TOWARDS THE ELECTRICAL STIMULATION OF PC12 CELLS

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ABSTRACT

The electrical stimulation of biological cells has been studied intensively since Galvani's experiment with a dead frog's leg muscle. Medical advances such as the pacemaker and the cochlear implants utilize some of the fundamentals of electrical stimulation as a result of these intensive studies. It is also a technique which has been explored in many research laboratories and has been shown to be able to play an important role in neurite outgrowth and the regeneration of transected nerves. Electric stimulation has also been shown to induce the differentiation of PC12 cells, faster than normal chemical means. The pheochromocytoma (PC12) cell line is derived from the pheochromocytoma of the rat adrenal medulla which can be differentiated to display characteristics similar to sympathetic neurons. By being able to induce differentiation faster, this opens the door to using complementary analytical techniques, such as a synchrotron-based Fourier Transform infrared spectromicroscopy to understand some of these molecular processes occurring during the differentiation process.

The long term objective of this project is to couple electrical stimulation and Fourier Transform infrared spectromicroscopy to study the electrically induced differentiation process. The primary focus of this work is to develop the methodology and background required to obtain this objective. This thesis focuses primarily on the PC12 cell line which has been reported in literature to differentiate both through chemical means (nerve growth factor, NGF) and electrical stimulation means. Using NGF, PC12 cells were able to show, within 24 hours, signs of initiating the differentiation process. Neurite outgrowths with a mean ± standard deviations of 10.0 ± 9.9, 14.0 ± 12.5, 21.4 ± 26.5, 21.6 ± 38.9 and 40.7 ± 49.1 μm corresponding to 24, 48, 72, 96 and 120 hours of NGF exposure was observed. PC12 cells grown on FTO conductive glass were electrically stimulated with a pulsing sequence of ±50 mV from the resting potential for 1 hour followed by 24 hours of incubation. These cells displayed a mean ± standard deviation neurite length of 9.35 ± 9.19 μm which is similar to PC12 cells exposed to 24 hours of NGF. The results of the electrical stimulation experiments are promising; however more experiments need to be conducted to determine the ideal electrical

stimulation parameters to induce differentiation. The promising results also bring us one step closer to coupling FTIR to better understand the differentiation process from a molecular viewpoint.

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DEDICATION

To my parents,

Terence and Jennifer Li

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LIST OF ABBREVIATIONS

AFM	atomic force microscopy
AUT	11-amino-1-undecanethiol
BS	blocking solution
BSA	bovine albumin serum
BDNF	brain-derived neurotrophic factor
CV	cyclic voltammograms
DAPI	4’-6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FTIR	Fourier-Transform infrared
FTO	fluorine-doped tin dioxide
GAP-43	growth associated protein-43
GFAP	glial fibrillary acidic protein
HI	heat-inactivated
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS	horse serum
Hz	Hertz
IDA	interdigitated array of electrodes
ITO	indium-doped tin dioxide
Ln	laminin
M	molar
MEA	microelectrode array
mV	millivolts
NGF	nerve growth factor
NgCAM	neuron-glia cell adhesion molecule
OCP	open circuit potential
PBS	phosphate buffer saline
PLL	poly-L-lysine

PKC	protein kinase C
RNA	ribonucleic acid
SAM	self-assembled monolayer
s.d.	standard deviation
trypsin-EDTA	trypsin-ethylenediaminetetraacetic acid

1. Introduction and Literature Review

1.1 General overview

The physiological responses to the electrical stimulation of biological cells, tissues, organs, and organisms have been of huge scientific interest.[1-3] The first reported electrical stimulation experiment was in 1791 by Galvani, who applied a current through a dead frog's leg muscle and observed it twitch.[4] From this initial electrical stimulation experiment by Galvani, there have been many advances in the understanding and use of electrical stimulation of neural cells and tissues including medical field applications.[5-7] The pacemaker, for example, is a well-known medical device which is used to treat individuals with bradyarrhythmia.[8-9] A pacemaker is a device which contains a battery and electronic circuits, which is implanted within the heart.[8-9] The pacemaker can monitor and detect the individual's heart rhythm and can stimulate the heart with electrical impulses (act as an electrode) to maintain or increase the heart rhythms.[8-9] According to Wood *et al.*, there were about 3 million individuals in the world as of 2002 with pacemakers, and 600,000 are implanted yearly.[8]

Electrical stimulation can also play an important role in neurite outgrowth and the regeneration of transected nerves which have been studied both vitro and in vivo.[10-13] Studies also indicate electrical stimulation of neuronal cells increases the rate of neurite outgrowth as compared to chemically induced differentiation.[13-16] While there have been many reports in the literature indicating the success of electrical stimulation to induce neuronal differentiation, much remains unknown about the molecular processes that occur within a differentiating neuronal cell. To acquire molecular information, it is desirable to couple traditional spectroscopic tools with in vitro cell biology techniques. In principle, Fourier Transform infrared spectroscopy (FTIR) can provide quantitative information on protein, lipid, and nucleic acid composition, conformation and concentration in biological cells as well as identifying small molecules that are produced and/or consumed in cellular processes.[17-18] FTIR can also be coupled with an optical microscope to afford spatially resolved, molecular spectroscopy of biological cells.[19-21] However, subcellular resolution with traditional FTIR instruments suffers from very poor signal to noise ratios due to the poor brightness of thermal IR sources as the diffraction limit is approached. Infrared

radiation from synchrotron sources can offer two to three orders of magnitude greater brightness at diffraction limits (ca. 10 μm) and in recent years, IR spectromicroscopy using synchrotron radiation has been employed for cell biology studies.[19-21]

A long term objective within the Burgess laboratory is to successfully study electrically-stimulated neuronal cell differentiation using synchrotron Fourier Transform infrared spectromicroscopy. While the laboratory has significant experience with the latter prior to the commencement of the work described in this thesis, there was no expertise or experience in cell biology within this group. The goal of this MSc thesis was to establish the methodologies needed to grow neuronal-like cells on conductive substrates and demonstrate that cell differentiation could be achieved via electrical stimulation of these substrates. The success of this project would provide an important stepping stone in the long term goal described above.

1.2 Biological Cells

All living organisms are made of either a single cell (prokaryotes) or multiple cells (eukaryotes).[22] At the time of conception, human beings start off initially as a single cell (zygote), which develops into a complex multicellular organism, with organs and tissues which work together to allow humans to function on a daily basis. Some of these specialized cells are the nerve or neuronal cells which make up the nervous system. These nerve or neuronal cells differ from non-nervous system cells by having two unique features: a single, long nerve fiber called an axon and numerous shorter processes called dendrites which extend from the cellular body (or soma).

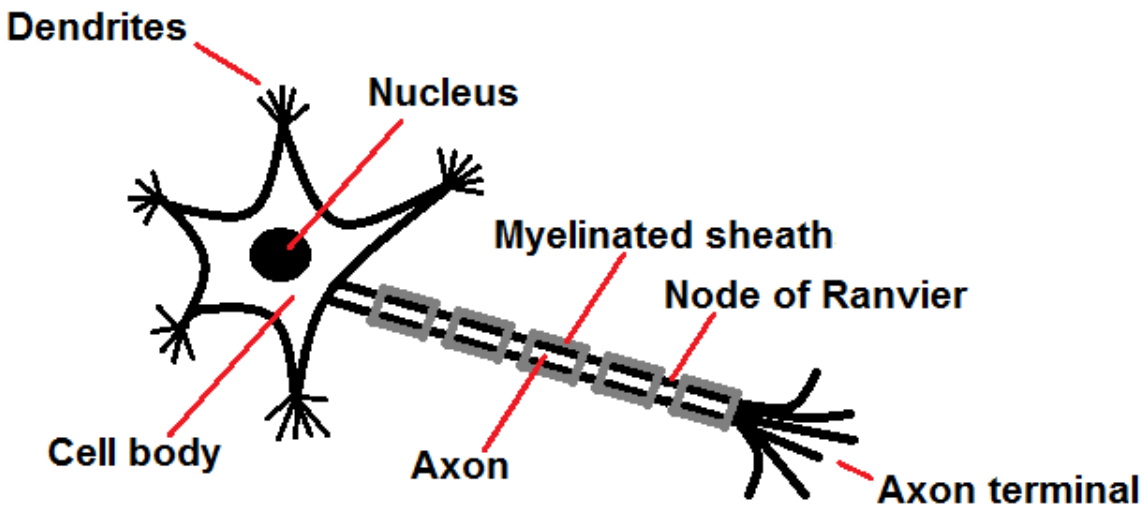

Figure 1.1. Schematic diagram of a neuronal cell.

Similar to all other cells, the cell body or soma of the neuronal cell contains the nuclei and other important organelles responsible for the function and survival of the cell. When stimulated, the dendrites receive signals from other neuron cells to its cellular body. The axon, which can be myelinated or unmyelinated, sends the signal from the cell body to another nerve or muscle cell. The action potential, which travels along the axon, when myelinated, can send the signals faster to its receptor cell. In between each myelinated sheath, there is an unmyelinated region called the nodes of Ranvier. It is at these nodes of Ranvier that the highest concentrations of ion channels are found. These ion channels allow for the influx of their respective ions into and out of the cells. It is the influx of these ions which polarize and depolarize the cell membrane, and cause an observable electrical current.

While at rest, the membrane potential is approximately -70 mV, as there are some ion channels which remain open and the ion concentrations in and outside of the cell are not in equilibrium.[23-24] When the nerve cell is sufficiently stimulated (threshold met), an action potential is sent along the axon resulting in the opening and closing of several ion channels through the process.

As the sodium channels open, an influx of sodium ions enters the cell, depolarizing the membrane potential to an approximate potential of +14 mV.[23] The membrane repolarizes when the potassium ion channels are open and the sodium ion channels close, causing an influx of potassium ions out the cell, overshooting to approximately -90 mV. The hyperpolarization of the membrane is due to more potassium channels remaining open after the action potential than compared to the membrane's resting state, allowing for potassium ions to continue leaving the cell. As these potassium channels begin to close, the membrane potential approaches the membrane's resting potential. During this hyperpolarization phase, no matter how intense a stimulus is, the neuronal cell cannot be stimulated again (refractory period). While some of these ion channels are leak channels (permanently open), some of the ion channels are voltage gated such as the voltage-gated sodium channels which can only be activated by depolarization of the membrane. When the action potential travels along the presynaptic axon to the postsynaptic axon, neurotransmitter release can be triggered which allows for signaling from one neuron to the next. The binding of the neurotransmitter on the postsynaptic cell's receptors

triggers various signaling and cascades dependent on the neurotransmitter. Both medical and recreational drugs take advantage of the knowledge and effects of a neurotransmitter to elicit a specific response, such as treatment for Parkinson's disease which uses medication which increase dopamine levels.[23]

1.3 Pheochromocytoma cells (PC12)

PC12 cells are a single cell clonal line derived from transplantable rat adrenal pheochromocytoma, which has been reported to be a useful model system for neurobiological and neurochemical studies.[25] In culture, undifferentiated PC12 cells typically are round in shape and have a tendency to grow in small aggregates. In 1976, it was first reported by Greene and Tischler, that when exposed to nerve growth factor (NGF), these cells can be observed to flatten and extend long, branching neuronal-like processes.[25]

In the same study, Greene and Tischler showed that while culturing PC12 cells in the presence of NGF, within one week of their study the PC12 cells cease to proliferate and instead branched varicose processes similar to sympathetic neurons.[25] Maintaining NGF in the PC12 cultures for several weeks led to process outgrowths extending up to 500-1000 μm in length. However, when they removed NGF from their media, it was observed that these process outgrowths would degenerate and return to their original undifferentiated state, where cellular proliferation would occur.[25]

Greene and Tischler also observed that newly dissociated PC12 cells did not adhere well to plastic culture dishes but adhered more firmly to collagen-coated culture dishes.[25] However, culturing these cells on at least two passages of collagen-coated culture dishes caused the weakly or non-adherent cells to adapt so that they could be cultured directly on plastic culture dishes. Undifferentiated cells can only be differentiated when adhered onto a substrate and the PC12 cells are no different. However this cell line can be cultured in either suspension or on a fixed polystyrene tissue culture plate pretreated with a chemical modification layer, usually in the form of an extracellular matrix protein or synthetic polymer.

Also in this study, Greene and Tischler determined that PC12 cells can synthesize and store sizable amounts of certain catecholamines, such as dopamine and norepinephrine but not epinephrine. This cell line has also been reported to express sodium action potentials.[25] From this study, Greene and Tischler have brought attention to PC12 cells as a potential model neuron system for neurobiology and neurochemistry, where preliminary experiments and understanding can be completed on this model neuron cell line prior to more expensive primary cultures.[25]

Since Greene and Tischler's first reported experiments with PC12 cells and NGF, PC12 cells have been studied for many purposes and various techniques have been employed. Many researchers have used the PC12 cell line to study cell responses as a result or during the chemical differentiation process, such as evaluating the changes in RNA and DNA metabolism, ionic responses, growth stimulation, and various proteins and neurotransmitters produced/involved during the differentiation process.[25-28]

Since then, PC12 cells have been coupled with electrochemical stimulation and various effects have been studied. In 1982, Dixey and Rein conducted experiments with PC12 cells stimulated under low-frequency magnetic fields which triggered the release of the neurotransmitter norepinephrine.[29] In 1998, Kimura *et al.* reported that PC12 cells can be electrically induced in the absence of NGF to differentiate while adhered on an electrode surface.[15] In this study, Kimura *et al.* used an alternating cathodic and anodic wave potential to stimulate differentiation.[15]

1.4 Nerve growth factor

Nerve growth factor (NGF) is the most used protein to drive neuronal differentiation of PC12 cells.[30-33] NGF was first isolated in the 1950s by Levi-Montalcini and Hamburger as a secreted protein which helps regulate the survival and development of neurons of the peripheral nervous system.[33] It is a part of the neurotropin family of proteins having similar structural homology with other protein members of the neurotropin class, such as brain-derived neurotrophic factor (BDNF) and neurotrophins NT-3, NT-4 and NT-5. These neurotrophins have two sets of transmembrane receptors in which they can bind to: TrkA and $p75^{NTR}$.[30,33] TrkA is a

single pass transmembrane receptor which when bound results in the phosphorylation of TrkA, triggering the activation of various signaling cascades, such as PI3 kinase, ras, and PLC.[34] Similarly, NGF and other neurotrophins can be bound to $p75^{NTR}$, a transmembrane glycoprotein, which triggers its own signaling cascades.[34] NGF can be bound to both TrkA and $p75^{NTR}$ while other neurotrophins may be selective to either or these receptors.[33]

Research with NGF has shown a positive contribution to the repair, regeneration, and protection of neuronal cells.[30-33] The addition of NGF to some cell lines, such as the PC12 cell line, can induce differentiation, in which a sympathetic neuronal phenotype is adopted and the extension of neurite outgrowths from the cellular body can be observed.[14-16,25,30,32-33]

1.5 Substrate choice

In tissue culture, biological cells are typically grown on polystyrene plastic or glass substrates. For electrical stimulation experiments, several criteria must be met: a conductive surface for which the cells can grow on and allow for electrical stimulation, and a substrate which does not affect the viability and cellular processes involved throughout the duration of the experiment. However, in the literature it has been reported in several studies, that the substrate chosen for culturing can affect the cells' viability and growth.[14, 34-35]

For the electric stimulation experiments, one goal of this work was to use gold electrodes or gold microelectrodes arranged in an array pattern (interdigitated array of electrodes) as a substrate. Using an interdigitated array (IDA) of microelectrodes or a microelectrode array would allow the experimentalist to control which cell or portion of a cell is electrically stimulated while monitoring a neighbouring cells' response to the stimuli.[36] This is an advantage of the interdigitated electrode or microelectrode arrays that a single working electrode cannot provide. With the use of the conductive substrates, non-native to cell culture (typically glass or polystyrene), the choice of the electrode becomes very important as it could directly affect the cells' viability and growth.

As the initial goal was to use gold electrodes, it was important to ensure that gold is a non-toxic substrate for cell growth and viability. At the present time, there is no reported literature indicating cells can grow directly on gold, but many electrochemical studies containing gold substrates utilize alkanethiol self-assembled monolayer anchors to promote cell adherence.[39] However, if bare gold can act as a substrate, any negative effects contributed by the chemical modification layer can be avoided.

The choice of substrate has been shown to have a direct effect on the proliferation rate, as reported by Lakard *et al.* who noted a higher proliferation rate for the rat neuronal cell line 13S124 when grown directly on fluorine-doped tin dioxide (FTO) conductive glass as opposed to glass, although the adhesion rate was the same.[34] There are other substrates which affect the viability of cells, according to Schmidt *et al.,* indium tin oxide (ITO)-conductive borosilicate glass was a poor substrate for PC12 cells to grow on.[14] However, other groups, such as Kimura *et al.* have reported success with ITO coated glass.[15]

1.6 Cellular adhesion molecules

Selective surface modifications have been used throughout various fields of biology since it has been shown that cellular adhesion molecules can play key roles in promoting cell-cell recognition, growth and maintenance of cells, tissue integrity, cell signaling, cellular differentiation, and cellular migration.[34,37-39] Extracellular matrix proteins and cellular adhesion molecules have also been reported to increase neurite outgrowth and axon development of various neuronal cell lines.[38] The choice of chemical adhesion layer also can affect both adhesion and the proliferation rate of the cell line.[34]

Synthetic polymers, such as polylysine and extracellular matrix proteins, such as collagen, laminin and fibronectin have been greatly studied and used throughout the literature as cellular adhesion molecules.[25,38] Esch *et al.* studied the effects of alternating strips of poly-L-lysine with extracellular matrix proteins laminin (Ln) or neuron-glia cell adhesion molecule (NgCAM) to determine if this alternating chemical environment has an effect on which neurite outgrowth can be predicted to develop into axons.[38] They reported finding preferential growth

along the Ln and NgCAM surfaces when their minor processes contacted both poly-L-lysine and extracellular matrix proteins, developing this minor process into an axon. For differentiating neuronal cells, the axon and dendrites cannot be identified until neurite outgrowth has reached a length of at least 10-15 μm which the neurite outgrowth is then defined as the axon.[38]

More recently, self-assembled monolayers and conductive polymers, such as 11-amino-1-undecanethiol and polypyrrole respectively, have also been used as cell-adhesion layers.[14,39] Long-chain alkanethiols can assemble into a monolayer onto a gold surface through a sulphur-gold bond and form compact films within a period of 1-5 hours.[39] An advantage to using SAM-modified gold is its stability in both air and water for extended durations (several months).[39] It also is reported to have sufficient stability in aqueous cell culturing media and their use as a cellular attachment has been reported throughout the literature.[38-40] The only disadvantage to using alkanethiol SAMs is its restriction to gold and other precious and coinage metal substrates.[39] In a study by Jans *et al.*, the researchers determined that SAMs alone are not sufficient to sustain neuronal adhesion and growth for hippocampal neuronal cells, but also changes in cell morphologies can be observed with different choices of SAMs used.[41]

Coupling extracellular matrix cell adhesion proteins, peptides and ligands have been used with alkanethiol SAMs to make mixed adhesion layers.[39-41] Jans *et al.* also found that SAMs alone are not enough to promote and maintain viable cells, but in fact proteins (synthetic or natural) anchored to a SAM-modified gold are required.[41] Nonetheless, the choice of cellular adhesion molecule can have a direct effect on the attachment and viability of cells due to various surface modifications and interactions such as, specific surface chemistry, surface hydrophobicity, surface topography (defects) and protein signaling and recognition.

