanned for their impedances, the scans were not completed through a single cell, but rather an equally concentrated field of cells.

Although normalizing the impedance data was the only manipulation which was completed between the cells and the media, in reality the electrodes also have an effect on the impedance measurements which has to be considered. While it is assumed that the cells are in series between the electrodes when the current passes through them, the phase of the AC sweep can effect this impedance magnitude. If the phases are equal for the scans of the cells in media and their respective media, this added impedance does not need to be accounted for in the calibration of data. It can be assumed that the cells in media and their respective media's differences in phases are statistically insignificant, due to the fact that the suspension of the cells in media is so dilute

for all conditions. Therefore the additional impedance caused by the electrodes is not included for the data calibration.

4.2 Statistical Analysis and Data Interpretation

At the default setting used for scanning, the LabView program completed a sweep from 1096 Hz up to 42.77 MHz in 75 separate frequency intervals. The scans produced a decreasing exponential curve consistently for all samples recorded. Prior to the recording of each sample of interest, the respective cell's media at the proper dilution had its impedance recorded a total of 5 times. After this was completed, 50 samples of the cells in media were scanned. As previously described, each cell type was scanned at three separate concentrations: 1:10, 1x, and 3x. After the data was collected, it was analyzed in an attempt to identify target frequencies for comparisons either within the α dispersion or β dispersion ranges. Within the α dispersion range (~10 Hz to 10 KHz), the current is being passed around the periphery of the cells of interest, providing feedback on the impedance of the membrane [21, 22, 25, 30]. Within the β dispersion range (~2 KHz to 1 MHz), the current flows through the membrane channels and through the cytoplasm and internal components of the cells of interest [21, 22, 25, 30].

Prior to completing any comparisons between the two cell types, the recorded impedance values of the cells in media and the respective media were tested for differences with a two tailed T-Test at a P value of < 0.1. There were numerous 'hits' ($P < 0.1$) for various frequencies of the three concentrations of

cells, but few situations of a 'hit' frequency across two or more of the 6 total samples. Two frequencies of interest which were further analyzed were 10782 Hz, and 0.903 MHz, as they contained two 'hits' of significantly different cells in media impedance values versus their respective media mixtures.

At the 10782 Hz frequency, the impedance values of N2A cells at 1:10 dilution and 3x concentration were significantly different from their respective medias. Although the astrocytes at these concentrations did not exhibit significance, the data showed a definitive trend with the cells in media mixture having a larger impedance value than the respective media. Therefore analysis of these samples continued for further comparison versus the N2A values. The first method used to analyze the data at this frequency was to subtract out the media impedance value (background) from all of the samples, and then compare versus the other cell type (N2A vs. astrocytes at 1:10, and N2A vs. astrocytes at 3x). It was found that the N2A cells in media exhibited statistically significant larger impedance values than the astrocytes in media for both the 1:10 dilution and the 3x concentration. The average impedance values at the respective concentrations can be seen in table 2, along with the P value comparing the N2A cell and astrocytes. However, the average impedance with background subtracted out of both the 3x concentration samples was lower than that of their respective 1:10 dilutions, which seemed counter intuitive. Therefore the data was analyzed by another method to calculate the percentage increase of impedance above its own background level. This method also provided that N2A cells had statistically significant larger impedance values (over background) than

the astrocytes. These values can also be seen in table 2. To further justify the use of percentage increase above background for cell type comparisons, the percentage increases over background of N2As at 1:10 dilution versus 3x concentration were completed via a two tailed T-Test, as well as between astrocytes at 1:10 dilution versus 3x concentration. The averaged values were relatively close as seen in figure 24 D, and both of these tests yielded that there was no statistically significant difference between the different concentrations of the same cell type. Therefore, it was determined that the best method to compare test results is to calculate the impedance percentage increase above background, and then to compare the sets of data. This is the recommended method of assessment for all future applications of this device.

Table 2 – Comparison of average impedance values with background removed for N2A and astrocyte samples at 1:10 and 3x, as well as comparison of impedance percentage above background for the same samples.