Conductive polymers such as polypyrrole have also been used as a cellular attachment layer to study the effects of electrical stimulation. Schmidt *et al.* has studied the effects of electrical stimulation of neuronal cells grown on polypyrrole coated electrodes while stimulating with electric fields.[14] They studied the effects of electrical field on neurite outgrowth of PC12 cells and reported observing faster neurite outgrowth (> 2x) than regular chemical means without electric stimulation.[14] However in a related study, oxidizing and reducing the

polypyrrole film would stall growth of aortic endothelial cells. It appears that the substrate and chemical modification layer used to anchor the cells on can differ widely from research group to research group.[44]

1.7 Electrical stimulation

In some neural applications and experiments, the electrical stimulation is applied in a biphasic pulsing sequence, alternating between anodic and cathodic polarizations while others stimulate using a constant cathodic or anodic potential.[14-15,43-44] In these electrical stimulation of cells and tissues an external potential is applied which can initiate the depolarization of the cell membranes. Depolarizing the cell membrane can result in the opening of some ion channels, which allow for an influx of ions into or out of the cell triggering the initiation of biological pathways or cascades.

Kimura *et al.* reported in 1998 that undifferentiated PC12 cells were able to be electrically induced to display the differentiated phenotype in the absence of nerve growth factor.[15] The researchers used a three electrode system, where the working electrode was an indium-tin oxide conductive glass electrode, with a platinum wire acting as a counter electrode and a Ag/AgCl reference electrode.[15] They used a rectangular potential wave with a peak to peak of 100 mV centered around a rest potential with a frequency of 100 Hz for 5, 14 and 60 minutes every 24 hours, which was repeated for 3 days, followed by 2 days of incubation.[15] In doing these experiments, Kimura *et al.* were able to induce differentiation of the PC12 cells and provided an understanding for the electrical induced mechanism.[15] They determined that during electrical stimulation there is an influx of calcium into the cell.[15] Calcium ions play a vital role in the potassium ion-induced differentiation of PC12 cells. According to Morgan and Curran, an increase in potassium concentration induces c-fos only when calcium is present in the extracellular environment.[44] The influx of calcium through the voltage-gated calcium channel into the cell triggers the production of c-fos gene expression when potassium concentrations are elevated. The protein kinase C, PKC, cascade was determined to play a role in the differentiation process. The role of the PKC cascade when electrically induced to differentiate was determined by Kimura *et al.*, when they introduced a PKC inhibitor, which shut down the

differentiation process.[15] It was determined that differentiation was not an immediate result of the activation of the PKC cascade. In NGF-induced differentiation, NGF is not known to activate the PKC cascades.[15]

As previously mentioned in the cellular adhesion molecules section, Schmidt *et al.* also studied the effects of electrical stimulation on neurite outgrowth of PC12 cells when grown on polypyrrole coated indium tin oxide (ITO)-conductive borosilicate glass.[14] The polypyrrole was electrochemically deposited using a three-electrode electrochemical set up, where the working electrode consisted of an ITO glass working electrode, a platinum gauze counter electrode and a Ag/AgCl reference electrode. For their electrical stimulation of the PC12 cells experiments, Schmidt *et al.* applied a constant potential of 100 mV for 2 hours. For their electric stimulation of the PC12 cells, the polypyrrole film was used as the anode and at the opposite end of the well, a gold wire served as a cathode and a silver wire served as a quasi-reference electrode. These researchers reported that neurite extension grew most notably for the PC12 cells grown on the polypyrrole film which was electrically stimulated than compared to those PC12 cells grown on the same substrate but unexposed to electrical stimulation.[13] Those PC12 cells grown on the polypyrrole film which saw electrical stimulation had an average neurite length of 18.14 um (n=5643) while those grown on the polypyrrole film which were not treated with electrical stimulation had average neurite lengths of 9.5 um (n=4440). It was also observed by these researchers that there was no directional growth towards either electrode.

Park *et al.* also performed their own electrical stimulation experiments with PC12 cells which were grown on a layer of gold nanoparticles coating the top of a positively charged cover glass (pretreated with polyethyleneimine) and observed electrically-induced neurite outgrowth.[46] Park *et al.* used a similar electric stimulation set up as Schmidt *et al.*, with their gold nanoparticle coating serving as the anode.[14,46] These researchers applied either a constant or alternating potential of 250 mV for 1 hour after 24 hours of incubation to allow the PC12 cells to adhere to their substrate.[46] They reported that the alternating potential led to higher levels of cell survival (90%) versus the constant potential (70%).

As previous success has been shown using electrical stimulation of PC12 cells to induce differentiation, this cell line looks promising to complete our goals of neuronal differentiation driven solely by electrical stimulation. As electrical stimulation has been shown to induce differentiation faster,[14-15] this technique shows promise for coupling other analytical techniques to study the differentiation process in-situ, such as using infrared spectroscopy. It has been reported by Gunning *et al.* that the DNA/RNA concentrations decrease and increase respectively during the differentiation process of the PC12 cells under chemically induced differentiation conditions. Using Fourier Transform infrared spectroscopy, these the phosphate backbone stretching bands of DNA could be ideal to monitor during the electrically induced differentiation process.

1.8 Objectives

The long term objective of this project is to study the molecular processes that occur within the electrically induced differentiation process with FTIR. To achieve this goal, this MSc thesis is broken into several short-term objectives. One objective is to establish and determine the methodologies and ideal parameters required to grow neuronal cells on a conductive surface. Another objective of this thesis is to successfully induce differentiation through both chemical and electrical stimulation means, which will assist in approaching the long term goal of studying the electrically induced differentiation process with FTIR.

The first objective of this MSc thesis requires the development of a system suitable for the comparison of electrically stimulated preneuronal cells with chemically induced differentiated cells. To achieve this objective, it was important to first learn the fundamental basics of cell culturing and find a preneuronal cell line differentiable by chemical means. In order to electrically stimulate a neuronal cell to differentiate, it was necessary to find a conductive substrate which the cells can either grow directly on top of or through the aid of a chemical modification layer. Both substrate and chemical modification tests must be completed to ensure viability of the cells.

The chemically induced differentiation system must be developed and understood well enough to allow for comparison with any successful electrochemically induced differentiation experiment. An electrochemical system also must also be developed to successfully complete the goal of electrically driven neuronal differentiation. The PC12 cell line looks promising for this project due to its well-studied neuron model system and abundance of reports in the literature documenting successful chemical (NGF) and electrical differentiation.

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2. Experimental

Chemical reagents were obtained from Sigma Aldrich (St. Louis, MO) and used without purification, unless otherwise stated. All biological cells were generously supplied by Dr. David J. Schreyer (University of Saskatchewan, Cameco MS Neuroscience Research Center). All biological media was stored at 4°C while serums were frozen at -20°C and thawed to room temperature at time of use. Poly-L-lysine and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were stored at 4°C after thawing from storage at -20°C. Laminin (Ln), nerve growth factor (NGF) and all primary and secondary antibodies were stored at -80°C and thawed to room temperature at time of use. All experimental procedures were carried out at room temperature, unless otherwise noted.

2.1 Cell Culture

All cell culture procedures were completed within a biological cabinet to maintain a sterile environment. The biological cabinet was sprayed and wiped down with 70% ethanol along with any other items entering the hood, such as 6- and 12-well BD Falcon plates, media bottles, etc. Pipette tips and media glassware were autoclaved at 121°C for 20 minutes and dried in the autoclave. Deionized water and PBS was vacuum or syringe filtered through a 0.22 μm filter.

All cells were thawed from frozen (stored in liquid nitrogen) and immediately split for cell culture. Cells were cultured on BD Falcon tissue culture plates (10 cm diameter), passaging cells at approximately 80% confluency. For adherent NIH/3T3, RN46A and PC12 cells, the media was removed from the culture plate using a sterilized pipette tip (or Pasteur pipette) attached to a vacuum system. Fixed cells were washed in 5 mL of sterilized 0.01 M sodium phosphate buffer solution (PBS, pH=7.4) followed by 1 mL of 0.05% trypsin-EDTA in PBS. The trypsin-EDTA in the petri dish was allowed to coat the entire surface to lift the adhered cells off the tissue culture plates for passaging or for seeding and excess trypsin was removed. 10 mL of media was added to the petri dish to inactivate the trypsin from further cell breakdown. Mechanical assistance was used to achieve dislodgement of NIH/3T3 cells followed by careful

pipetting and releasing media to detach cells (avoiding the formation of bubbles in the media). Cultures were passed onto a new dish at a dilution of 1:2, 1:5, 1:10, and 1:20 with a total volume of 10 mL. NIH/3T3 and PC12 cultures were kept in a CO_2 incubator set at 37°C and 5% CO_2, while undifferentiated RN46A cultures were kept at 33°C and differentiation attempts were done at 39°C, both with 5% CO_2.

For seeding and subculturing PC12 cells grown in suspension, the 10 mL of detached cells were placed in a 15 mL falcon tube and centrifuged at 800 rpm and 4°C for 5 minutes to generate a small pellet of cells. The media was carefully removed through the pipette/vacuum system to avoid disrupting the cell pellet. Based on the pellet size, a small volume of media was added (typically up to 5 mL) and redistribution of the cells in the media was accomplished by repeated pipetting/repipetting. 80 μL of cell suspension was removed from the stock solution and placed into a 0.65 mL microcentrifugal tube. 20 μL of nigrosine or trypan blue was then added, both of which act as a dye exclusion or viability test, where live cells can exclude the dye and dead cells absorb the dye. The cell and dye mixture was mixed with light tapping of the microcentrifugal tube. 10 μL of the cell/dye mixture was added to each chamber of the hemocytometer and the cells inside the outer squares were counted.

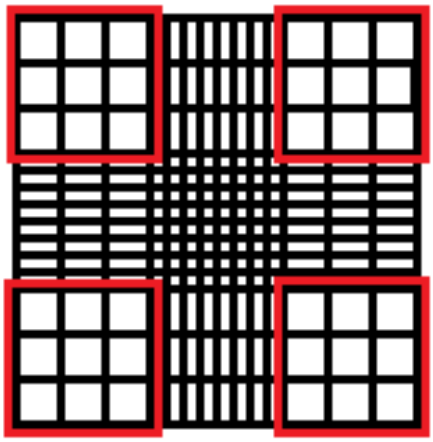

Figure 2.1. Example of a hemocytometer with the corner squares used for counting cells.

To obtain the most accurate cell count, both chambers were counted and verified to be within 90% consistency. The total cells per sample was calculated using the average of the total cells in the two chambers.

$$\text{Total cells per sample} = \frac{\text{Total cells counted}}{\text{Total area counted (mm}^2\text{)}} \times \frac{\text{Total dye dilution (uL)}}{\text{Volume of cells in dye dilution (uL)}} \times \text{Total dilution (mL)} \times 1000\ \text{mm}^2/\text{mL}^2$$

For seeding cells onto gold electrodes, both modified and unmodified gold surfaces were employed (see self-assembled monolayers section). Cells were then plated at densities of 2,500 or 5,000 (3T3), 20,000 (RN46A) and 75,000 cells/well (PC12) unless otherwise indicated. To fix the cells, the media was removed from the tissue culture plate and the tissue culture plate was washed with 3x1 mL of PBS, followed by fixation with cold methanol (-20°C, >99.98%).

2.1.1 3-Day Transfer, Inoculum 3x10^5 cells (NIH/3T3)

The growth medium for NIH/3T3 cells (CRL 1658, ATCC) consisted of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). NIH/3T3 cells were maintained directly on tissue culture petri dishes. Stock NIH/3T3 cells were harvested at 80% confluency and passaged at 1:10 or 1:20 dilutions.

2.1.2 RN46A cells

The growth medium for undifferentiated RN46A cells consisted of DMEM:F12 (1:1) with 10 % FBS with 250 µg/mL antibiotic G418. Cells were maintained at 33°C and 5% CO_2. To induce differentiation, the temperature was raised to 39°C and the medium was changed to serum free DMEM:F12 (3:1) with 1% bovine serum albumin (Boehringer-Mannheim), 1 µg/mL transferrin, 5 µg/mL insulin, 6.3 ng/mL progesterone, and 16.1 µg/mL putrescine.[1]

2.1.3 *Pheochromocytoma cells (PC12)*

The growth medium for the pheochromocytoma (PC12) cells (CRL 1721, ATCC) consisted of BioWhitter's™ RPMI 1640 with L-glutamine, 10% heat-inactivated horse serum (HI HS, H1270, donor grade, USA origin) and 5% fetal bovine serum (FBS). Heat-inactivation of the horse serum was completed by heating the serum at 57°C for 30 minutes. All experiments in this thesis involving the PC12 cell line were completed using passages 7-13 unless otherwise stated. Some experiments consisted of culturing PC12 cells in suspension, while others consisted of PC12 cells being cultured on laminin (L2020, from Engelbreth-Holm-Swarm murine basement) coated tissue culture plates. To induce (and maintain) differentiation of the adhered PC12 cells, 2.5S nerve growth factor (NGF, Cederlane Laboratories, Burlington, ON) prepared from male mouse submandibular gland was added to the medium. NGF was reconstituted as directed by manufacturer: dissolved in 0.02% acetic acid and diluted to a stock concentration of 10 µg/mL with culturing media, aliquoted and frozen at -80°C until time of use. In any experiment using chemical induced differentiation, the NGF was thawed and stock media containing 10 ng/mL was made at the time of experiment.

For control experiments where PC12 cells were induced to differentiate with NGF, cells were allowed to adhere overnight (Day 0), and NGF was introduced on Day 1. Media changes were completed on Days 3 and 5 for experiments going to Days 5 and 6, respectively. Optical images and immunocytochemistry were used to determine successful differentiation and neurite outgrowth. For electrical stimulation experiments using the interdigitated array of electrodes chip, the chip was coated in laminin for a minimum of 2 hours, followed by washing of excess laminin with PBS. Cells were then seeded and allowed to adhere overnight, followed by electrical stimulation the following day. For the electrical stimulation experiments using the FTO coated glass, following Ln coating the FTO coated glass was incubated in the incubator for 72 hours in a
6 cm tissue culture petri dish with the PC12 media. Cells were then seeded and incubated for another 3 days prior to any electrical stimulation experiments. Both incubation steps of the FTO coated glass was to ensure there was no signs of contamination.

2.2 Substrates/electrodes

Throughout the course of this thesis, many different substrates were used. For the culturing of the biological cells, tissue culture treated polystyrene dishes were used. In order to perform electrical stimulation experiments, a conductive electrode was required, which is non-native to the cell culturing procedure. Cell viability of these cells grown on these conductive surfaces, e.g. gold or FTO had to be determined prior to electrical stimulation.

2.2.1 Polystyrene tissue culture plates

The polystyrene 6-, 12-, and 24- well plates (BD Falcon) were sprayed down with 70% ethanol prior to entering the biological cabinet and opened inside to maintain manufacturer sterility. Tissue culture petri dishes packages were opened outside the hood taking care not to open any individual petri dish. For culturing PC12 cells, tissue culture plates which were not precoated with laminin, were incubated with laminin at 4°C for a minimum of 2 hours and used immediately after washing with 3x10 mL PBS. Precoated laminin coated tissue culture plates (BD Falcon) were kept at 4°C until time of use.

2.1.3 Glass coverslides

Round glass coverslides were sonicated in 20 mM sodium dodecyl sulfate (SDS) for 15 minutes, followed by sonication cycles of water and 70% ethanol each for 15 minutes. In between sonications, the glass coverslides were washed with deionized water (MilliQ, 18.2 mΩ) and stored in 70% ethanol solution. After each experiment using glass coverslides, the glass coverslides were discarded.

2.1.4 Polycrystalline gold electrodes

Polycrystalline gold electrodes (substrates) were prepared by melting gold in a graphite crucible under vacuum, followed by cooling to room temperature. The gold nuggets were placed in a polypropylene cup and then fixed with epoxy. The melted gold samples were then polished

to a flat surface using 120 or 180 grain sandpaper, followed by 400 and 600 grain sandpaper. The gold electrodes were then polished to a mirror-like shine using 6 micron diamond suspension followed by 3 and 0.5 micron diamond suspensions (Leco® Corporation, St. Joesph, MI) manually on a polishing pad. Chloroform was used to dissolve the epoxy and remove the gold electrodes from the polypropylene holders.

2.2.4 Interdigitated array of electrodes

Experiments performed on interdigitated array (IDA) of electrodes (ALS, Japan) required the housing of a single chip that had been lithographically printed with individually addressable counter, reference, and working electrode arrays (see **Figure 2.2**).

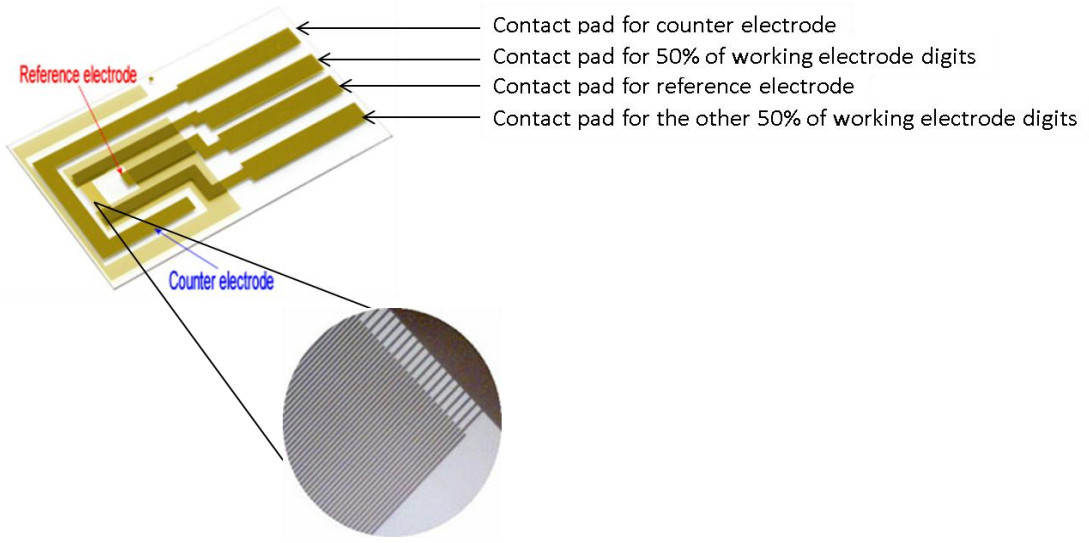

Figure 2.2. Interdigitated array of electrodes used for electrical stimulation experiments.[2]

This IDA of electrodes contained 65 pairs of 3 μm wide by 2 mm long Au working electrodes. The IDA of electrodes was cleaned through 15 minute sonication cycles of 20 mM SDS, deionized water, and 70% ethanol, with rinsing (deionized water) in between each step.

2.2.5 Fluorine-doped tin dioxide coated glass

Fluorine-doped tin dioxide (FTO) coated glass was a generous gift from the Paige Group at the University of Saskatchewan. Square foot sheets of this conductive glass were purchased from Pilkington which had 3.0 mm thickness and have resistivity of 8 Ωcm. All experiments using FTO-coated glass in this thesis made use of approximately 1 inch by 1 inch pieces. FTO coverslides were cleaned in Piranha solution (3:1 H_2SO_4:HOOH) (for a minimum of 30 minutes) followed by autoclaving at 121°C for 20 minutes and drying in an autoclave (90 minutes). Conductivity of the FTO coated coverglass was measured after each cleaning step to ensure no loss of the conductive dopant layer

2.3 Self-assembled monolayers & cellular adhesion promoting layers

11-amino-1-undecanethiol (AUT, >99%) was purchased and used without purification from Asemblon INC. (Redmond, WA, US) and Sigma Aldrich (St. Louis, MO). Polycrystalline gold electrodes (~40 mm^2) were immersed in 1 mM AUT in 95% ethanol for a minimum of 2 hours, followed by rinsing of excess (non-covalently attached) AUT with 95% ethanol. Gold electrodes and glass/plastic coverslips were coated in 10 μg/mL Ln in sodium phosphate buffer solution (PBS, 10 mM, pH 7.4) for 90-120 minutes (4°C) followed by 5x 1 mL sterile PBS washes. After rinsing, Ln coated substrates remained immersed in either PBS or culture medium to prevent drying out. Gold electrodes and glass/plastic coverslides coated with poly-L-lysine (PLL) were coated at 0.1 mg/mL (in sterile deionized water, MilliQ, 18.2 mΩ cm) overnight, followed by 5x 1 mL sterile water washes, and allowed to air dry in the biological cabinet.

For Ln-coating of FTO coverslides, these glass coverslides were placed in a 10 cm tissue culture plate and approximately 15 mL of 10,000 μg/mL Ln in PBS was added and allowed to

incubate for a minimum of 2 hours. The coverslides were then washed with 3x 15 mL PBS, and placed into individual 6- cm BD Falcon tissue culture plates. Media was added to these plates and allowed to incubate for a minimum of 3 days to ensure that no contamination had occurred and then seeded with the PC12 cells.

2.4 Immunocytochemistry

For cell counting purposes, 4'-6-diamidino-2-phenylindole (DAPI) was used; DAPI is a fluorescent molecule which can bind to DNA. A 300 nM dilution of DAPI in blocking solution (PBS with 2% horse serum and 0.5% bovine albumin serum (BSA)) was added to the sample and allowed to incubate in the dark for 30 minutes, followed by 3x 1 mL washing with PBS prior to imaging. For experiments where DAPI staining was coupled with immunocytochemistry, DAPI was added at the fluorescent tag labeling (secondary antibody) step. All immunocytochemistry was studied using a Zeiss Axiovert 100 inverted microscope which was connected to a Nikon Super High Pressure Mercury lamp. All optical images were captured using a Qimage Retiga Exifast camera and recorded with Northern Eclipse software (Empix Imaging Inc.).

For confirmation and visualization of differentiated PC12 cells, the fixed samples were placed in blocking solution for a minimum of 1 hour at room temperature. The role of the blocking solution is to block non-specifically all the receptors on the cell membrane so primary antibodies of interest can be added to specifically bind to their receptors. The primary antibodies were then detected using a fluorophore-conjugated secondary antibody.

Primary antibodies used in the completion of this thesis were: β-III-tubulin, anti-GAP-43 monoclonal antibody 9-1E12 ascites fluid, anti-glial fibrillary acid protein ascites fluid (anti-GFAP), anti-S-100 or anti-MAP2 monoclonal antibody which were diluted to: 1:400, 1:5000, 1:400, 1:1000 and 1:500 in blocking solution respectively. The samples were allowed to sit for a minimum of 2 hours. Samples were then washed 3x
1 mL in PBS, followed by adding the secondary fluorescent antibodies (Alexafluor® 555 or 488 (1:400 dilution in blocking solution), Fluorescein isothiocyanate or RhoD as specified and 3 nM DAPI in blocking solution samples for a minimum of 2 hours (dark). For transparent substrates,

optical images of PC12 cells were obtained and analyzed for differentiation: those PC12 cells which displayed process outgrowths at least ≥ 2x the cell body diameter were considered differentiated.

2.5 Atomic force microscopy

Atomic force microscopy (AFM) measurements were carried out in contact mode on a Dimension Hybrid Nanoscope system (Veeco Metrology Group, Santa Barbara, CA) with the microscope mounted in an acoustic-vibration isolation system. AFM measurements of methanol-fixed biological cells were completed in air and room temperature using a cantilever tip with a force constant of 0.06 N/m. A scan rate of
0.502 Hz or 1.00 Hz was used with a resolution of 512 samples/line.

2.6 Electrochemical Preparation

Electrochemical experiments were carried out in a specially designed electrochemical cell. Prior to electrochemical experiments, the electrochemical cell was cleaned in an acid bath (1:2 HNO_3:H_2SO_4) followed by rinsing in deionized water (MilliQ, 18.2mΩ cm). Electrochemical experiments with the IDA of electrodes, was completed using its internal counter reference as a counter, one working electrode as a reference and the other as a working electrode (unless otherwise stated). Electrochemical experiments completed with FTO and indium-doped tin oxide (ITO) were completed using a three electrode electrochemical cell where the working electrode consisted of either FTO or ITO coated glass electrodes, a platinum coil counter electrode, and a Ag/AgCl reference electrode. The platinum coil counter electrode was flame annealed prior to each experiment. A comparison of the electrochemical set up was also conducted with a hanging gold electrode. Testing of each electrochemical set up was completed using hexammineruthenium(III) chloride (99%) in sodium perchlorate which was also degassed in argon.

Cyclic voltammograms (CVs) were obtained using a homemade LabVIEW program which also included the HEKA Potentiostat PG590 (HEKA, Mahone Bay, NS, Canada) and a

multifunction DAQ card to test the electrochemical set up and each electrode. For electrical stimulation of PC12 cells, the HEKA Potentiostat PG590 in conjunction with another homemade LabVIEW program and the DAQ card was used to control the pulsing profile.

2.7 Electrical stimulation of PC12 cells

For electrical stimulation experiments, the electrochemical cell was housed in a waterbath maintained at 37°C (physiological temperature). The electrolyte used in the electrical stimulation of PC12 cells was the RPMI 1640 with 10% HI HS and 5% FBS. The pH of the media electrolyte was found to be stable enough to withstand electrochemical experiments less than 4 hours and no gas mixer was used in these experiments.

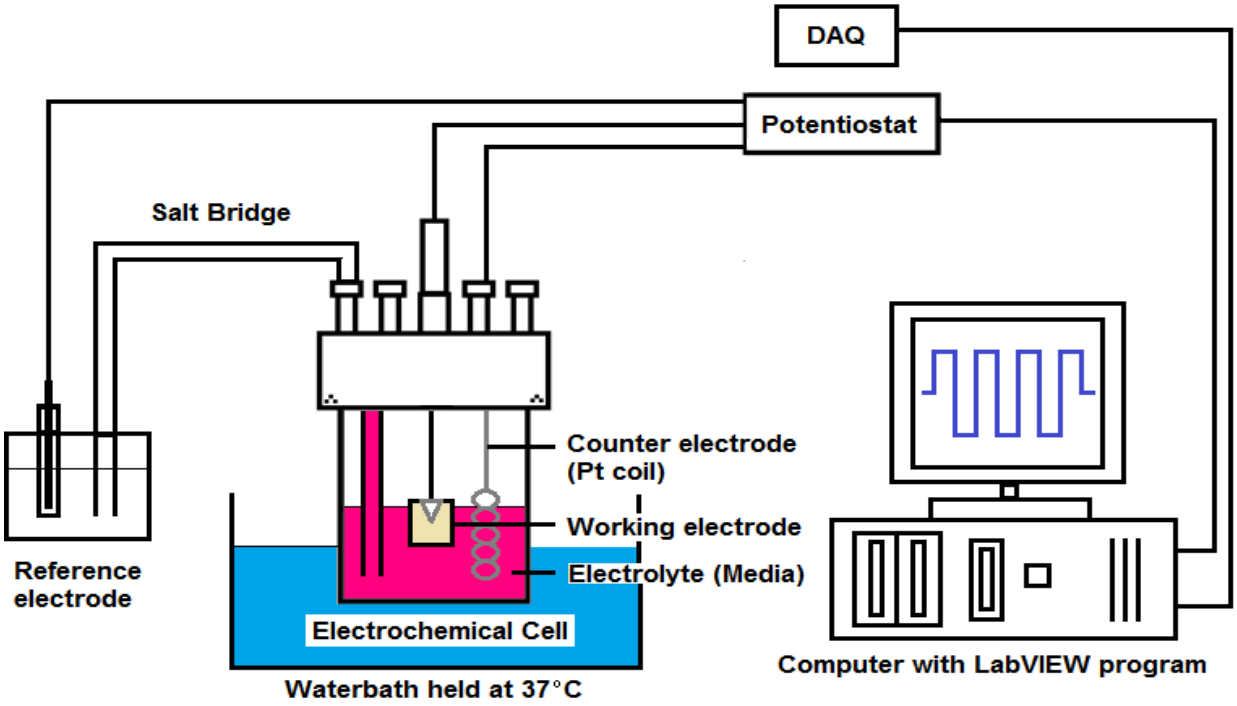

Figure 2.3. A schematic diagram of the electrical stimulation set up.

For the final electrical stimulation experiments with FTO, the RPMI 1640 with L-glutamine and 25 mM HEPES was used (Hyclone®) in place of BioWhitter™ (exhausted BioWhitter™ supply).

The homemade LabVIEW pulsing program allowed for the user to control several parameters: the frequency of the potential pulses, the type of the potential waveform (ex. square, triangle, sine, and sawtooth), the potential pulse amplitude, the duty cycle (only relevant if the user is applying a square wave excitation), and the potential offset due to the open circuit potential (OCP). Through the completion of this thesis, unless otherwise stated, a square waveform of 50% duty cycle was applied with amplitudes of 25, 50, 100, 250 and 500 mV from the OCP (see **Figure 2.4**). The frequency used for all electrical pulsing stimulation experiments was 0.50 Hz

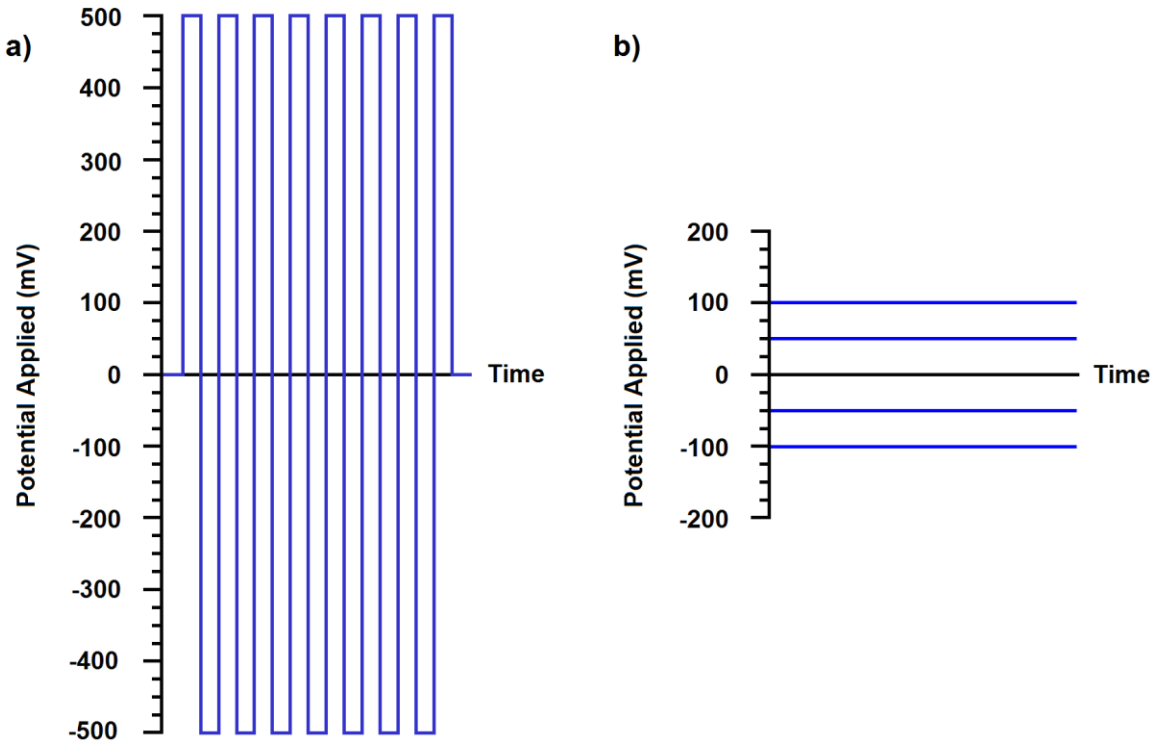

Figure 2.4. A sample waveform illustrating the electrical stimulation parameters used in this thesis: **a)** square waveform with amplitude of ±500 mV from the OCP at a frequency of 0.50 Hz (only the ±500 mV pulse is shown here). **b)** An applied constant cathodic or anodic potential of either ±50 or ±100 mV.

2.8 References

1 Andersen, P. L., et al. (2000). "Divergent regulation of GAP-43 expression and CNS neurite outgrowth by cyclic AMP." J Neurosci Res **61**(6): 626-635.

2 ALS Co. "IDA (Interdigitated Array) Electrode 3 um." Retrieved January 11, 2012, from http://www.als-japan.com/ida_3um.html.

3. Non-electrochemical Studies of Biological Cells

Three different cell lines: NIH/3T3 (fibroblast cells), RN46A and PC12 cells (preneuronal cells) were studied for their adhesion to conductive substrates that would be suitable for eventual electrical stimulation studies. Although NIH/3T3 cells are not neuronal precursor cells, the initial substrate studies were completed using this cell line since these immortalized fibroblast cells are relatively simpler to use to learn the basics of cell culturing and fundamental cellular biology techniques required throughout the completion of this MSc thesis. RN46A cells were initially used when moving toward the goal of electrical stimulation of neuronal and neuronal-like cells. RN46A cells can be induced to differentiate with temperature and biological media changes. After repeated unsuccessful differentiation attempts, the cell lines was changed once again to PC12 cells, a model neuronal-like cell line. A further advantage of switching to PC12 cells is the abundance of literature for the electrical stimulation of these cells in comparison to RN46A cells. In the literature, many differentiation studies with PC12 cells upon the addition of nerve growth factor and electrical stimulation have been successful.[1-4] As it was possible to differentiate PC12 cells through NGF addition (**Section 3.5.2**) this cell line should make it feasible to compare electrically and non-electrically induced PC12 cell differentiation.

3.1 Substrates

The role of the substrate upon which biological cells are grown can play an important role in the culturing, growth and survival of the cells. In the culturing of biological cells, the most common substrates for cells growth are either polystyrene tissue culture plates or glass coverslides. However, to induce neuronal differentiation through electrical stimulation a conductive surface is required. While there are reports of other research groups using gold as a conductive surface, these reports have all indicated that a self-assembled monolayer is required to anchor or attach the cells to the gold support.[5-8] Prior to the work reported in this thesis, there were no reports in the literature of successful cell attachment and growth on unmodified gold surfaces. In a review presented by Ostuni *et al.*[8] it was also reported that many mammalian cell types will undergo apoptosis if the cells are not grown on a substrate which allows them to

adhere and spread. The use of self-assembled monolayers (SAMs) which promote adherence becomes important in maintaining cell survival and growth. For all the cell lines studied in this thesis, attempts were made to grow cells directly on bare gold (unmodified) as well as modified gold (**Section 3.2**).

3.2 Self-assembled monolayers & cellular adhesion promoting layers

Throughout the literature, examples are provided demonstrating the need to modify the surface of conductive substrates such as gold with a self-assembled monolayer (SAM) or the use of a cellular adhesion promoting peptide to ensure cell immobilization and viability.[5-12] A natural compound, Ln, a synthetic polymer, poly-L-lysine and a self-assembling monolayer, 11-amino-1-undecanethiol (AUT) were utilized in an attempt to ascertain which was most efficacious for cellular adhesion. AUT which has a thiol headgroup, will covalently bind to the gold surface through Au-S bonding interaction while the amino head group will interact with the cellular membrane of the cells (**Figure 3.1**).

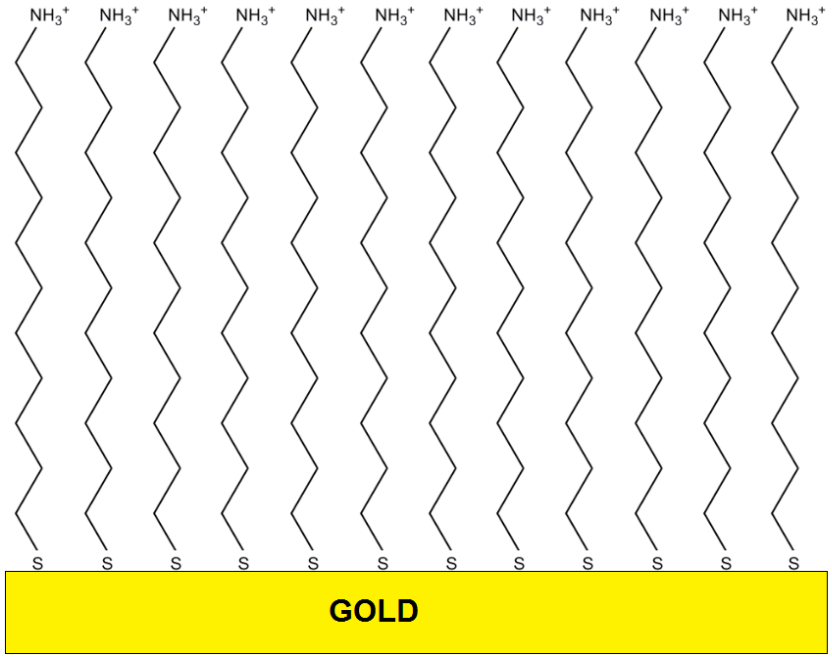

Figure 3.1. Schematic representation of a SAM of AUT on gold.

It has been reported in literature that adhering peptides which promote cellular adhesion onto a SAM-functionalized gold will result in both cellular attachment and improved viability of the cells grown on these SAM-functionalized gold surfaces.[6] The following combinations of adhesion layers were tested to find the optimal substrate for cell adherence and growth: PLL coated gold, Ln coated gold, PLL and Ln coated AUT-modified gold and unmodified gold. By testing each chemical adhesion layer it is possible to determine the ideal conditions necessary for future electrical stimulation experiments. Ideally, if it were possible to adhere the cells directly onto unmodified gold or a single chemical adhesion layer, there would be fewer chances for bacterial or fungal contamination. Atomic force microscopy and optical microscopy were used to qualitatively compare the cells grown on different substrates to ensure the growth substrate and unmodified gold did not negatively affect the cells, as there have been reported studies where gold nanoparticles and other metal substrates can cause cellular cytotoxicity.[13-14]

3.3 NIH/3T3 Cells

Although the majority of this thesis will focus on the PC12 model neuronal cell line, the initial experiments were completed on NIH/3T3 cells to learn the cell culturing techniques required to complete this thesis and to determine potential candidates for substrate and chemical modification layer for the neuronal cell line. NIH/3T3 cells are a cell line derived from NIH Swiss mouse embryos which display fibroblast cell morphology.[15] These cells have been well studied and characterized since the mid 1960s.[16-18] NIH/3T3 cells have a relatively quick turnover rate (less than 24 hours for doubling was observed) and can grow directly on polystyrene tissue culture plates.[15] The quick turnover rate and adherence properties of this cell line make it an ideal candidate for substrate and chemical modification layer tests.

3.3.1 Chemical modification layers and substrate study

After learning the cell culturing techniques, the project progressed towards substrate and chemical modification layer studies with the NIH/3T3 cells. NIH/3T3 cells were seeded onto modified and unmodified gold substrates for 2 and 3 days, respectively, at approximately 3,200 and 25,000 cells/well to determine the ideal seeding density as well as a method testing cellular viability to each of chemical modification layers and the gold substrate.

To confirm attachment, the NIH/3T3 cells grown on the modified and unmodified gold samples were fixed with cold methanol and counted using DAPI which fluorescently labels the nuclei of the cell. Using immunocytochemistry along with an inverted microscope allowed for the recording of the images and the counting of the number of cells (nuclei) attached to each sample. The DAPI staining confirmed that NIH/3T3 cells could grow on unmodified gold and in one trial there was preferential growth on bare gold versus the other growth substrates (**Figures 3.2** and **3.3**).