Cell Type	Concentration	Average Impedance (Ω) - Background	P Value
N2A	1:10	10685	0.000163
Astrocyte	1:10	4342	
N2A	3x	4937	0.000978
Astrocyte	3x	1260	
Cell Type	Concentration	Percentage Above Background	P Value
N2A	1:10	49.45	~ 0.0
Astrocyte	1:10	11.12	
N2A	3x	45.37	0.000166
Astrocyte	3x	7.69	

The frequency of 10782 Hz which was used to capture these data points has been previously linked to both the α dispersion and β dispersion range. Therefore it is undetermined if the scan at this frequency was measuring the membrane or cytosol impedance values [21, 22, 25, 30]. As there is no EIS data

regarding neural cell types, a deduction had to be reached with the data available. As previously noted in S. Hernandez's thesis, typically the point at which the impedance value begins to drastically drop is when the scan begins to transition between the membrane and the cytosol [60]. This is due to the fact that at this frequency, the membrane becomes polarized and stops resisting the current, and it flows freely through the pores in the membrane [26]. As seen in figure 22, the drop off for the astrocytes at the 1:10 dilution occurs after the frequency reaches ~10 KHz, suggesting that at the frequency used, the membrane impedance was being sensed. Overall, according to the data collected the membrane impedance of N2A cells is significantly greater than that of astrocytes, when recorded at 10782 Hz.

At the 0.9 MHz frequency, the impedance values of N2A cells at 1:10 dilution and 1x concentration were significantly greater than that of their respective medias. Much like the samples analyzed at 10782 Hz, the astrocytes at these concentrations did not exhibit significance. However, this data also showed a definitive trend for the 1:10 diluted samples with the cells in media mixture having a larger impedance value than the respective media. Therefore analysis of these samples continued for further comparison versus the N2A values. The first method to analyze the data described previously was used to subtract out the media impedance value (background) from all of the samples, and then compare versus the other cell type (N2A vs. astrocytes at 1:10 only). When the sets of data were analyzed with a two tailed T-Test, there was no significance found, suggesting that the impedance values with the background

subtracted out were not different. Although there was no significance found, the positive results found while comparing the percentage increases over background at the 10782 Hz frequency level warranted further analysis for these samples found at 0.9 MHz. Therefore the data was analyzed to calculate the percentage increase of impedance above its own background level. However when this set of data was analyzed via a two tailed T-Test, there was no significance found, suggesting there is no difference between the cell types at 0.9 MHz. This frequency lies within the β dispersion range found for other cell types, and although there was no statistically significant difference between the two cell types, this does not mean that the cytosols of the cells do not differ in contents or byproducts. A more precise device or scanning method to differentiate the two types of cells within this range may be required.

4.2.1 Theory Behind the Lower Membrane Impedance of Astrocytes

As previously described, the membrane impedance recorded at 10782 Hz of the astrocytes was significantly lower than that of the N2A cells. According to previous research, malignant tissue exhibits lower membrane impedance values, opposite to what was found during research for this thesis. The reasoning for this discrepancy may be due to the unique nature of the central nervous system, and specifically the functionality and coexistence of the various cell types found in the brain. As the brain is a very unique organ compared to the rest of the body – it has a barrier between the tissue and the vasculature which is particularly impermissible, its primary functioning cell types (neurons) do not undergo mitosis

and will not regenerate, its main function of signaling is unlike that of any other – the cell types found within this organ may contain aspects which lead to their own unique set of characteristics regarding impedance spectroscopy [6].

Astrocytes, the non-malignant cell type used as a comparison to N2A cells, have numerous functions – as seen in the introduction. One task of these cells which garners great interest regarding this thesis is their ability (and necessity) to uptake various ions and molecules which have been secreted by neurons as transmission signals [6]. After a neuron transmits a signal, the signal compounds must be quickly voided from the neural tissue, otherwise cell death will occur by excitotoxicity [13]. Some of these molecules which must be sequestered by the astrocytes include inhibitory ions like potassium, and excitatory molecules like glutamate and calcium. Due to the fact that neurons can transmit signals at a pace of up to 1000 times per second, the rate at which the astrocytes prevent transmission signal molecule saturation in the neural fluid must be extremely quick. This is likely accomplished by highly efficient and abundant cellular channels which cover the membrane of the astrocytes – those which uptake ions and those which uptake large molecules such as glutamate. One such channel found on astrocytes that is activated by glutamate presence, NMDA, must be strongly depolarized to function, lending to the idea that astrocyte membranes are easily permeated by electrical current [1]. Astrocytes have also been documented as cells with high resting membrane potentials, within the range of -75 to -95 mV [67, 68]. This probable ample presence of channels on the membranes of the astrocytes, as well as the necessity for a

highly depolarized membrane for NMDA channel functionality leads to the belief that astrocyte membranes are highly permissible to electrical current, and therefore have significantly lower impedance than like malignant cells such as N2A. There is also evidence which suggests that the membranes of astrocytes exhibit significantly lower resistance to electrical current than neurons [68]. Although it is possible for glioma/neuroblastomas such as N2A cells to arise from astrocytes, these malignant cell types will likely not maintain the highly permissible nature of astrocytes as their newly acquired functioning is entirely unrelated to sequestering neural transmission signal ions and molecules.

4.3 Future Work

As the scope of a master's thesis project is not as in depth as one of a PhD candidate, along with the limited budget for this project, all potential device redesign and testing possibilities could not be completed. While the data acquired for this thesis is a significant source of information usable for interpretation, there is much more work which can be completed – regarding the study of neural cancer diagnostics with EIS, as well as other electrical impedance spectroscopy sensing at Cal Poly by using the device which has been produced.

For continued testing with the produced device, there are numerous things which have been discovered during this research which can be utilized and also explored further. Beginning with basic suggestions regarding the use of the device as it was used for this thesis, scanning cells while at a concentrated state such as the 3x used during this thesis or greater is recommended. The device

exhibits functionality, but the overall controls are limited to the point where attempting to isolate an individual cell between the electrodes is a burden. As the data collected (primarily the background impedance) had major differences between the days of recording, completing any data recording for a set of values which will be compared (i.e. N2A at 3x and astrocytes at 3x) should be completed on the same day without any detachment of the device. Although recording the data on separate days did not seem to affect the percentage increase of cell sample impedance above background values, it may have affected the data analysis from being completed comparing straight impedance values with background impedance subtracted out. To improve the visibility of cells within the microfluidic chamber, a method to stain or highlight the cells without compromising their natural functions and viability could be looked into.

Regarding the functionality of the device and the settings for recording within the LabView program, a computational model of the electrodes with various amplitude settings could be completed. This would help ensure the most efficient setting of amplitude is used, which potentially could decrease variability within the data samples. More research studying the frequencies found as 'hits' with significant difference between the cell mixtures and the media, could be completed to see which of these frequencies correlate to regions of interest within the realm of neuronal cells, or other cell types. This would require further testing of different cell types, and analysis of their impedance data. Using this information, a more specific spectrum of target frequencies to analyze could be documented for future research regarding EIS.

Although the astrocytes did not exhibit strong impedance signals (membrane or cytosol) that differed from their own media, N2A cells are prime candidates for future research comparing neuronal cancers to other benign central nervous system tissues. Future research could be completed studying the differences in impedance of N2A cells to other nervous system cells such as neurons or oligodendrocytes, or to particulate matter found in the nervous system such as myelin. Comparing the impedance values of various types of nervous system matter and cells to neural cancers such as neuroblastomas would help expand the knowledge of the intricate central nervous system, and could potentially be used in the future for improved diagnostics.

There are also various design considerations which should be taken into account if a future device for electrical impedance spectroscopy is to be created for use within this lab. When pouring the layer of PDMS to form the microfluidic channel, it is very important to make it relatively thick (3+ mm) so that the tubing can be inserted easily into the device without worrying about it falling out. Also if multiple ports are required for inflow and outflow, designing the device so that a more firm seal can be applied to the unused regions is important. When a port is not ideally sealed, it can disrupt the flow of the fluid within the device, as it typically provides a pathway of least resistance. Designing the region near the electrodes (and the electrodes themselves) to capture or isolate minimal amounts of cells would be greatly beneficial. As the current device has its electrodes in the center of the channel, there is no way to physically trap the cells. With use of a redesigned mold for the PDMS or by using a syringe pumps

to alter flow of the cells, single cells or small masses of cells could be captured for more specified recording. Another potential design consideration, if possible, would be to use silver as the metal of choice for the electrodes. Although gold does add a small amount to the recorded impedance, silver will add a lesser amount to this impedance magnitude. By using these suggestions, as well as the previously described protocol, an improved device can be created.