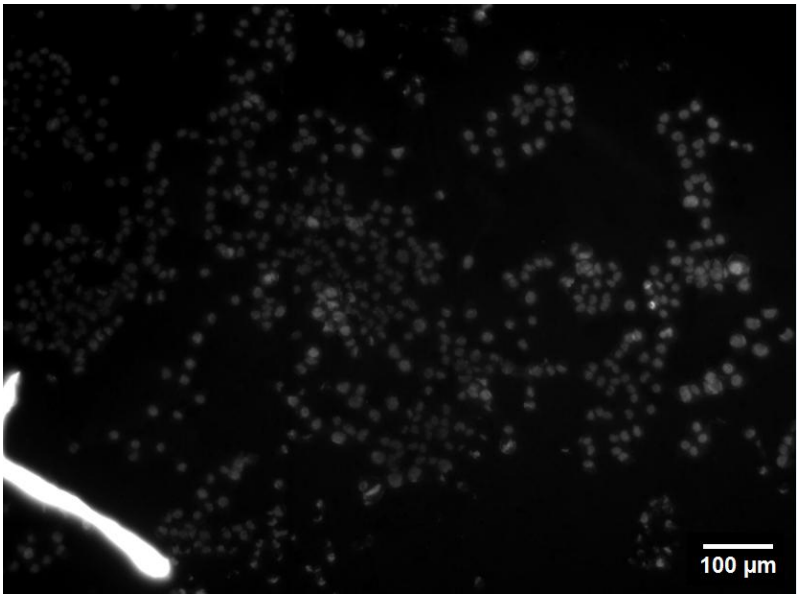

Figure 3.2. A DAPI-labelled image of a NIH/3T3 cells grown on bare gold which shows a large population of NIH/3T3 cells.

Counting and normalizing the DAPI-labelled NIH/3T3 cells grown on the various modified and unmodified gold surfaces shows no significant preference for any particular chemical modification layer (**Figure 3.3**).

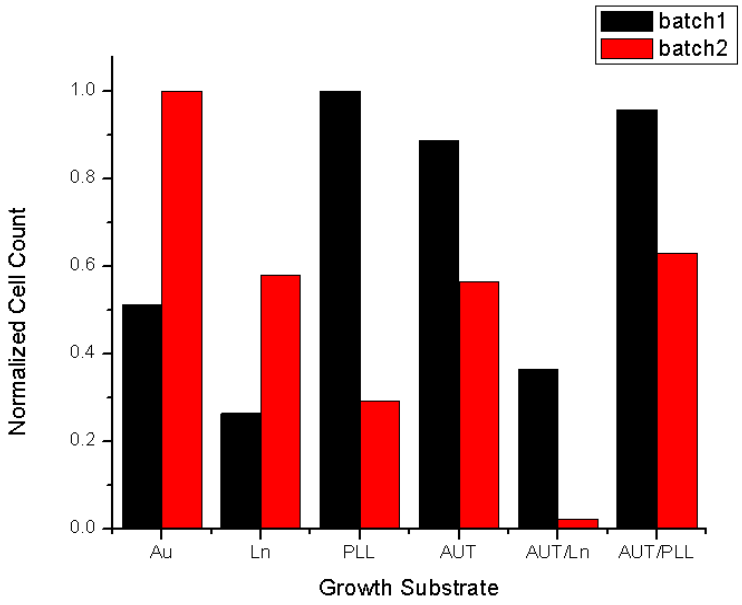

Figure 3.3. Normalized cell counts of NIH/3T3 cells as grown on various substrates to test substrate viability (individual images captured approximately 0.84 mm^2; random sampling was employed for cell counts, approximately 9 images per sample were taken to a total of 7.6 mm^2 of the overall ~40 mm^2): Ln = laminin, PLL = poly-L-lysine, and AUT = 11-amino-1-undecanethiol. Batch 1 and batch 2 were completed at an initial seeding density of 3,200 and 25,000 cells/well, respectively.

In both trials of different seeding densities, NIH/3T3 cells showed attachment onto all of our modification layers. While two different seeding densities were initially chosen to determine the ideal seeding density for the duration of the experiment, the chemical modification study must be repeated using the same seeding density and durations. While progressing towards a preneuronal cell line, attempts at repeating the chemical modification layer preference study were attempted but not aggressively pursued.

3.3.2 Atomic force microscopy

Atomic force microscopy (AFM) images of the NIH/3T3 cells were obtained which were initially collected with the intention of confirming cellular adherence to the gold substrate as well as the smoothness of the melted and polished polycrystalline gold substrates. It has been previously reported that biological cells prefer smooth surfaces for cell adhesion and viability.[19]

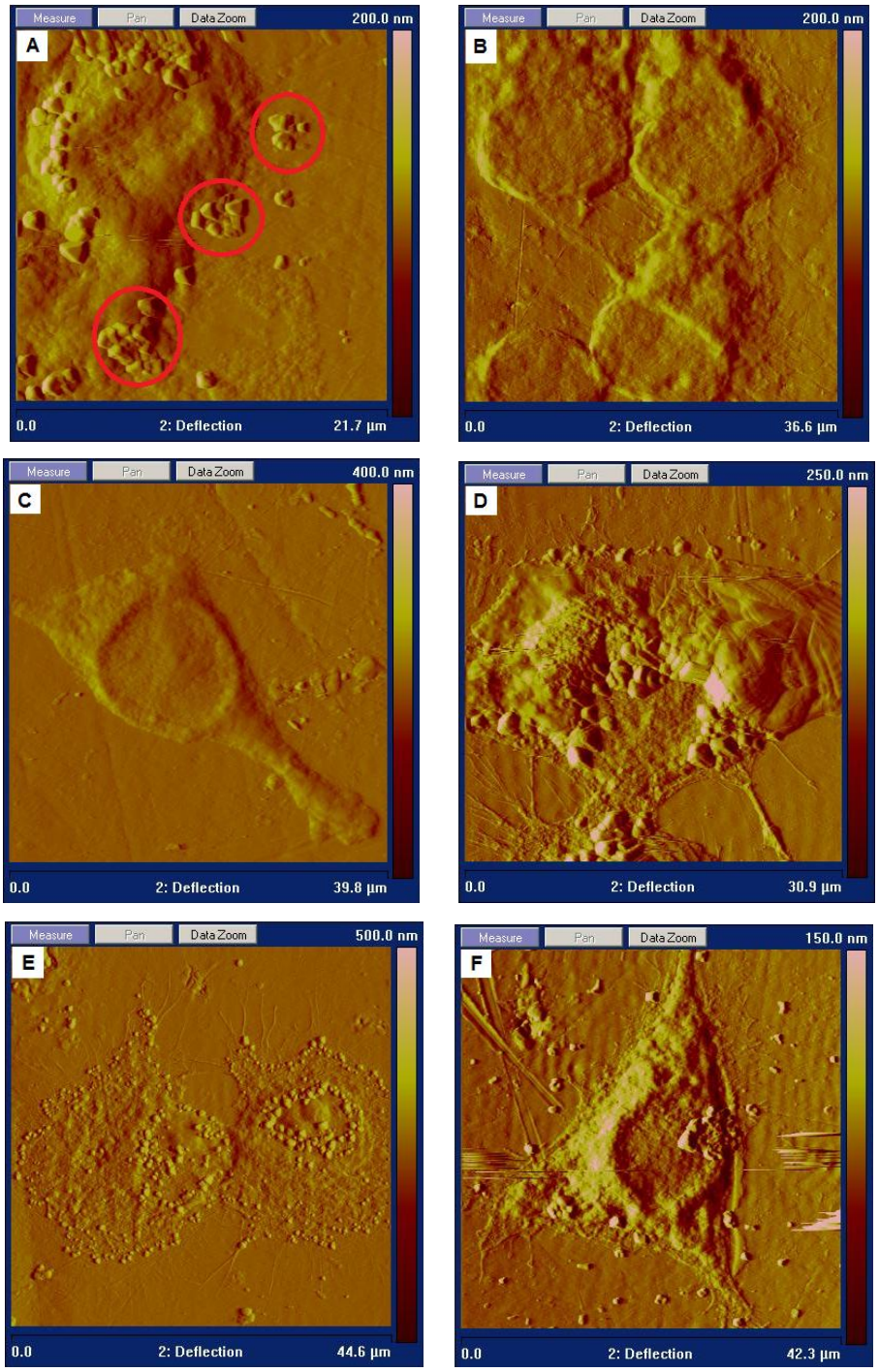

Figure 3.4. AFM images of methanol-fixed NIH/3T3 cells grown on **(a)** unmodified gold with red circle identifying selected salt crystals, **(b)** poly-L-lysine coated-gold, **(c)** laminin-coated gold, **(d)** AUT-modified gold, **(e)** poly-L-lysine coated AUT-modified gold, **(f)** laminin-coated AUT-modified gold.

Analyzing AFM images of the NIH/3T3 cells, morphological defects were observed that varied upon chemical modification layer choice. It can be seen from **Figure 3.4a** (unmodified gold), that with insufficient PBS washing of the cells prior to methanol fixation, salt crystals remained on the gold substrate (red circles). Comparing the NIH/3T3 cells grown on unmodified gold, PLL coated gold and laminin coated gold, these cells were circular (cells in **Figure 3.4a**) or elongated in shape (cell in **Figure 2.4c**)**.** In **Figure 3.4c** a clearly defined circle in the center of the cell can be seen which is believed to be the nucleus. The size and shape of the suspected nucleus is comparable to a fluorescently stained image of a NIH/3T3 cell reported by immunocytochemical supplier, abcam®. It was necessary to compare the AFM image to a separate sample that had been fluorescently stained and imaged since at the present time, immunocytochemistry data and AFM topographical data can not be obtained simultaneously.

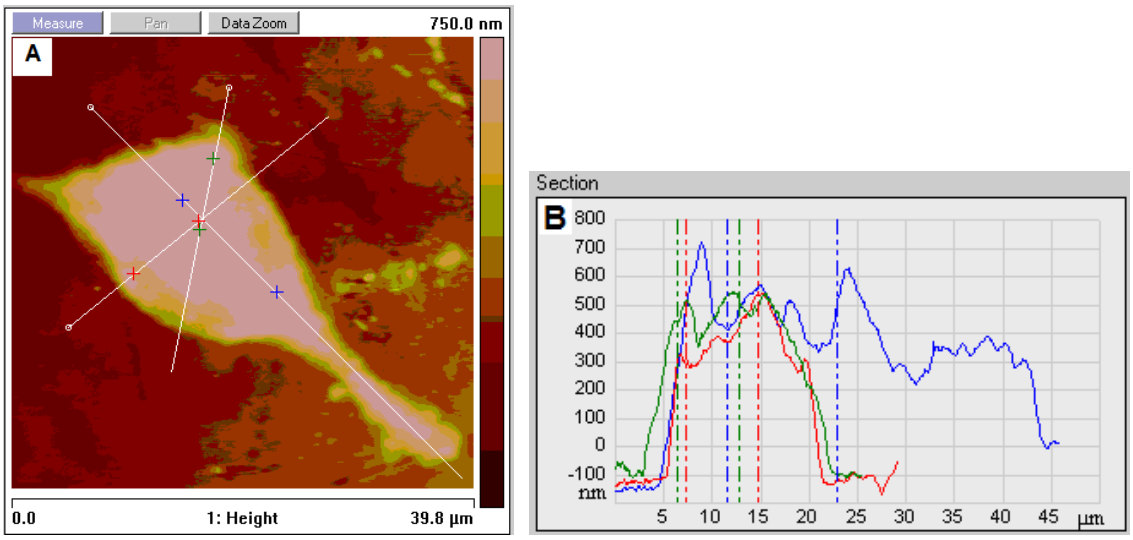

Figure 3.5. (a) Height-mode AFM image of a NIH/3T3 cell grown on laminin-coated gold and **(b)** the corresponding height profile: the nucleus contributes to the increase in height found in 10-20 um range.

Inspection of the images of the NIH/3T3 cells grown on AUT modified gold (**Figure 3.4d**), reveals the presence of perinuclear blebs or swelling which was unobserved in bare, PLL- and Ln-coated gold samples. The perinuclear blebs were found in the majority of the cells grown on AUT-modified gold with fewer cells observed with the circular and typical fibroblast cell appearance compared to those adhered on unmodified, PLL- and Ln-coated gold surfaces. The NIH/3T3 cells with the perinuclear blebs can be colloquially referred to as "unhealthy" cells. A closer look at the NIH/3T3 cells grown on AUT-modified gold images (**Figures 3.4 d** and **f**) also appear to have small flat processes (finger like extensions) which are not apparent on unmodified, PLL- or Ln-coated gold.

In 2009, Jans *et al.* reported SAM-functionalized gold alone is not sufficient enough for cells to grow on gold.[6] However, their mouse hippocampal neurons (primary cultures) could not survive past 1-2 days on SAM-modified gold,[6] whereas in this work NIH/3T3 cells (immortalized cultures) were seen to grow for several days (minimum 4 days) although they had an unhealthy appearance. NIH/3T3 cells also showed adherence directly onto gold, without the aid of a chemical modification layer in both trials. In the second trial, the NIH/3T3 cells showed preference towards the bare gold substrate vs. the other substrates. Avoiding the use of a chemical modification adhesion layer to promote cellular adhesion could be advantageous: there is less opportunity for contamination during the duration of the experiment, as well as cost benefit and avoiding misinterpretation of chemical signatures in spectroscopic data.

Jans *et al*.'s results also suggested that linking a peptide which supports cellular adhesion to the SAM-functionalized gold greatly supports the growth and viability of their cultures.[6] Although my results suggest there is more preference for AUT-modified gold vs. Ln- coated AUT-modified gold, my results are not as conclusive as more trials need to be conducted. Jans *et al.* also reported that coadsorption of the cell medium proteins could cause altered cell morphology. This could be linked to the observed perinuclear blebbing and assist in the visibility of the cytoplasmic processes for my NIH/3T3 cells. However more experiments, such as using mixed SAMs would be required to determine this conclusively. For NIH/3T3 samples grown on PLL- or Ln-coated AUT-modified gold, there were less signs of unhealthy cells, however, the perinuclear blebbing and cytoplasmic processes common to AUT-modified gold was still seen.

While in some of the AFM images (such as **Figures 3.4 a, b,** and **d**) it is difficult to identify the subcellular components of the cell, it is possible to potentially identify some of these subcellular components in the two cells (potentially undergoing cellular division) grown on PLL coated AUT-modified gold (**Figure 3.4e** and **Figure 3.6**).

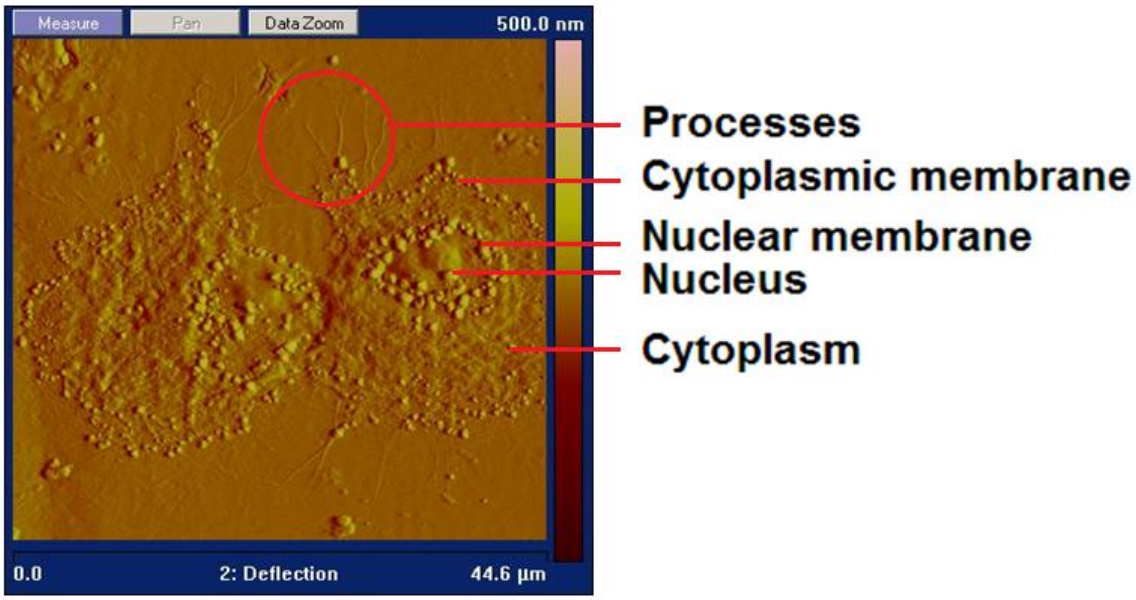

Figure 3.6. AFM image of two NIH/3T3 cells grown on PLL-coated AUT-modified gold.

Starting from the outside of the cell, processes are observed extending from the plasma membrane which is believed to be the beading observed around the cell. While it is impossible to identify the organelles in the cytoplasm (interior of the cell) using AFM, it is possible to identify the nuclear membrane as the thicker interior circular beading which encloses the nucleus (smaller circle).

3.3.3 Conclusions to NIH/3T3 study

Using NIH/3T3 cells, the initial cell culturing techniques required for the completion of this thesis were learned. Testing different potential chemical modification layers of gold substrates, it was determined that NIH/3T3 cells attached onto each of the chemical modification layers as well as bare gold. Randomly imaging of NIH/3T3 cells grown on each substrate with AFM, a potential morphological defect (perinuclear swelling) was observed on those cells grown on AUT modified gold substrates. The AUT-modified gold results are similar to those of Jans *et al.*, however my NIH/3T3 cells were able to proliferate and survive for a minimum of 4 days. The correct choice of modified substrates thus appears important in regulating cellular morphology which may in turn reflect chemical or biochemical changes within cells detectable by electrochemical and IR spectroscopic techniques.

3.4 RN46A cells

As one of the main goals in this thesis is to electrically stimulate neuronal cells to induce differentiation it was imperative that to the cell biology progressed towards a preneuronal cell line, as the NIH/3T3 cells are a fibroblast cell line. Efforts were shifted toward the RN46A cell line which are derived from embryonic rat raphe nucleus neuron precursors.[20] RN46A cells have a temperature-sensitive mutant of the SV40 large T-antigen which at 33°C drives cellular proliferation.[21] When the temperature is increased to 39°C, SV40 large T-antigen can no longer drive cellular proliferation and instead adopts a neuronal phenotype which includes the extension of long processes.[21]

3.4.1 Chemical modification layers and substrate study

Similar to the NIH/3T3 cells, the adherence of RN46A cells was tested on modified and unmodified gold substrates to determine the ideal substrate for future electrical stimulation experiments (see **Figure 3.7**).