5 Conclusion

Across various industries including biotechnology, food production, environmental engineering and biomedicine, cell system investigation systems are utilized for different diagnostic needs [20]. Within the realm of biomedicine and biomedical engineering, a particular method of cell system investigation which is currently being studied is electrical impedance spectroscopy (EIS). The devices, by using the principles of electrical circuit theory to quantify differences in impedance, have been able to detect viral infections, drug responses occurring, and various living cell traits – such as the incidence of cancer [20, 21, 32, 36]. As cancer can progress quickly throughout a host's body and cause irreversible damage, early and accurate detection of the presence of cancer is extremely important. Previous studies with EIS have shown promise regarding the use as a highly accurate cancer diagnostic device, specifically as cancerous cell membranes exhibit lower impedances than like benign tissue, as the membranes display higher permittivity to electronic current [32, 36]. However, many current EIS devices scan through a small chunk of excised tissue. This method may be more variable than scanning through a single cell or small amount of cells, resulting in escalated risk of the diagnoses [32, 36].

This thesis aimed at studying the capabilities of isolated single cell electrical impedance sensing as a means of diagnosing disease. Specifically, the goals of the thesis were to: 1) manufacture a suitable platform for cell introduction and scanning, 2) develop a reproducible protocol for future cell analysis to be completed with the manufactured device, 3) to perform impedance

measurements on enough samples of cancerous and non-cancerous cells to complete analysis with statistical validity, and 4) to complete statistical analysis on the recorded data to determine if the membrane and cytosol impedances between neural cancerous cells and non-cancerous cells are significant. A device capable of completing impedance recording on cells in media was successfully manufactured. After creating the device, N2A and astrocytes were grown up in tissue culture flasks to provide the comparison between cancerous and non-cancerous cells. The cells were introduced into the device in various concentrations, and the electrical impedance of the samples was recorded at frequencies between 1096 Hz and 42.77 MHz. After analyzing the results, it was determined that the membranes of N2A cells exhibit a larger impedance than that of astrocytes when scanned at a frequency of 10.8 KHz. The results of this study are opposite from other types of cancers studied via electrical impedance spectroscopy, as cancerous cell membranes have been shown to have lower impedance values than that of like benign tissues [32, 36]. While the results seem to be counterintuitive, there are no documented studies of neural cancer impedance comparisons, so the results should not be discounted. The behavior of the astrocytes as neural support cells and their specific function to sequester transmission signal molecules may lead to the permittivity of their membranes. While the data acquired from this thesis suggests that the membrane impedance of neural cancerous cells is larger than that of astrocytes, there was no conclusive evidence regarding the comparison of these two cell types' cytosols. This thesis has laid a minute basis for future neural cancer studies with

electrochemical impedance spectroscopy, as well as suggestions for future use of the device by future students in the Biofluidics lab group of Dr. David Clague. Hopefully this thesis will promote the exploration of continued single cell impedance as use as a diagnostic device, and will help improve healthcare across the world.

REFERENCES

1. Alberts, B., et al., *Molecular Biology of the Cell*, 5th ed. 2008, New York: Garland Science.
2. Cancer.org. [Learn About Cancer: Cancer Basics] 2011
<<http://www.cancer.org/Cancer/CancerBasics/index>>
3. Hoelzl-Wallach, D.F., et al., *Cellular Membrane and Tumor Behavior: a New Hypothesis*. Proceedings of the National Academy of Sciences, 1968. **61**(15): p. 868-874.
4. Anghileri, L.J., et al., *Mechanisms of Gallium-67 Accumulation by Tumors: Role of Cell Membrane Permeability*. The Journal of Nuclear Medicine, 1988. **29**(5): p. 663-668.
5. Wang, F., et al., *Small mitochondria-targeting molecules as anti-cancer agents*. Molecular Aspects of Medicine, 2010. **31**: p. 75-92.
6. Saladin, K.S., *Anatomy & Physiology, The Unity of Form and Function*, 5th ed. 2010, New York: McGraw-Hill.
7. Simon, T., et al., *Short- and long-term outcome of patients with symptoms of spinal cord compression by neuroblastoma*. Developmental Medicine & Child Neurology, 2012. P. 1-6.
8. Ellingson, B., et al., *High Order Diffusion Tensor Imaging in Human Glioblastoma*. Academic Radiology, 2011. **18**(8): p. 947-954.
9. Cancer.org [Learn About Cancer: Brain/CNS Tumors in Adults] 2011
<<http://www.cancer.org/cancer/braincnstumorsinadults/index>>
10. Cancer.org [Learn About Cancer: Brain/CNS Tumors in Children] 2011
<<http://www.cancer.org/cancer/braincnstumorsinchildren/index>>
11. Lee, J-H., et al., *Schwann cell-like remyelination following transplantation of human umbilical cord blood (hUCB)-derived mesenchymal stem cells in dogs with acute spinal cord injury*. Journal of the Neurological Sciences, 2011. **300**: p. 86-96.
12. Faulkner, J., et al., *Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury*. Transplant Immunology, 2005. **15**: p. 131-142.
13. Arundine, M., et al., *Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity*. Cell Calcium, 2003. **34**: p. 325-337.
14. Stupp, R., et al., *Radiotherapy plus Concomitant & Adjuvant Temozolomide for Glioblastoma*. New England Journal of Medicine, 2005. **352**: p. 987-996.
15. Cancer.org [Learn About Cancer: Neuroblastoma] 2011
<<http://www.cancer.org/cancer/neuroblastoma/index>>

16. Poretti, A., et al., *Neuroblastoma with spinal cord compression: is there an emergency treatment of choice?* Developmental Medicine & Child Neurology, 2012. P. 1.
17. Modak, S., et al., *Neuroblastoma: Therapeutic strategies for a clinical enigma.* Cancer Treatment Reviews, 2010. **36**: p. 307-317.
18. Castlberry, R.P., *Paediatric Update: Neuroblastoma.* European Journal of Cancer, 1997. **33**(9): p. 1430-1438.
19. Fukushima, S., et al., *Clinicopathological analysis of olfactory neuroblastoma.* Brain Tumor Pathology, 2012.
20. Iliescu, C. et al., *A microfluidic device for impedance spectroscopy analysis of biological samples.* Sensors and Actuators B, 2007. **123**: p. 168-176.
21. Jao, J.Y., et al., *Electrical characterization of single cell in microfluidic device.* Microelectronics Reliability, 2011. **51**: p. 781-789.
22. Zou, Y., et al., *A review of electrical impedance techniques for breast cancer detection.* Medical Engineering & Physics, 2003. **25**: p. 79-90.
23. Bai, S.J., et al., *In vivo electrochemical impedance measurements on single cell membrane.* Microelectronic Engineering, 2011. **88**: p. 3094-3100.
24. Gilchrist, K. H., et al., *Sensitivity of cell-based biosensors to environmental variables.* Biosensors and Bioelectronics, 2005. **20**: p. 1397-1406.
25. Brown, B.H., et al., *Relation between tissue structure and imposed electrical current flow in cervical neoplasia.* The Lancet, 2000. **355**: p. 892-895.
26. Gawad, S., et al., *Dielectric spectroscopy in a micromachined flow cytometer: theoretical and practical considerations.* Lab Chip, 2004. **4**: p. 241-251.
27. Das, S., et al., *Effects of Conductivity Variations within the Electric Double Layer on the Streaming Potential Estimation in Narrow Fluidic Confinements.* Langmuir, 2010. **26**(13): p. 11589-11596.
28. You, Xue-Yi, et al., *Analysis of EDL Effects on the Flow and Flow Stability in Microchannels.* Journal of Hydrodynamics, 2010. **22**(5): p. 725-731.
29. Wang, H., et al., *Simulation of electric double layer capacitors with mesoporous electrodes: Effects of morphology and electrolyte permittivity.* Electrochimica Acta, 2011. **56**: p. 6189-6197.
30. Wang, J.W., et al., *Effects of electrode geometry and cell location on single-cell impedance measurement.* Biosensors and Bioelectronics, 2010. **25**: p.1271-1276.