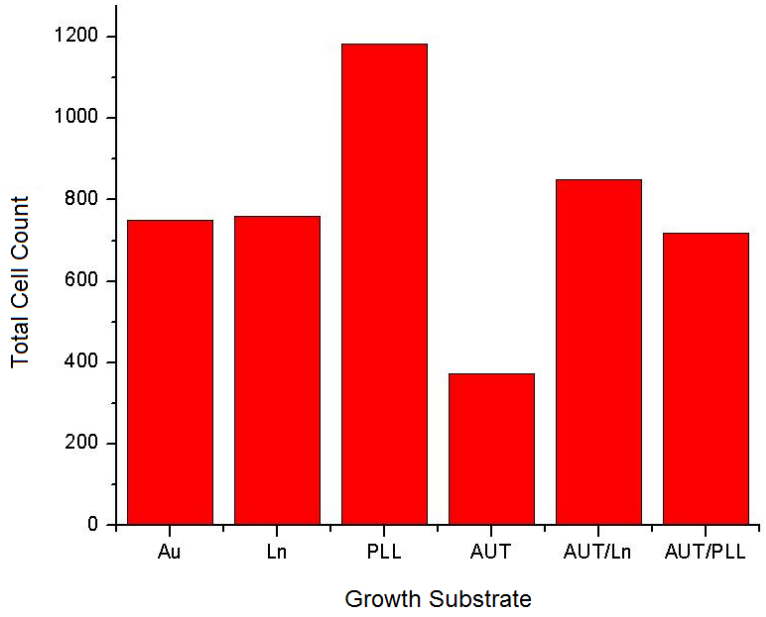

Figure 3.7. Total cell counts of RN46A cells as grown on various substrates to test substrate viability (random sampling of 10 images per sample, a total of 8.4 mm^2 of cells counted from the overall ~40 mm^2): Ln = laminin, PLL = poly-L-lysine, and AUT = 11-amino-1-undecanethiol

Similar to NIH/3T3 cells, RN46A cells displayed cellular adherence to all tested modified and unmodified gold substrates. RN46A cells had preferential growth on PLL coated gold which agrees with observations noted in literature, as most experiments are performed on polylysine or collagen coated glass or plastic substrates.[20-22] However, concurrent attempts to differentiate the RN46A cells were problematic (**Section 3.4.3**) which limited the number of experiments of this nature that were performed. Consequently, this aspect of thesis work was not aggressively pursued, but it would be desirable to conduct more trials to confirm these results.

3.4.2 Atomic force microscopy

AFM images of the undifferentiated RN46A cells were collected to determine if the choice of modified substrates affects the RN46A cell morphology in an analogous fashion to what was observed for the NIH/3T3 cells (see **Figure 3.8**). If a morphological effect is observed this could lead to future problems when trying to differentiate the RN46A cells (due to an unknown effect on the cell itself).

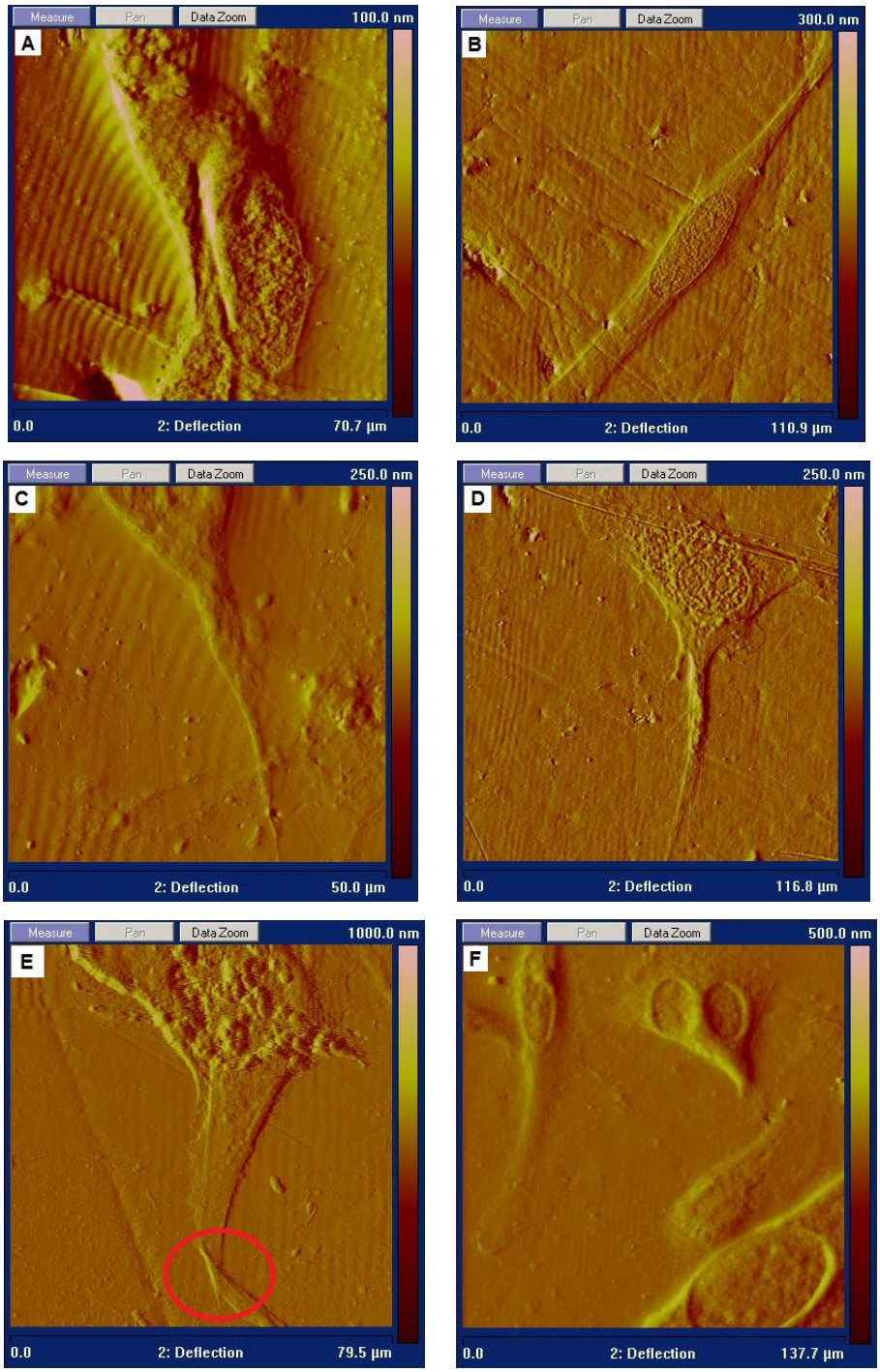

Figure 3.8. AFM images of methanol-fixed RN46A cells grown on **(a)** unmodified gold, **(b)** poly-L-lysine coated-gold, **(c)** laminin-coated gold, **(d)** AUT-modified gold, **(e)** poly-L-lysine coated AUT-modified gold, **(f)** laminin-coated AUT-modified gold.

The nuclei of the RN46A cell are more easily identifiable for these neuronal cells compared to the fibroblast (3T3) cells. The nuclei are characterized by the circular or oval shape in the approximate center of the cell body. The cytoplasmic processes are characteristic of the AUT modified gold as they were in the NIH/3T3 cells however they are slightly difficult to see perhaps due to the size difference between cell lines (**Figure 3.8**).

Figure 3.9. Two cells grown on laminin coated AUT-modified gold showing the processes extending from the plasma membrane (enlarged from **Figure 3.8f**).

Several RN46A cells grown on AUT and AUT-PLL coated gold did contain perinuclear blebs (**Figure 3.8e**) which was not present when grown on unmodified, PLL- and laminin-coated gold, or even on normal PLL coated glass growth conditions (**Figure 3.10**). The cell grown on PLL-coated AUT-modified gold (**Figure 3.8e**) also showed a foldover of the process outgrowth. The cause of this foldover could be due to an artifact of the fixation process or an AUT-induced morphological defect; however more experiments need to be conducted to determine the direct cause of the foldover.

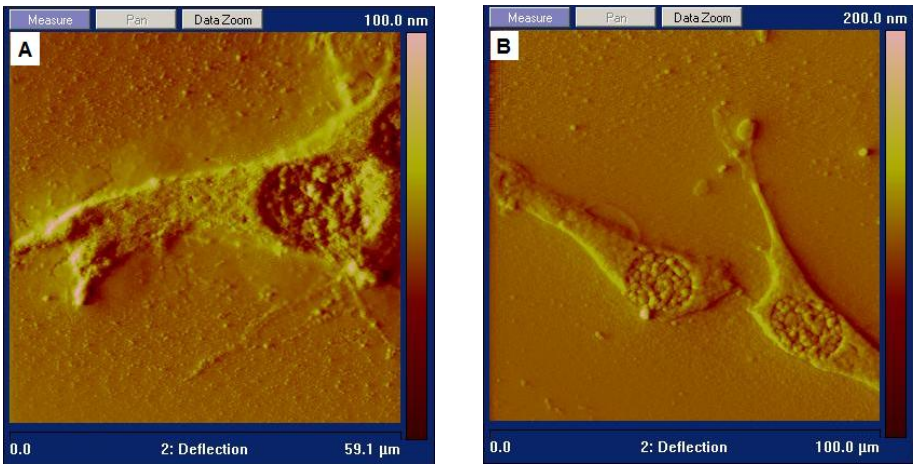

Figure 3.10. AFM images of RN46A cells plated on **(a)** glass and **(b)** PLL-coated glass coverslides.

The shape of the RN46A cells grown on unmodified or PLL-coated is similar to cells grown under normal PLL coated glass conditions. It was also found that while surface defects do exist on the gold substrates, it did not hinder cellular adherence for either cell lines.

From immunocytochemistry and AFM images, it was determined that NIH/3T3 and RN46A cells, which are both adherent cell lines, can attach directly onto unmodified gold surfaces. It was decided to approach any future experiments with NIH/3T3 and RN46A cells with unmodified gold to avoid any potential effect the monolayer may have on the cells. In addition, electrostimulation is most effective when the cell is in intimate contact with the conductive electrode. The presence of a dielectric or ion charged modifying layer could greatly diminish the effective potential drop across the cell during electrostimulation.

3.4.3 Differentiation attempts

RN46A cells are reported to differentiate upon a media change and with thermal activation.[20-22] Once differentiation occurs, cellular proliferation should cease and process outgrowth should begin. However, it was not possible to get the RN46A cells to differentiate on both gold and poly-L-lysine coated glass coverslides under previously reported differentiation conditions.[21] Instead, it was found that RN46A cells did not survive the media and thermal changes. After several attempts to differentiate the RN46A cells, it was concluded that a new cell line, PC12 cells, which are a model neuronal cell line often used to understand the biological processes associated with various stimulation would be better suited for this project.

3.4.4 Conclusions to the RN46A study

Similar to the NIH/3T3 cells, RN46A cells were able to adhere to all of the chemical modification layers as well as bare gold, however significant preference was found for poly-L-lysine coated gold. Morphological defects (perinuclear swelling) were also observed by AFM imaging when the RN46A cells were adhered on an AUT-modified gold substrate. Although only one trial of the chemical modification layers and substrate test was completed due to problems getting the RN46A cells to differentiate through chemically induced means, these cells

were shown to also provide competitive adherence on bare gold. Later attempts at repeating trials were completed; however the RN46A cells would not survive the thawing (or transportation) process. The novelty of the result that cell lines properly adhere to bare gold is of general interest to the field of cell engineering and future studies should be completed to confirm this result.

3.5 PC12 cells

As previously mentioned in **Section 1.3**, PC12 cells are derived from transplantable rat adrenal pheochromocytoma. These cells are a model neuronal system, and have been used widely to understand biological processes involved in the differentiation of neuronal cells. These cells were an ideal candidate after the unsuccessful differentiation attempts with the RN46A cells, since cellular differentiation can be induced by the addition of a single chemical compound (as opposed to the complexity of the differentiation conditions (both a thermal and nutrition change) required of the RN46A cells.

3.5.1 Chemical modification layer and substrate studies

The major disadvantage of PC12 cells are their poor adherence on solid surfaces. While NIH/3T3 and RN46A cells are both adherent cell lines, PC12 cells are only weakly adherent and need cellular adhesion layers to become attached to substrates. Once again, the same modified and unmodified gold surfaces used for the previous cell lines were tested and it was found that little to no viable cells were attached to unmodified gold (**Figure 3.11**). Surveying the literature, it was found that many successful attempts to study PC12 cells use laminin, polylysine (D- or L-) or collagen to promote cellular adhesion of the cells onto glass or polystyrene substrates otherwise the PC12 cells simply remain in suspension.

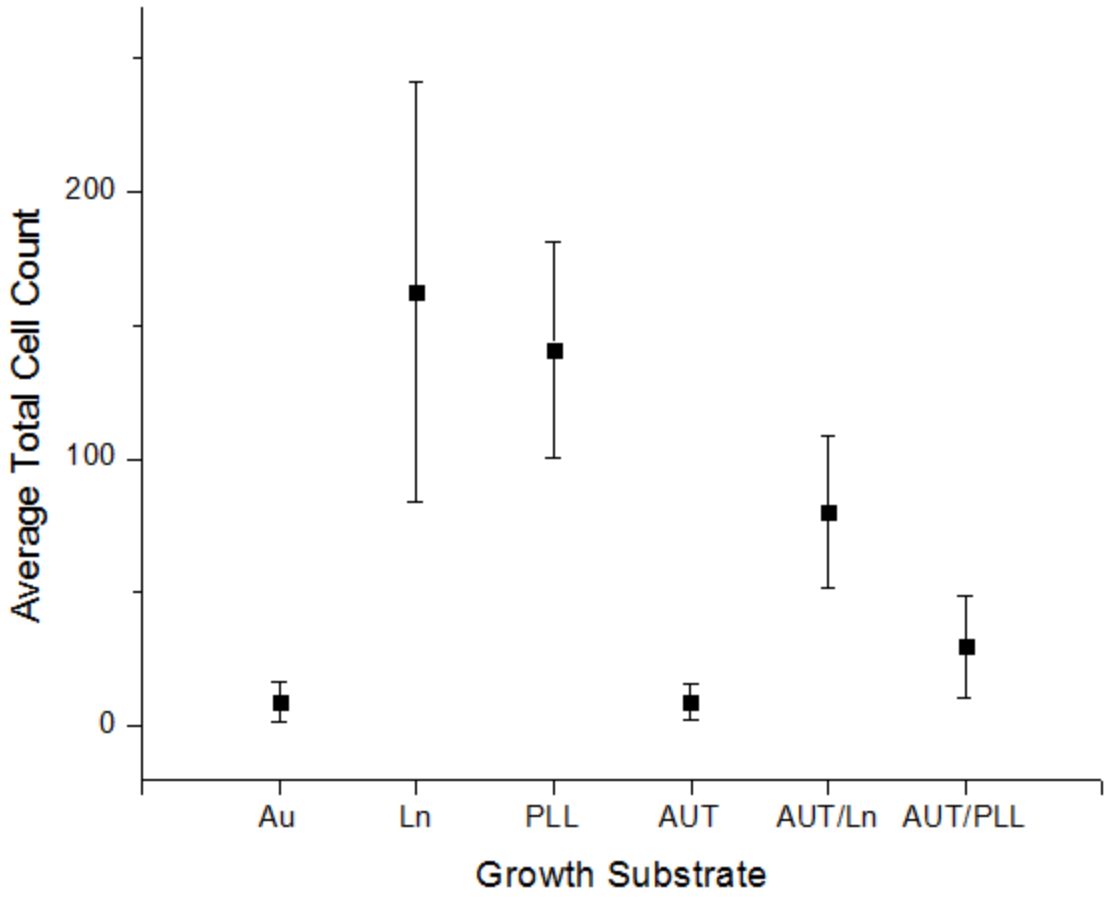

Figure 3.11. Average total cell count of PC12 cells on each 40 mm^2 modified and unmodified gold electrode, measured using DAPI-labelled nuclei. PC12 cells were seeded at a density of 20,000 cells/well and counted after 4 days: Ln = laminin, PLL = poly-L-lysine, and AUT = 11-amino-1-undecanethiol, n = 3 trials.

With significant adherence preference for laminin-coated gold, all future experiments used laminin coated substrates (glass, gold, IDA of electrodes, and FTO). No attempts were made to differentiate PC12 cells on bare (unmodified) gold, AUT-PLL coated and AUT modified gold due to low undifferentiated population counts. Although substrate studies were completed using a seeding density of 20,000 cells/well, all future studies were completed using a seeding density of 75,000 cells/well unless otherwise stated, as this seeding density allowed an ideal confluency of PC12 cells (without overgrowth) over the duration of experiments (~ 6 days). Initial experiments were conducted using PC12 cells subcultured in suspension.

3.5.2 Chemically induced differentiation of PC12 cells

PC12 cells are reported to differentiate upon the addition of NGF to their media mixture.[1-2,23] When these cells differentiate, the cells change from circular shaped to a stretched and flatted cell body with neurite extensions which proceed from the cell body. Since the first reported NGF induced differentiation (50 ng/mL) by Greene and Tischler in 1976 to present time, varying concentrations of NGF (from 5 to 100 ng/mL) have been reported.[2,24-26]

With assistance from Tangyne Berry (Tissue Engineering, University of Saskatchewan), a dose-response curve was obtained based on increasing concentrations of NGF: 0, 5, 10 and 30 ng/mL. PC12 cells were grown on laminin coated on top of poly-L-lysine coated chambers slides. Optical images were obtained of the PC12 cells showing varying levels of differentiation. Only cells which had neurite extensions or process outgrowths to be at least double the length of the cellular body were considered to be differentiated.

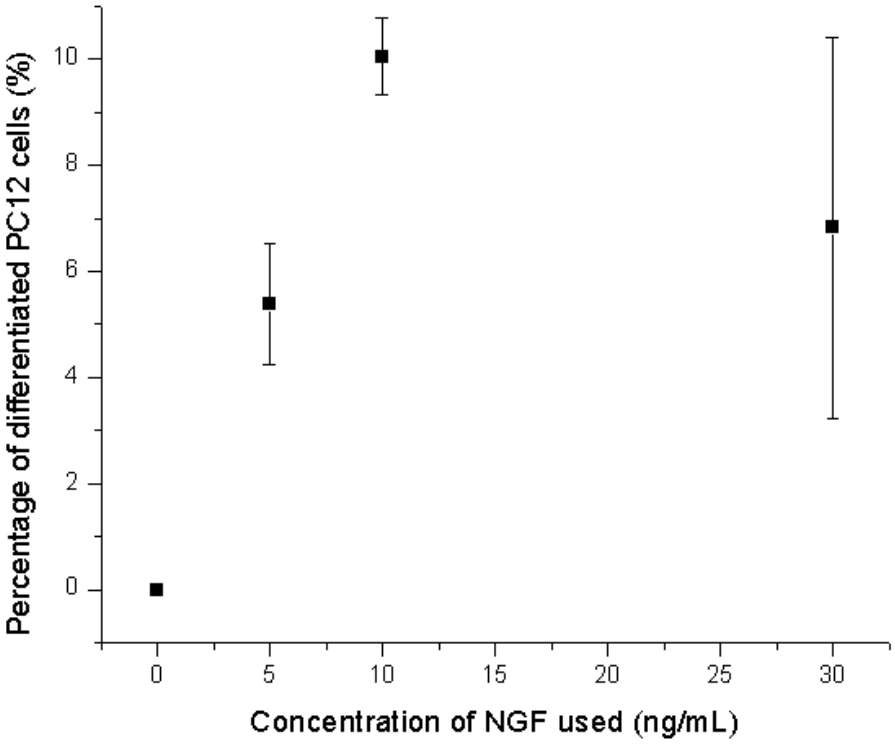

Figure 3.12. NGF-dose response curve to determine ideal NGF concentration to drive PC12 differentiation, n=3 trials.

The data shown in **Figure 3.12** is comparable with the NGF dose-response curve reported by Wu *et al.*.[26] In Wu *et al.*'s NGF response curve, similar concentrations of NGF were used and a similar plateau effect was observed [26] However, their curve extended to 100 ng/mL where the maximum stimulation of neurite outgrowth was seen. Since 10 ng/mL NGF for 5 days seems to be at maximum in my dose-response curve, this NGF concentration was chosen for chemically-induced differentiation experiments.

PC12 cells not exposed to NGF were circular in shape with diameters of approximately 10-20 µm. Within 24 hours of NGF exposure, the flattening of the cell body and the beginning of the process outgrowth were observed. Significant process outgrowth was observed with higher concentration of NGF for the same duration of time. For those differentiated PC12 cells, the cellular body extended up to approximately 25-30 µm with outgrowth extensions of approximately 1-5 µm width (length depended on both NGF dosage and duration of NGF exposure).