31. Wang, M.H., et al., *A systematic investigation into the electrical properties of single HeLa cells via impedance measurements and COMSOL simulations*. Biosensors and Bioelectronics, 2009. **24**: p. 2830-2935.
32. Aberg, P., et al., *Skin cancer identification using multifrequency electrical impedance-a potential screening tool*. IEEE Transactions on Biomedical Engineering, 2004. **51**(12): p. 2097-2102.
33. Park, H., et al., *Single-cell manipulation on microfluidic chip by dielectrophoretic actuation and impedance detection*. Sensors and Actuators B, 2010. **150**: p. 167-173.
34. Yin, H., et al., *Microfluidics for single cell analysis*. Current opinion in Biotechnology, 2012. **23**: p. 110-119.
35. Werdich, A., et al., *A microfluidic device to confine a single cardiac myocyte in a sub-nanoliter volume on planar microelectrodes for extracellular potential recordings*. Lab Chip, 2004. **4**: p. 357-362.
36. Halter, R., et al., *Electrical Impedance Spectroscopy of Benign and Malignant Prostatic Tissues*. The Journal of Urology, 2008. **179**: p.1580-1586.
37. Thein, M., et al., *Response characteristics of single-cell impedance sensors employed with surface-modified microelectrodes*. Biosensors and Bioelectronics, 2010. **25**: p. 1963-1969.
38. Asphahani, F., et al., *Influence of cell adhesion and spreading on impedance characteristics of cell-based sensors*. Biosensors and Bioelectronics, 2008. **23**: p. 1307-1313.
39. Wheeler, A.R., et al., *Microfluidic Device for Single-Cell Analysis*. Analytical Chemistry, 2003. **75**(14): p. 3581-3586.
40. Xia, Y., et al., *Soft Lithography*. Annual Review of Material Science, 1998. **28**: p. 153-184.
41. Jiminez, V., et al., *Transient dynamics of a MEMS variable capacitor driven with a Dickson charge pump*. Sensors and Actuators A, 2006. **128**: p. 89-97.
42. Wang, W. and S. Soper, *Bio-MEMS: Technologies and Applications*. W. Wang and S. Soper. 2007, Boca Raton: CRC Press.
43. Ernst, H., et al., *High resolution flow characterizations in Bio-MEMS*. Sensors and Actuators A, 2002. **100**: p. 54-62.
44. Grigorov, A.V., *Suspended channel MEMS fabrication process for advanced device design and bio-analytical functionalization*. Microelectronic Engineering, 2008. **85**: p. 2290-2298.
45. Rivet, C., et al., *Microfluidics for medical diagnostics and biosensors*. Chemical Engineering Science, 2011. **66**: p. 1490-1507.

46. Uchiyama, K., et al., *Development of a Bio-MEMS for Evaluation of dioxin toxicity by immunoassay method*. Sensors and Actuators B, 2004. **103**: p. 200-205.
47. Bouaidat, S., et al., *Plasma-polymerized coatings for bio-MEMS applications*. Sensors and Actuators A, 2004. **110**: p. 390-394.
48. Lueke, J., et al., *MEMS-Based Power Generation Techniques for Implantable Biosensing Applications*. Sensors, 2011. **11**: p. 1433-1460.
49. Whitesides, G.M., *The origins and the future of microfluidics*. Nature, 2006. **442**: p. 368-373.
50. Byun, I., et al., *Fabrication of a new micro bio chip and flow cell cytometry system using Bio-MEMS technology*. Microelectronics Journal, 2008. **39**: p. 717-722.
51. Smith, G.D., et al., *Gamete and embryo isolation and culture with microfluidics*. Theriogenology, 2007. **68S**: p. S190-S195.
52. Bashir, R., *BioMEMS: State-of-the-art in detection, opportunities and prospects*. Advanced Drug Delivery Review, 2004. **56**(11): p. 1565-1586.
53. Lan, S., et al., *Surface modification of silicon and gold-patterned silicon surfaces for improved biocompatibility and cell patterning selectivity*. Biosensors and Bioelectronics, 2005. **20**: p. 1697-1708.
54. Voskerician, G., et al., *Biocompatibility and biofouling of MEMS drug delivery devices*. Biomaterials, 2003. **24**: 1959-1967.
55. Lee, Y-H., et al., *Modified titanium surface with gelatin nano gold composite increases osteoblast cell biocompatibility*. Applied Surface Science, 2010. **256**: p. 5882-5887.
56. Fadrique, J-J., *Design, Fabrication, and Implementation of a Single-Cell Capture Chamber for a Microfluidic Impedance Sensor*, Master's Thesis in Biomedical Engineering 2009, California Polytechnic State University, San Luis Obispo.
57. Deng, X., et al., *A novel approach for the preparation of PMMA-PDMS core-shell particles with PDMS in the shell*. Applied Surface Science, 2007. **253**: p. 4823-4829.
58. Kim, E-H., et al., *Composite binders for precision casting shell molds*. Ceramics International, 2012. **38**: p. 1205-1209.
59. Locquet, J-P., *Local electrochemical oxidation/reduction: first step towards a new lithography?* Physica C, 1996. **263**: p. 160-163.
60. Hernandez, S., *Single Cell Impedance Measurements Using Microfabricated Electrodes and Labview Graphical Programming*, Master's Thesis in General Engineering 2009, California Polytechnic State University, San Luis Obispo.

61. Madou, M., *Fundamentals of Microfabrication: the Science of Miniaturization*, 2nd ed. 2002, CRC Press.
62. Bodas, D., et al., *Formation of more stable hydrophilic surfaces of PDMS by plasma and chemical treatments*. Microelectronic Engineering, 2006. **83**: p. 1277-1279.
63. Bodas, D., et al., *Hydrophilization and hydrophobic recovery of PDMS by oxygen plasma and chemical treatment-An SEM investigation*. Sensors and Actuators B, 2007. **123**: p. 368-373.
64. Kim, H.T., et al., *PDMS surface modification using atmospheric pressure plasma*. Microelectronic Engineering, 2011. **88**: p. 2281-2285.
65. Efimenko, K., et al., *Surface Modification of Sylgard-184 Poly(dimethyl siloxane) Networks by Ultraviolet and Ultraviolet/Ozone Treatment*. Journal of Colloid and Interface Science, 2002. **254**: p. 306-315.
66. Wu, Z., et al., *One-stage fabrication of sub-micron hydrophilic microchannels on PDMS*. Applied Surface Science, 2009. **255**: p. 4702-4704.
67. Jensen, D., *The Human Nervous System*. 1980, New York: Appleton-Century-Crofts.
68. Willis, W.D., et al., *Medical Neurobiology*. 1973, Saint Louis: The C.V. Mosby Company.

6 Appendices

Appendix A – Silicone Wafer Master Mold Fabrication

- 1) Obtain negative transparency mask.
- 2) Clean 100 mm diameter silicon wafers by soaking in Piranha solution (98% concentration Sulfuric Acid & 30% concentration Hydrogen Peroxide mixed to 9:1 ratio) for 15 minutes.
- 3) Rinse with de-ionized water.
- 4) Rinse wafers with BOE solution (HF acid/H₂O) for 15 minutes.
- 5) Rinse with de-ionized water.
- 6) Dry with nitrogen gas.
- 7) Bake at 205° C for 10 minutes.
- 8) Cool to room temperature.
- 9) Spin coat with SU-8.
- 10) Bake on hot plate at 85° C for 3 minutes.
- 11) Cool to room temperature.
- 12) Place in mask aligner with negative transparency mask placed above it.
- 13) Expose UV light at 125 mJ/cm² for 100 seconds.
- 14) Bake on hot plate at 85° C for 5 minutes.
- 15) Cool to room temperature.
- 16) Place in propylene glycol monomethyl ether acetate solution for 3 minutes.
- 17) Rinse with de-ionized water.
- 18) Allow to dry – finished product.

Appendix B – PDMS Pouring Procedure

- 1) Mix Dow Corning Sylgard 182A and 182B in a 1:10 volume ratio in a plastic cup with a metal stir rod until bubbles form.
- 2) Degas mixture in vacuum chamber at -22 inches Hg for 25 minutes (or until all bubbles are removed).
- 3) Place SU-8 master mold geometry side up in a 140 mm diameter petri dish with forceps.
- 4) Slowly pour PDMS over master mold to desired thickness and remove any air bubbles with a needle.
- 5) Bake cure for 4 hours at 65° C.
- 6) Cool to room temperature.
- 7) Cut out region of PDMS with scalpel, then place clear tape over formed geometry until device fabrication.