For completeness it should be noted that although the extent of differentiation (neurite extension length) was further progressed for PC12 cells exposed to 30 ng/mL NGF, identification of which neurite outgrowth belonged to which cell body at the higher concentration was extremely difficult to determine. However, preliminary experiments were completed using 100 ng/mL NGF to ensure that both the PC12 cells and the NGF stock solution used were suitable for inducing neuronal differentiation.

3.5.3 Atomic force microscopy

Similar to the NIH/3T3 and RN46A cell lines, AFM images of both undifferentiated and differentiated PC12 cells were obtained. However these studies were only performed on a subset of the chemical adhesion layers. Due to the low population of PC12 cells grown on bare gold, AUT-modified gold and PLL-coated AUT-modified gold, these AFM images were not collected.

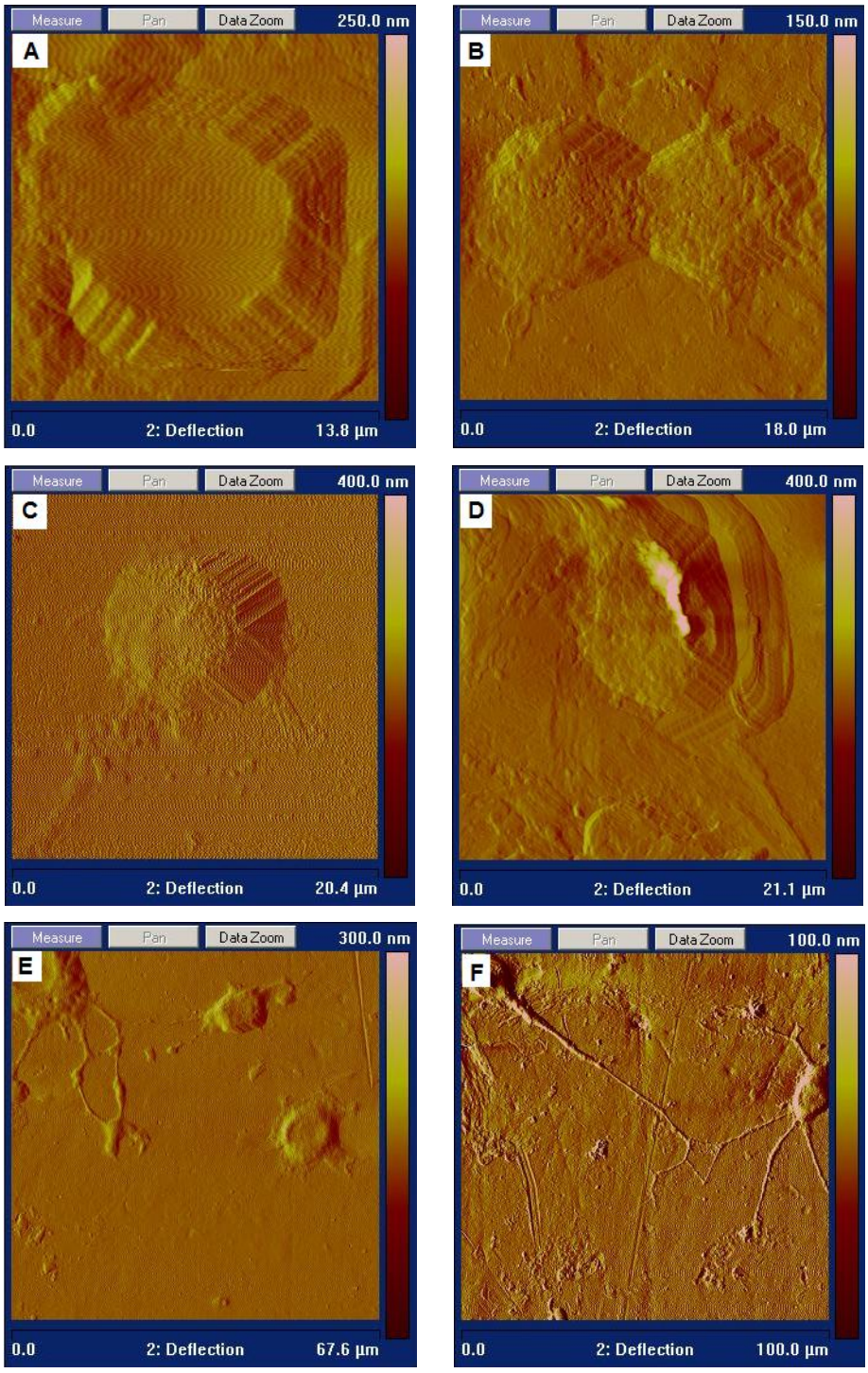

Figure 3.13. AFM images of undifferentiated PC12 cells grown on **(a)** PLL-coated gold, **(b)** laminin-coated AUT-modified gold, **(c)** laminin-coated gold; differentiated PC12 cells grown on **(d)** PLL-coated gold, **(e)** laminin-coated AUT-modified gold, **(f)** laminin-coated gold exposed to 100 ng/mL of NGF for 6 days

On the remaining substrate surfaces undifferentiated PC12 cells are seen to be circular in shape (approximately 10 μm) and in images of differentiated cells (especially **Figure 3.13f**) the cell body becomes elongated and there is a clear indication of neurite extensions that were absent in undifferentiated PC12 cells.

It is not possible to conclusively determine if AUT has an unfavourable morphology effect on the PC12 cells other than noting the correlation that AUT modified gold decreased the number of viable cells. Similarly, no observations of processes extending from the plasma membrane and perinuclear swelling of PC12 cells on AUT modified surfaces could be made due to difficulties in obtaining AFM images of samples with small population counts. However it is possible to conclude that for both NIH/3T3 and RN46A cell lines, AUT did have a negative effect on the morphology of these cells and AUT-modified gold negatively affects the viability of PC12 cells.

3.5.4 Immunocytochemistry & cell counts

For PC12 cells, DAPI staining is especially useful since these cells are naturally found in clusters. Counting groups of clustered cell is very difficult especially when using the bright field microscope alone (**Figure 3.14a**). As shown in **Figure 3.14a**, some cells are found in clumps and for counting purposes, approximations would have to be made, whereas in **Figure 3.14b**, DAPI allows for accurate cell counts even in clustered cellular aggregates.

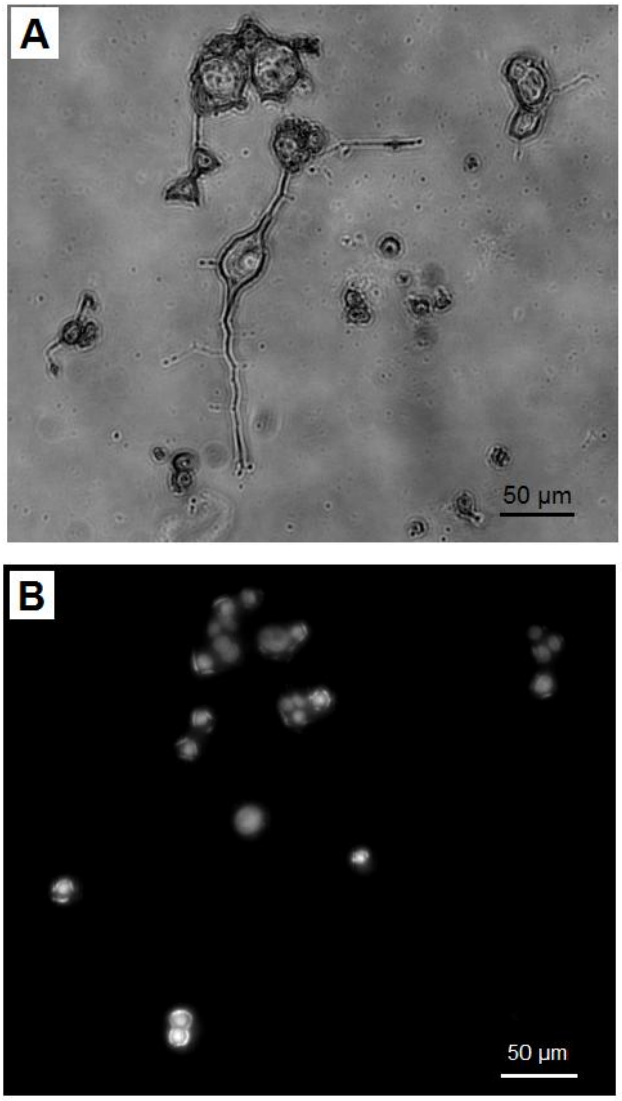

Figure 3.14. (a) Optical image of PC12 cells grown on glass coverslides exposed to 100 ng/mL NGF for 6 days **(b)** DAPI stained cells.

While DAPI may help identify each individual cell (nuclei), DAPI cannot be used to identify differentiated cells. Instead, one must combine DAPI with a primary antibody based on proteins found in high concentrations in neuronal cells. For the intended electrical stimulation experiments, initial plans were to use either solid gold electrodes or gold interdigitated array of electrodes. These solid and opaque substrates prevent the use of bright field images for cell counting and assessment of neurite differentiation, thus immunocytochemistry is essential to confirm morphological and number density changes in those experiments.

To confirm differentiation, GAP-43, β-III-tubulin, GFAP and S-100 immunocytochemistry staining was utilized as primary antibodies as these proteins are often found in high levels in neuronal cells (**Figure 3.15**). To be able to visualize the antibodies, secondary antibodies which had a fluorescent label attached to the antibody must be attached to the primary antibody. Secondary antibodies used in these experiments included: FITC, Rhod, Alexa Fluor® 488 and Alexa Fluor® 555.

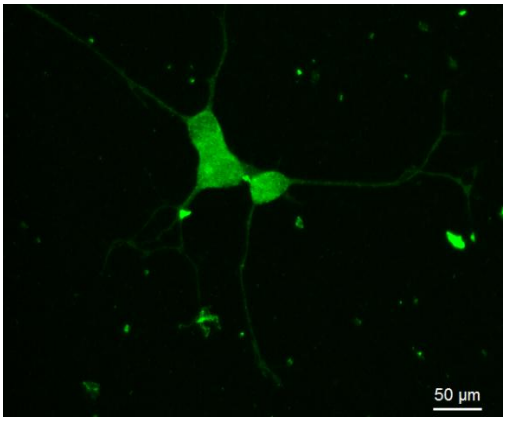

Figure 3.15. Differentiated PC12 cell grown on glass coverslides after 6 days of exposure to 100 ng/mL NGF, GAP-43 stained.

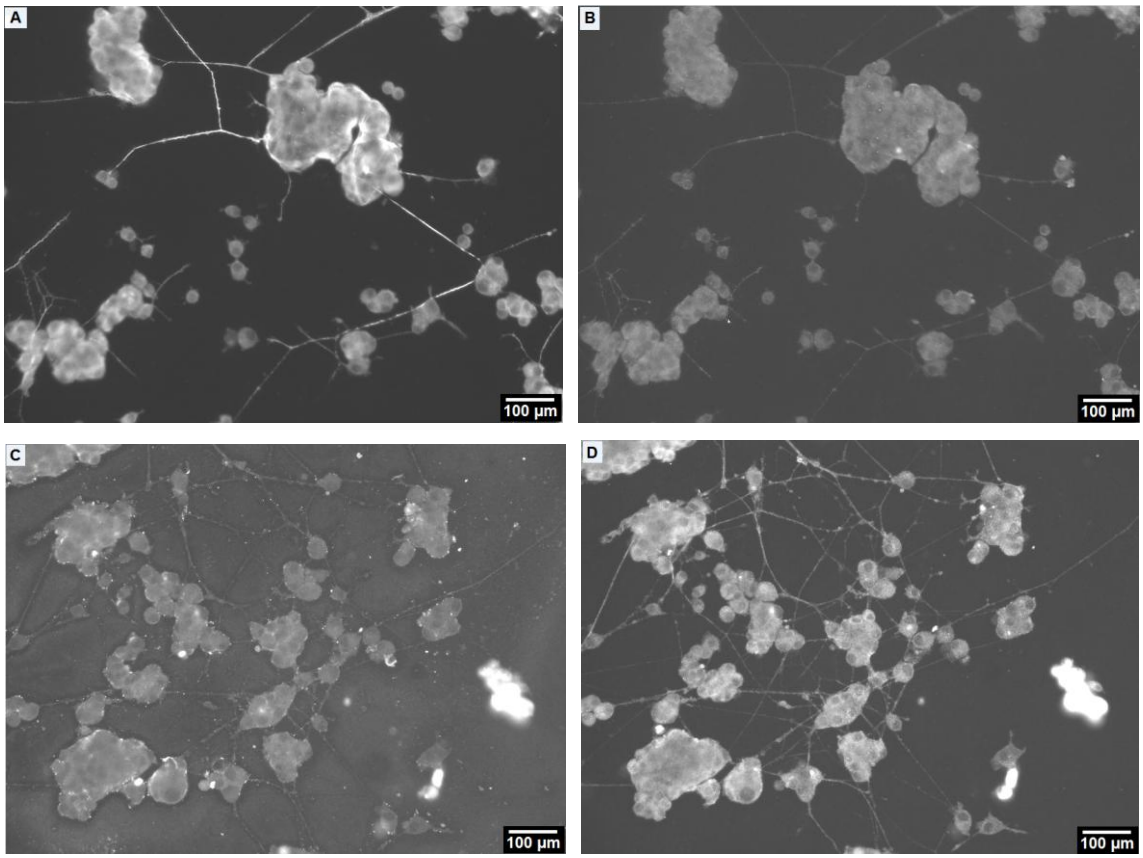

Figure 3.16. Immunocytochemistry of differentiated PC12 cells after 5 days of 30 ng/mL NGF exposure using primary antibodies: **(a)** β-3-tubulin, **(b)** S-100, **(c)** GAP-43 and **(d)** GFAP - **a** and **c** were stained using Rhod while **b** and **d** were stained using FITC as secondary antibodies.

All of the chosen primary antibodies tested positive in the differentiated PC12 cells. For future gold-based substrate experiments, β-III-tubulin and GFAP (or GAP-43) will be used as the process outgrowth appeared to have highest intensity using these immunocytochemical stains.

3.6 Conclusions to PC12 cells study

Unlike the NIH/3T3 and RN46A cell lines, the nonadherent PC12 cells showed little to no adherence on bare gold, AUT-modified gold and PLL-coated AUT-modified gold. There was however, significant notable preference for laminin and PLL-coated gold. This observation is consistent within literature as these two adhesion layers are often commonly used to promote the attachment of PC12 cells onto polystyrene or glass substrates. Due to the lack of attachment of the PC12 cells onto bare gold, AUT-modified gold, it was not possible to observe morphological defects common to the chosen self-assembled monolayer, AUT. The morphological defects as a result of the AUT-modified gold are inconclusive for this cell line. Successful differentiation was could be achieved for the PC12 cells by the addition of NGF (confirmed both optically and with immunocytochemistry). Upon addition of NGF, the cell body of the PC12 cells were observed to flatten and neurite extension from the cell body was seen within 24 hours (depending on the NGF dose).

Successful chemically induced differentiation of this cell line will serve as a comparison for any successful electrically induced differentiation. As laminin-coated gold appeared to allow the highest number of PC12 cells to attach on electrodes, all future experiments will be conducted with laminin-coated conductive substrates. Using laminin-coated polystyrene substrate along with 10 ng/mL exposure to NGF will serve as a control for comparisons with our electrical stimulation experiments. These results are the subject of the next chapter.

3.6 References

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4. Electrochemical Stimulation of PC12 Cells

While electrical stimulation has been shown in literature to induce differentiation of PC12 cells,[1-4] prior to any electrical stimulation attempts, it is important to establish a set of controlled chemically induced differentiation data to serve act as a comparison.

4.1 Chemical induced differentiation control study

To determine the chemical rate of cell differentiation as induced by 10 ng/mL NGF exposure, PC12 cells were grown on laminin-coated BD Falcon 12-well plates (polystyrene) at a density of 75,000 cells/well and exposed to 10 ng/mL NGF. PC12 cells were allowed to adhere overnight (day 0), while media and NGF changes were completed on days 1 and 3. As a control, one sample was cultured in the absence of NGF to ensure differentiation was not occurring. After 24 hrs, 48 hrs, 72 hrs, 92 hrs and 120 hrs of NGF exposure and 120 hours of media exposure (control), cells were fixed in cold methanol. Cells were then DAPI labeled for counting and optical images were taken to determine the rate of differentiation. Six random fields on the well for each sample were imaged using light microscopy and analyzed. In this rate determination study, cells with no neurite extension were excluded with the exception of the media control samples.

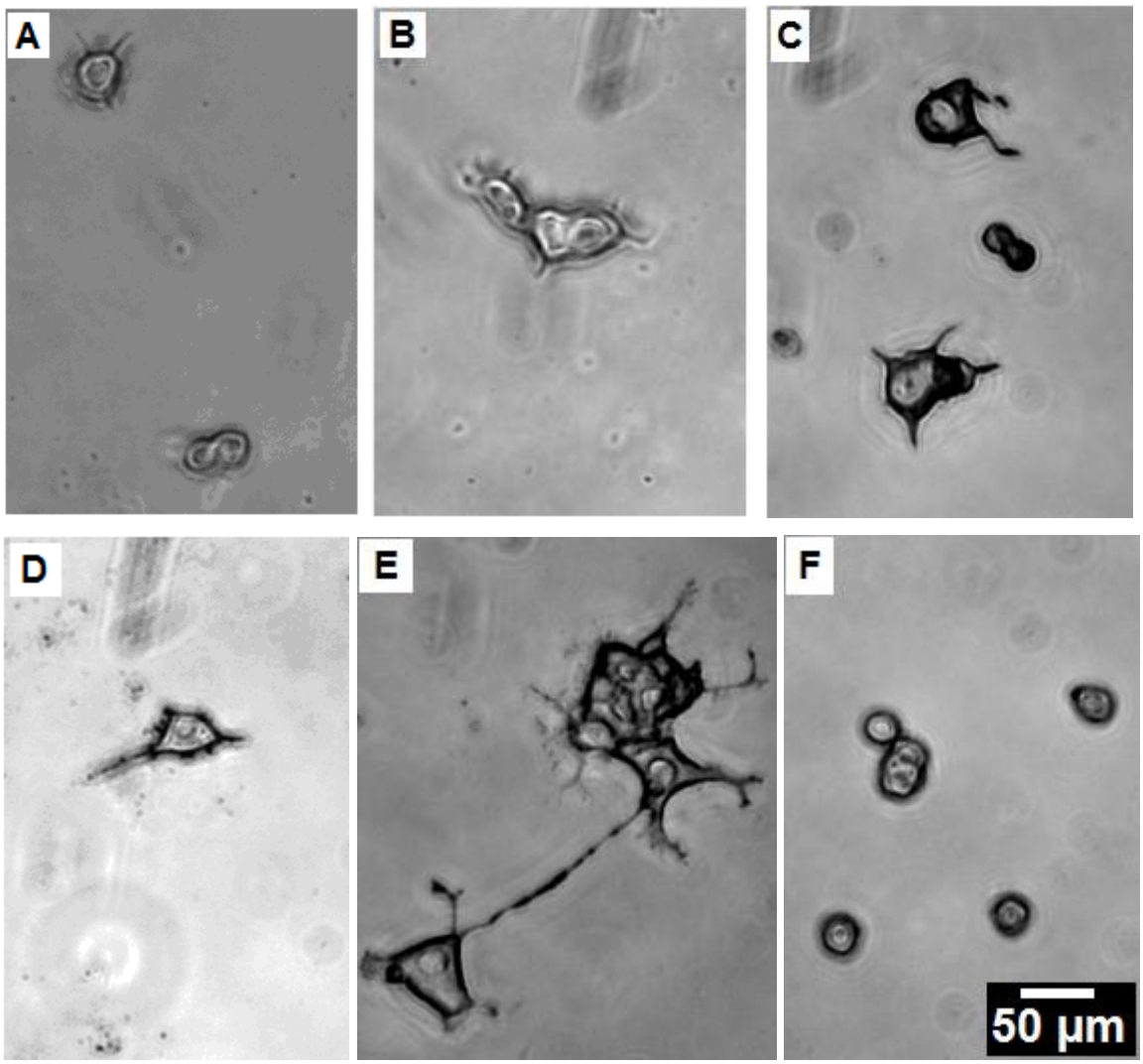

Figure 4.1. Optical images of the PC12 cells (passage 9) exposed to 10 ng/mL of NGF for (a) 24 hrs, (b) 48 hrs, (c) 72 hrs, (d) 92 hrs, (e) 120 hrs and (f) one control sample grown in the absence of NGF for 120 hrs.