Appendix C – Murine Primary Astrocyte Harvesting

- 1) Sterile filter 1000 mL of Dissociation Media with final concentrations of: Na₂SO₄ at 81.8 mM (Sigma-Aldrich S9627), K₂SO₄ at 30 mM (Sigma-Aldrich P0772), MgCl₂ at 5.8 mM (Sigma-Aldrich M8266), CaCl₂ at 0.25 mM (Sigma-Aldrich 53704), HEPES buffer at 1 mM (Sigma-Aldrich 83264), Glucose at 20 mM (Sigma-Aldrich D9434), Phenol Red at 0.001% (Sigma-Aldrich P0290), and NaOH at 0.16 mM (Sigma-Aldrich 43617 FLUKA).
- 2) Sterile filter 1000 mL of Kynurenic Acid mixture with final concentrations of: Kynurenic Acid at 10 mM (Sigma-Aldrich K3375), Phenol Red at 0.0025%, HEPES buffer at 5 mM, and MgCl₂ at 100 mM
- 3) Mix ice cold 90% Dissociation Media/10% Kynurenic Acid mixture and pour into 5 tissue culture petri dishes, place on ice.
- 4) Prepare papain solution by mixing 6 mL papain (Sigma-Aldrich 76216) per 5 cortices into 15 mL conical tube.
- 5) Activate papain solution by adding a few L-cysteine crystals (Sigma-Aldrich C7880) and adjust pH to 7 by addition of 0.1 N NaOH.
- 6) Sterile filter papain solution with 0.22 µM syringe 30 minutes prior to use.
- 7) Sacrifice mother via cervical dislocation.
- 8) Spray abdominal area with 70% EtOH, open with surgical scissors.
- 9) Remove uterine sac, place into tissue culture petri dish with 90/10 DM/KY.
- 10) Discard mother in biological waste bag.
- 11) Remove pups from uterus, place into next tissue culture petri dish.
- 12) Remove heads of pups, and cut skull in middle region and create a transverse cut behind the eyes then peel skull away.
- 13) Scoop out brain with brain spatula and place into next tissue culture petri dish.
- 14) Remove olfactory bulbs from brains with surgical scissors, and pull away cortexes with fine forceps.
- 15) Remove meninges from cortical lobes and place cortical lobes into final culture dish.
- 16) Mince cortices into ~ 1 mm³ segments with razor blade cleaned with 70% isopropanol.
- 17) Transfer brains and fluid to 15 mL conical tube and allow to settle.
- 18) Remove media slowly, and add 6 mL/5 brains of papain solution.
- 19) Slowly invert tube every 2 minutes for 15 minutes total.
- 20) Allow tissue to settle, and remove all media.
- 21) Add 2mL/brain trypsin inhibitor solution.

- 22) Invert slowly every minute for 5 minutes.
- 23) Remove all of media and wash with 1 mL/brain of Opti-MEM/Glucose and invert tube several times.
- 24) Allow tissue to settle, and remove all media.
- 25) Add 5 mL fresh Opti-MEM/Glucose and gently grind remaining tissue with sterile pipette tip.
- 26) Add cell solution to 50 mL conical tube and dilute to 50 mL total volume with Opti-MEM/Glucose solution.
- 27) Mix cell suspension briefly, then remove 10 uL and add to 10 uL of DM/KY in an Eppendorf tube, also add 10 uL Trypan Blue.
- 28) Take 10 uL of mixture and add to hemocytometer.
- 29) Count 16 squares from 2 separate regions for viable (not blue) cells.
- 30) Average amount of cells and multiply by 30,000 to identify number of cells per mL of cell solution.
- 31) Dilute cell solution to 1×10^6 cells/mL then plate into T175 flask.
- 32) Remove media after flask rests for 2 hours.
- 33) Fill flask with 50 mL of astrocyte specific media: DMEM + GlutaMax (GIBCO 10569), 10% Fetal Bovine Serum (Hyclone ASJ 30474), and 1% Penicillin-Streptomycin (Sigma-Aldrich P4458).
- 34) Place flask into 37° C & 5% CO₂ incubator for 24 hours.
- 35) Remove flask, slap firmly on sides of flask and aspirate all media.
- 36) Replace with fresh astrocyte media and allow to grow to confluency.