From the optical images, a growth in neurite extension is directly related to the duration of NGF exposure. In the case where the PC12 cells were not exposed to NGF, a significantly smaller population of cells displayed neurite outgrowth. The total number of cells per spot size was counted using the phase-contrast micrographs and these counts were also confirmed using DAPI staining. The optical images also allowed for the measurement of the neurite extension length as a function of NGF exposure time.

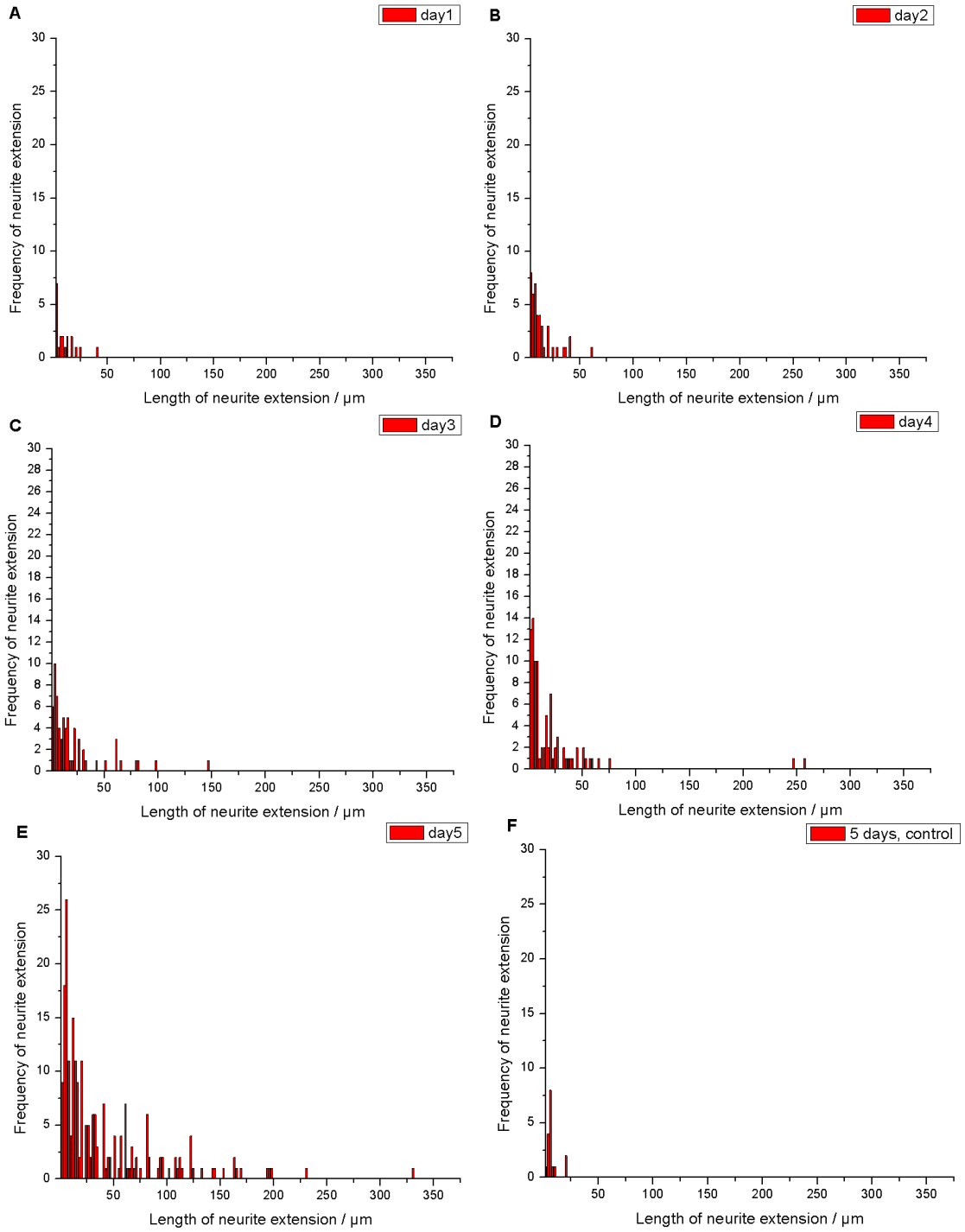

Figure 4.2. Frequency of neurite extension per day of exposure to NGF, **(a)** through **(e)** is 24, 48, 72, 96, and 120 hours respectively and **(f)** is the control sample grown in the absence of NGF for 120 hours.

The optical images provided a means to determine the average neurite length as a function of NGF exposure time. The mean ± standard deviation (s.d.) neurite length for days 1 through 5 was found to increase with time and had values of 10.0 ± 9.9, 14.0 ± 12.5, 21.4 ± 26.5, 21.6 ± 38.9 and 40.7 ± 49.1 μm respectively. When the PC12 cells were cultured in the absence of NGF (control), very few cells (< 3%) possessed neurite outgrowths and those that did had a much smaller average length of 7.44 ± 5.2 μm (n= 17 displaying neurite extension from a total of 578 cells).

Using the definition of differentiation, where the neurite outgrowth must be at least 2x the cell body, it can be seen that the percentage of differentiated cells increases as the interval of NGF exposure is increased (**Figure 4.3**).

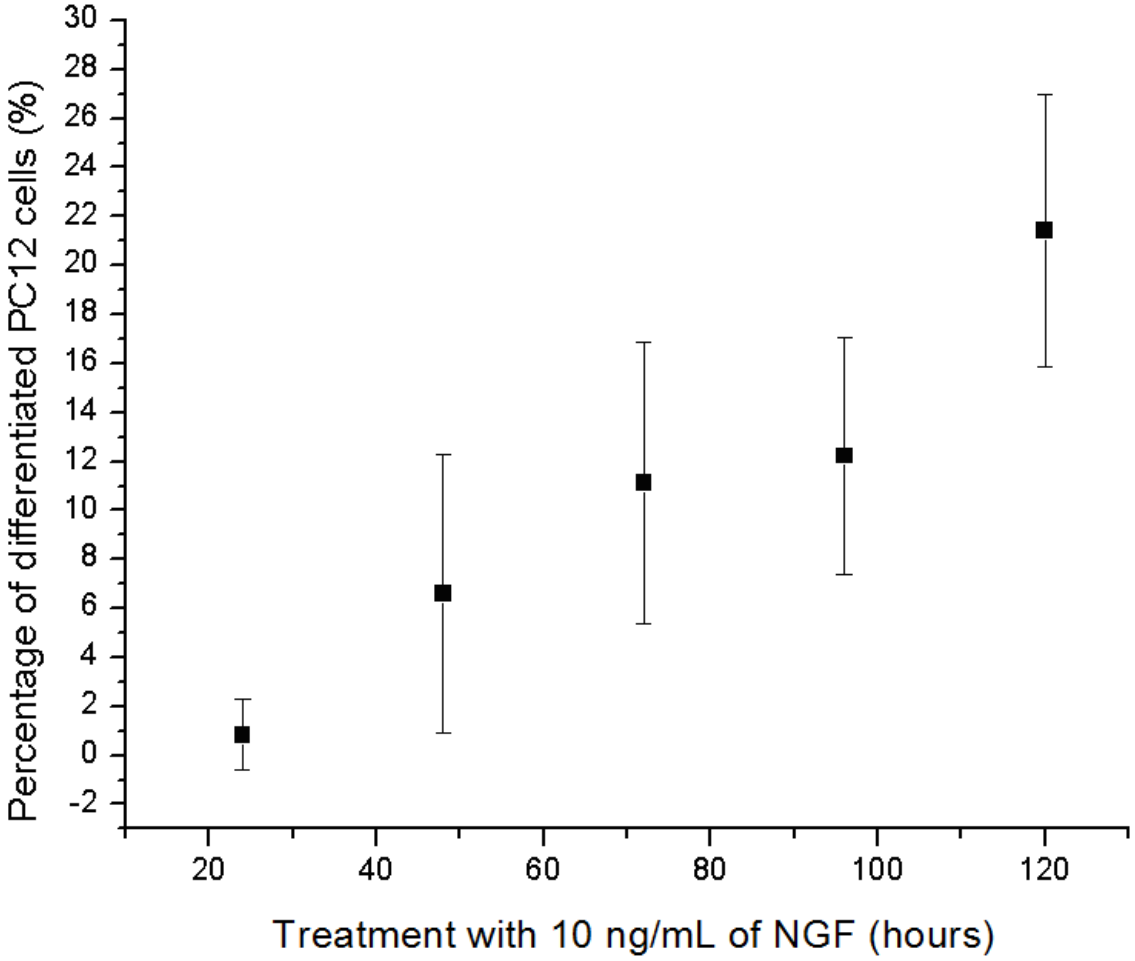

Figure 4.3. Percentage of the differentiated PC12 cells as a function of treatment with 10 ng/mL of NGF.

This confirms that PC12 cells responded positively towards NGF and differentiation increases over the duration of the NGF exposure. A parallel control study was also completed where the PC12 cell samples were grown in the absence of NGF for 120 hours to ensure no spontaneous differentiation or external NGF source contributed to PC12 cell differentiation. In these control samples, no differentiated PC12 cells (neurite outgrowth $\geq$ 2x cell body) were observed over the length of the experiment.

It was also observed while culturing the PC12 cells in their undifferentiated media (i.e. absence of NGF), that sometimes fixed PC12 cells began to display differentiated phenotypes (**Figure 4.4**). It was subsequently realized that this spontaneous differentiation only occurred for PC12 cells that were cultured to higher passages (>18).

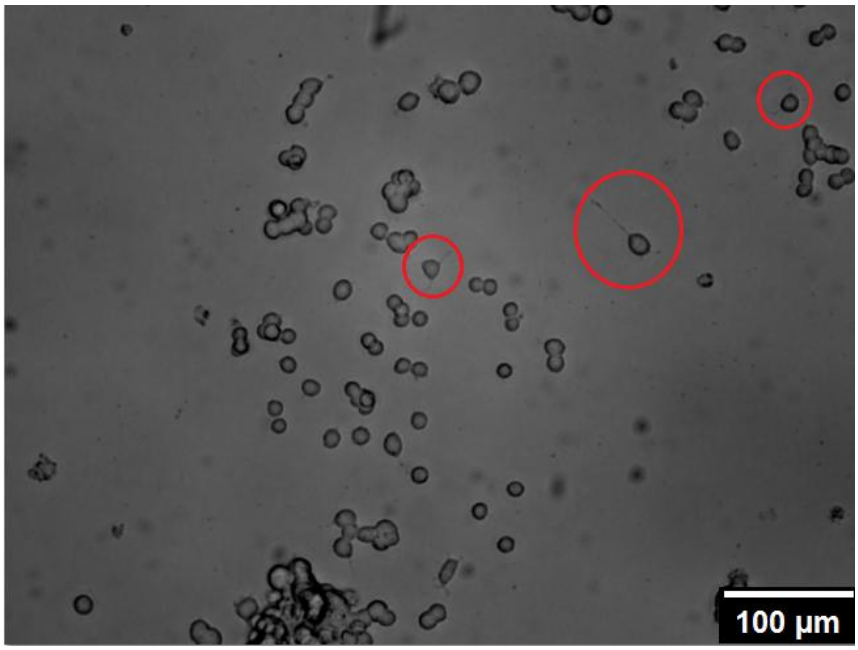

Figure 4.4. PC12 cells (passage 23) grown on a laminin-coated tissue culture petri dish for 4 days in the absence of NGF, displaying initial signs of differentiation.

This effect was very much attenuated in early passages and if the number of passages was smaller than 13, less than 1% of the cells showed evidence of spontaneous differentiation. For this reason, all experiments were completed using passages 7-13.

4.2 Interdigitated array of electrodes

The use of interdigitated array of electrodes and microelectrode arrays while electrically stimulating biological cells are becoming increasingly popular.[5-7] These electrodes allow for the study of a single cell and upon stimuli either various parts of the cell and/or the effect on neighbouring cells can be monitored. The ability to electrically stimulate a single electrode in an array or a single set of interdigited microelectrodes is a huge advantage not accesible to traditional macroelectrodes, where everything on the conductive surface is stimulated.

4.2.1 Cyclic voltammograms

After attempts to differentiate the PC12 cells using NGF were successful, the project was ready to move towards the goal of using electrical stimulation to induce differentiation of the PC12 cells using an IDA of electrodes.[3,8] To proceed, the electrochemical behavior of the IDA of electrodes was first tested by using a simple redox active molecule, hexaammineruthenium trichloride in a standard electrochemical experiment. Cyclic voltammograms (CVs) of 0.100 mM hexaammineruthenium trichloride in 50 mM sodium perchlorate as an electrolyte were obtained comparing the working electrode of the IDA of electrodes to that of bare gold (**Figure 4.5a**). These CVs were obtained using a Pt coil counter electrode and a Ag/AgCl reference electrode. Another CV was obtained using the internal working, reference and counter electrodes of the IDA of electrodes (**Figure 4.5b**).

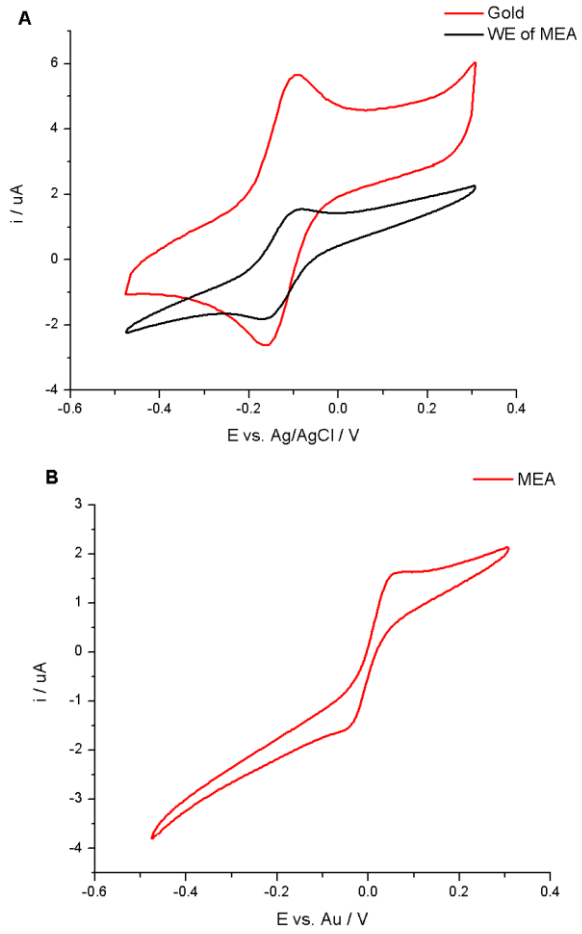

Figure 4.5. Cyclic voltammetry of 0.1 mM $Ru(NH_3)_6Cl_3$ in 50 mM $NaClO_4$ using **a)** a single working electrode from the IDA of electrodes and a hanging gold electrode with a Pt coil counter and Ag/AgCl reference electrode and **b)** all three electrodes are from the internal accessible working, counter and reference electrodes [scan rate 20 mV/s].

The excellent quality of the CV of $Ru(NH_3)_6Cl_3$ in $NaClO_4$ ensured that the IDA of electrodes was both electrochemically active and a suitable substrate for electrical stimulation of neuronal cell experiments (**Figure 4.5, Table 4.1**).

Table 4.1. Formal potential of $Ru(NH_3)_6^{3+}$ in 50 mM $NaClO_4$ as per gold electrodes:

Electrode	Oxidation Potential (V)	Reduction Potential (V)	Formal Potential (V)
Hanging Gold	-0.08899	-0.1630	-0.1260
Microelectrode Array			
1 Working Electrode	-0.08112	-0.1715	-0.1263
Microelectrode Array	0.05081	-0.03415	0.008330

As expected, the formal potential of the $Ru(NH_3)_6^{3+}$ is comparable (-0.1260V) for both the hanging gold and a single gold working electrode when the same reference and counter electrodes were used (Ag/AgCl and Pt-coil counter electrodes respectively). When the internal counter and reference electrodes, both gold, are used, along with a single set of interdigitated electrodes, the formal potential of $Ru(NH_3)_6^{3+}$ is 0.008833 V. This shift in formal potentials between the two electrochemical set ups is due to the difference in reference electrodes (Ag/AgCl vs. gold).

4.2.2 Electrical stimulation attempts

Attempts at inducing PC12 cell differentiation through electrical stimulation were completed using the square pulsing sequence similar to Kimura *et al.*[2] and shown in **Figure 4.6**. Instead of using an amplitude of ±100 mV from the rest potential or open circuit potential (OCP), a larger amplitude of ±250 mV was used which is in the range Kimura *et al.* reported was appropriate to induce differentiation (200 and 400 mV from the rest potential). The amplitude of the pulse was equal to the DC potential of +250 mV used by Park *et al.* to induce differentiation.[9-10] A low frequency (0.5 Hz) was chosen to prevent electroporation from occurring which is reported to occur using extremely short pulses in the nanosecond range at very high voltages.[11] Electroporation is the process whereby pores or holes are created which allow ions and molecules to transfer through without causing the membrane to be permeated.[11]

For the electrical stimulation experiments, one set of interdigitated electrodes was used as the working electrode, the other set served as a reference electrode and the counter electrode was a third gold pad on the integrated IDA chip. The electrochemical cell was housed in a water bath preset at 37°C and the PC12 undifferentiated media acted as the electrolyte.

4.2.3 Electrical stimulation parameters

Preliminary experiments were completed to test the electrical stimulation set up. The electrochemical cell was housed in a water bath at 37°C and a mixture of 5% CO_2:95% purified air (SierraTek flowmeter) was supplied into the cell with the PC12 cells seeded on a laminin-coated IDA of electrodes. Electrical stimulation was attempted to differentiate the PC12 cells by using a homemade LabVIEW program which allowed for the custom application of a desired square waveform potential pulse. The desired total pulse period, resulting frequency, amplitude, potential offset and square wave duty cycle (%) were all controlled through this software. Initial electrical stimulation conditions used were as follows: a rectangular wave function with amplitude of 250 mV from the OCP of 250 mV with a resulting frequency of 0.50 Hz for 4 hours.

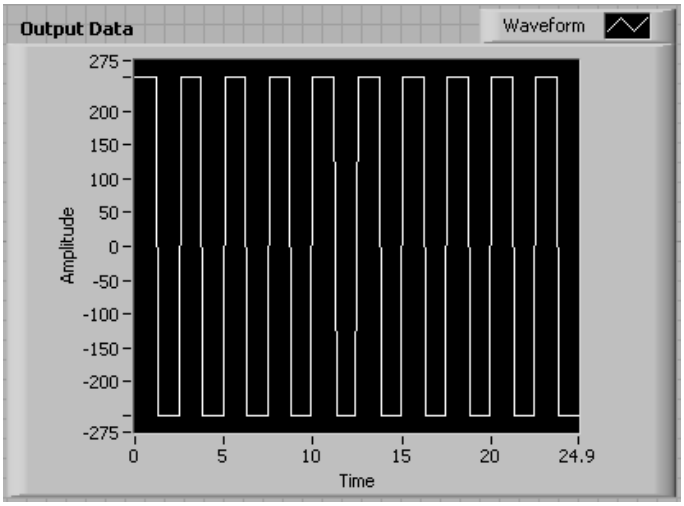

Figure 4.6. Sample voltage pulsing waveform (amplitude of 250 mV from the OCP of 250 mV with a resulting frequency of 0.50 Hz).

After electrical stimulation, the cells grown on the IDA of electrodes were fixed in cold methanol. Using the inverted microscope to visualize the interdigitated electrodes, no signs of successful differentiation on the IDA of electrodes were observed. However, this was caused by cellular detachment from the IDA of electrodes when compared to the IDA of electrodes prior to electrical stimulation (**Figure 4.7**). There were also signs of spontaneous differentiation occurring for some of the PC12 cells grown on the laminin-coated glass portions of the electrode chip. The spontaneous differentiation is believed to correlate to the high passage number of the PC12 cells (passage 18) and not a result of the electrical stimulation.

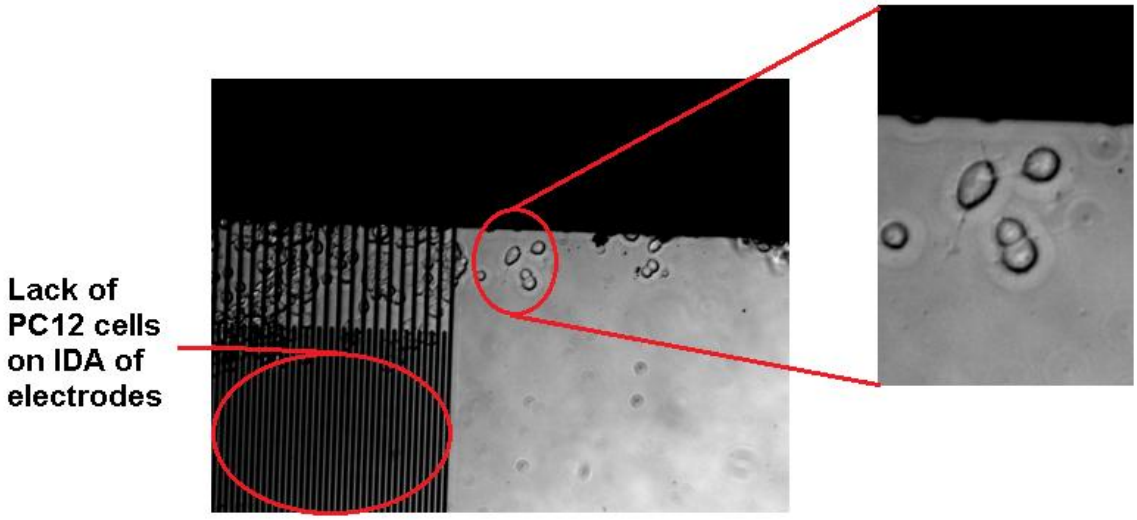

Figure 4.7. PC12 cells grown on the IDA of electrodes stimulated under electrical pulsing for 4 hours in slightly basic conditions. PC12 cells had detached from the IDA of electrodes during the electrical stimulation process is highlighted and some spontaneous differentiation was also observed for several PC12 cells on the laminin coated glass portion of the electrode chip.

Although this initial attempt to use electrical stimulation to induce differentiation was unsuccessful, several problems were noted. In this experiment, a short bubbler was used to feed the 5% CO_2 mixture into the cell (above the media electrolyte), which led to the media turning slightly basic over the duration of the experiment. This was most likely caused by the 5% CO_2 gas mixture not diffusing into the liquid media quickly enough or an insufficient fraction of CO_2 was used in the gas purge mixture.

4.2.4 Improvements to electrochemical set up

Experiments were next performed to determine the ideal means to bring the seemingly required air/CO_2 gas mixture into the cell. Two limiting approaches were tested: 1) a long bubbler which would flow the gas mixture directly into the biological media or 2) a short bubbler which would flow the gas over top of the media and the CO_2 would then diffuse into the media. It was found that using the long bubbler to feed the 5% CO_2 mixture directly into the media led to acidic electrolytes relatively quickly (pH decreased from 7.2-7.4 to < 6.8 within two hours) and rates of cell survival were less than the congruent experiments performed using the short bubbler. However, as mentioned above, using the short bubbler to feed the 5% CO_2 mixture into the air portion of the cell caused the media to become slightly basic over time (pH>8 over 4-6 hours). More detailed experiments of this nature revealed that the PC12 cells could survive under these experimental conditions for time periods up to 4 hours (**Figure 4.7**).

4.2.4.1 pH stability tests

The lack of pH stability was perplexing and another buffer system was investigated to see if it had better buffering capacity under these conditions. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES (a zwitterionic organic chemical buffering agent often used in cell culture to help maintain physiological pH), was chosen. HEPES has been reported to be better than bicarbonate buffers at maintaining physiological pH.[12] Sterilized 20 mM HEPES was added (starting pH was 6.20 which was adjusted to pH 7.2 by the addition of 6.69 M NaOH) to the media mixture which raised the pH slightly (+0.1) of the media (7.55). However, using 20

mM HEPES, the pH remained stable over a longer period as compared to the media alone (**Figure 4.8**).

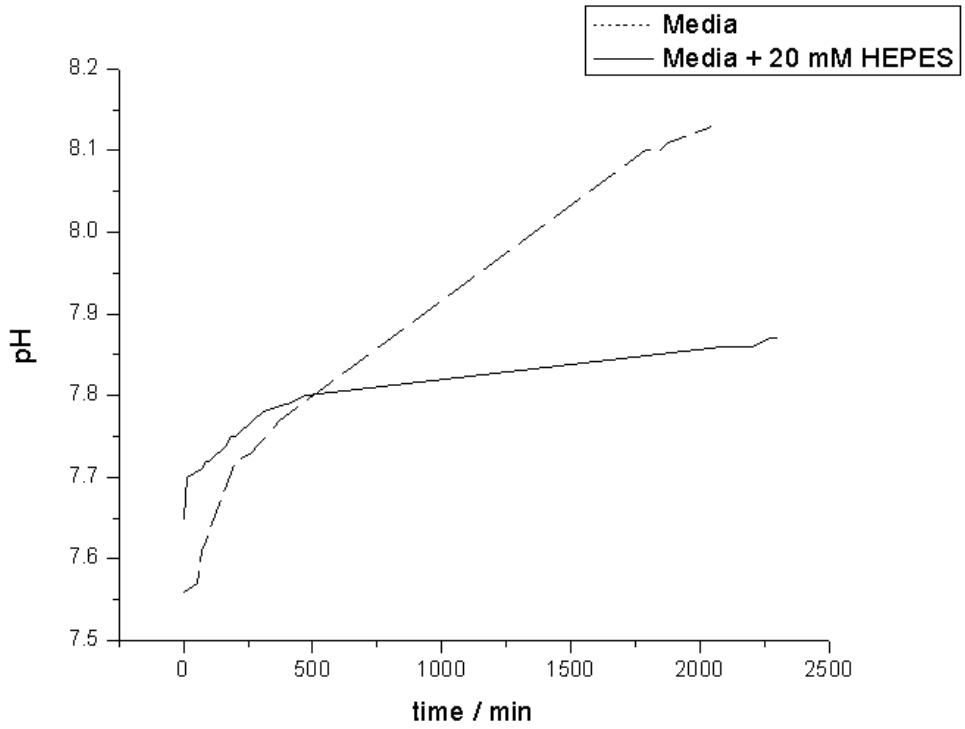

Figure 4.8. Comparison of the pH levels between PC12 media and media with 20 mM HEPES as a function of time.

During the pH studies it was also noticed that the media was evaporating from the electrochemical cell, so a pre-bubbler was placed after gas mixing and prior to the electrochemical cell. In an incubator, there is a pan of water to keep the incubator humid to prevent the evaporation of culture medium; this pre-bubbler serves this role in the electrical stimulation experiments.

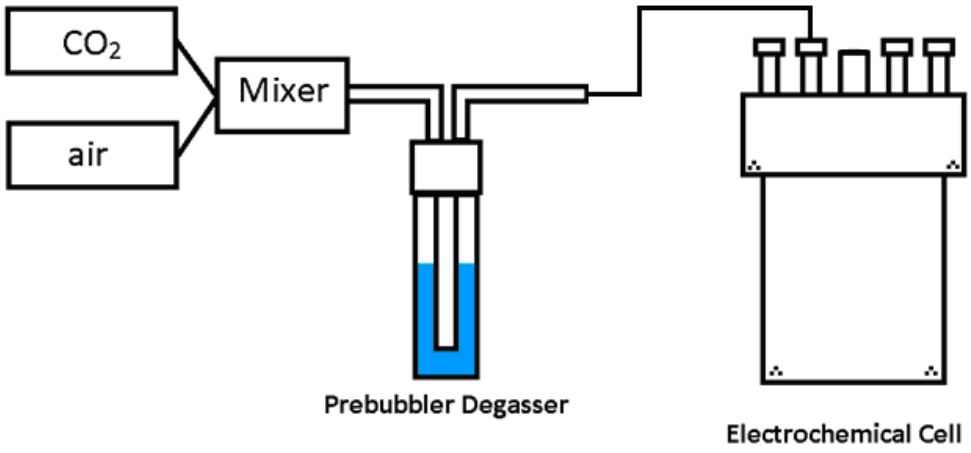

Figure 4.9. Simplified schematic of the electrical stimulation experiment, showing just the gas mixture portion connecting to the electrochemical cell.

Prior to proceeding with HEPES, one last attempt to maintain the bicarbonate buffer by incubating the media overnight inside a T25 flask which has a vented 0.20 µm filter screw cap was made. By incubating overnight and allowing 5% CO_2 to diffuse into the media, the media was closer to biological pH and remained stable for at least 7.5 hours in a closed falcon tube. For long-term electrochemical stimulation experiments (beyond 4 hours), both the media incubation method along with a closed electrochemical cell or adding 20 mM HEPES to the media to maintain biological pH could be used. For short durations, <4 hours, it was determined that the media which had been incubated in 5% CO_2 overnight or the 20-25 mM HEPES in media can resist pH changes without the requirement of a constant 5% CO_2 stream into the cell.

4.2.4.2 Adherence issue

While looking at the IDA of electrodes chip under the optical microscope, it was observed that a significant degree of detachment of the PC12 cells occurred, mainly at the IDA portion of the chip (see **Figure 4.7**). Repeating the electrical stimulation experiments with the IDA chip, it was observed that common to all trials there was detachment of PC12 cells centrally localized on the interdigitated array. Clearly a means to enhance PC12 adherence would be tremendously advantageous.

In Greene and Tischler's 1976 paper, the researchers demonstrated increased adherence after seeding PC12 cells onto collagen-coated tissue culture plates for a minimum of two passages.[8] After culturing their PC12 cells onto collagen-coated tissue culture plates for a minimum of two passages, the PC12 cells could adhere directly onto the polystyrene tissue culture plates.[8] Using this result as a basis, a comparison was completed using laminin in place of collagen-coated petri dishes thereby culturing the PC12 cells adhered to the culture plate as opposed to suspension. To determine whether PC12 cell adherence increases after seeding PC12 cells onto the gold electrode substrates used in this study, a comparison between cell counts per approximately 40 mm^2 were obtained after seeding and maintaining cells for 4 days on each of the chemical modification layers (**Figure 4.10**). PC12 cells were grown on laminin-coated tissue culture petri dishes for two passages followed by repeating the chemical modification layer study.

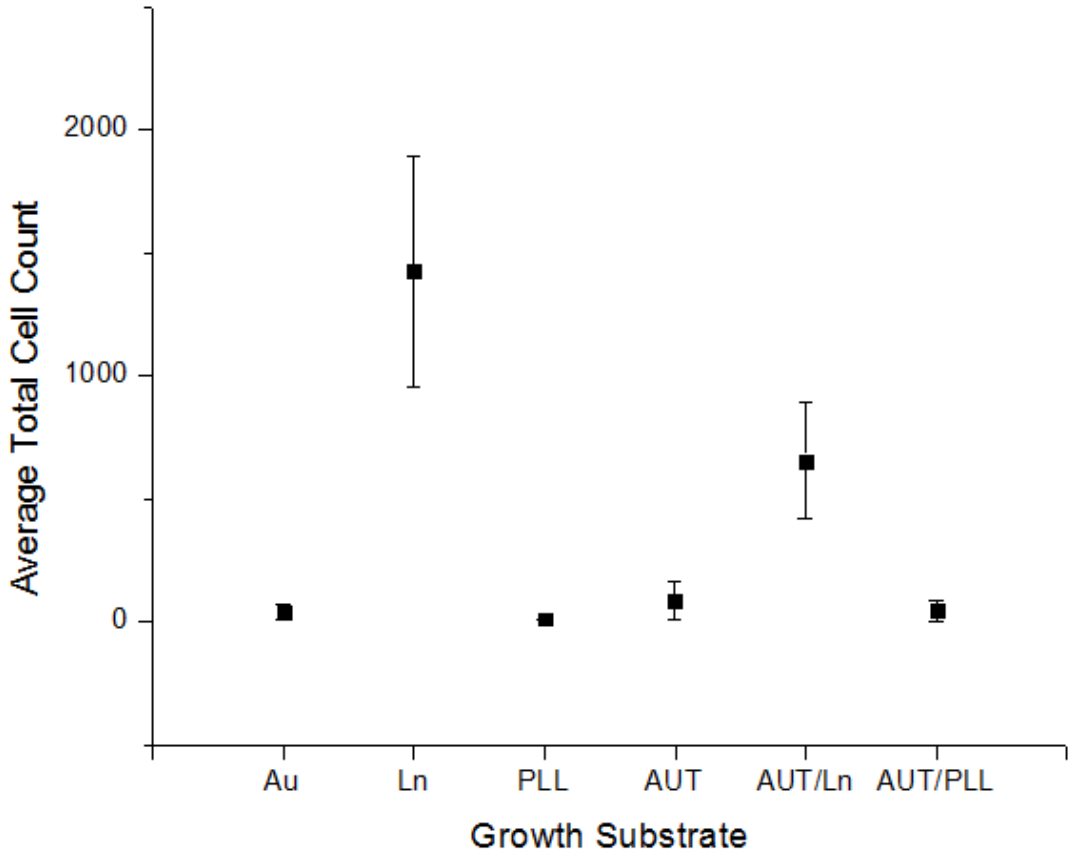

Figure 4.10. Average total cell count of PC12 cells on each 40 mm^2 modified and unmodified gold electrodes, subcultured on laminin-coated tissue culture plates (2x). PC12 cells were seeded at a density of 33,000 cells/well and counted after 4 days: Ln = laminin, PLL = poly-L-lysine, and AUT = 11-amino-1-undecanethiol.

Figure 4.10 reveals a significant increase in the number of adhered cells as compared to PC12 cells grown in suspension and seeded to attachment at the time of the experiment (see **Figure 3.11**). Comparing **Figures 3.11** and **4.10**, a substantial enhancement in the total cell counts per substrate with preferential adherence for those chemical modified with layers with laminin can be seen. These results illustrate that the adherence technique that Greene and Tischler used in 1976 with collagen can be replicated with laminin to gain preferential adherence of the PC12 cells.[8]

To compare the increase in preferential growth on laminin, based on culturing the PC12 cells in suspension or on laminin-coated tissue culture petri dishes, a ratio of their total cell counts to their seeding densities was completed. Although different seeding densities, a significant increase (approximately four times stronger adherence) in counts is observed for cells exposed to laminin for a minimum of two passages prior to seeding compared to cells that received no laminin passages (**Figure 4.11**).

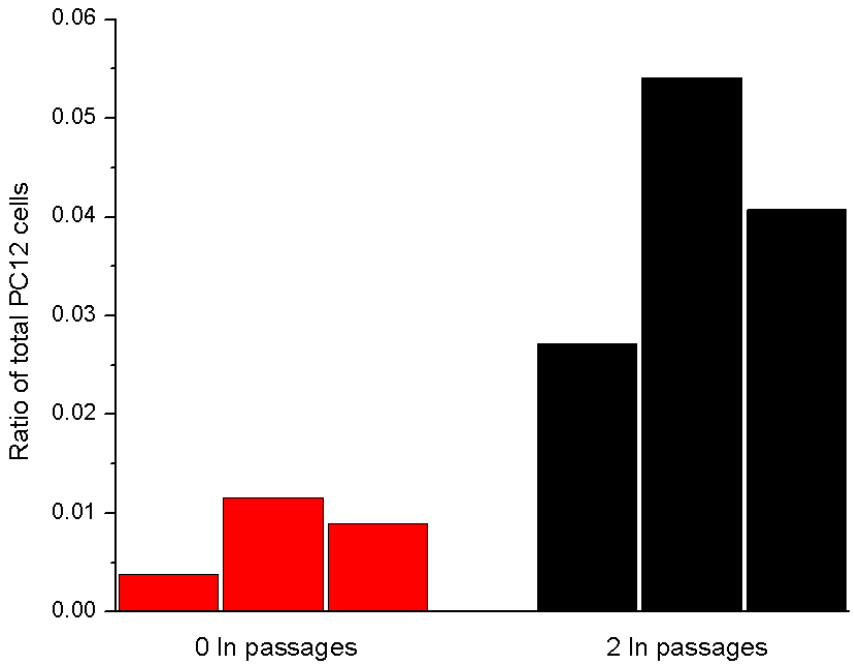

Figure 4.11. The ratio of the total PC12 cells grown on laminin-coated gold substrates to their overall seeding densities, illustrating increased adherence of the PC12 cells subcultured on laminin-coated tissue culture plates.

These results confirm Greene and Tischlers' earlier observations, that growing PC12 cells on a extracellular matrix protein coated substrate, PC12 cells mutate to a more adherent cell line.[8] Thus, all future subculturing with PC12 cells was completed on laminin coated petri dishes to increase cellular attachment onto the electrodes. The greater adherence afforded through this methodology should significantly enhance the chances of successful differentiation through electrical stimulation means and minimize cellular detachment from the electrode.

Despite the modified methodology designed to improve adherence, additional attempts at using the interdigitated array of electrodes continued to suffer from PC12 cell detachment. For unknown reasons, it seems that these substrates are not suitable for these purposes and this electrode array was abandoned in favour of conductive glass electrodes. Additionally, as the latter materials are optically transparent they are hugely advantageous for observation of the outcome of the electrical stimulation experiments because they do not require the use of immunocytochemistry to confirm differentiation.

4.3 Conductive Glass Electrodes

Conductive glass, such as indium-doped tin oxide coated glass has been used in literature as a substrate for PC12 cells to grow and allow for electrical stimulation.[2] In these electrical stimulation attempts, FTO and ITO conductive glass electrode were chosen. FTO was ultimately chosen as the conductive substrate for these electrical stimulation experiments due to having an abundance of uniform sized FTO coverslides.

4.3.1 Cyclic voltammograms

To test the FTO and ITO conductive glass electrodes, cyclic voltammograms were obtained using 0.1 mM $Ru(NH_3)_6^{3+}$ in 50 mM $NaClO_4$ electrolyte, a Pt-coil counter electrode and Ag/AgCl reference electrode.

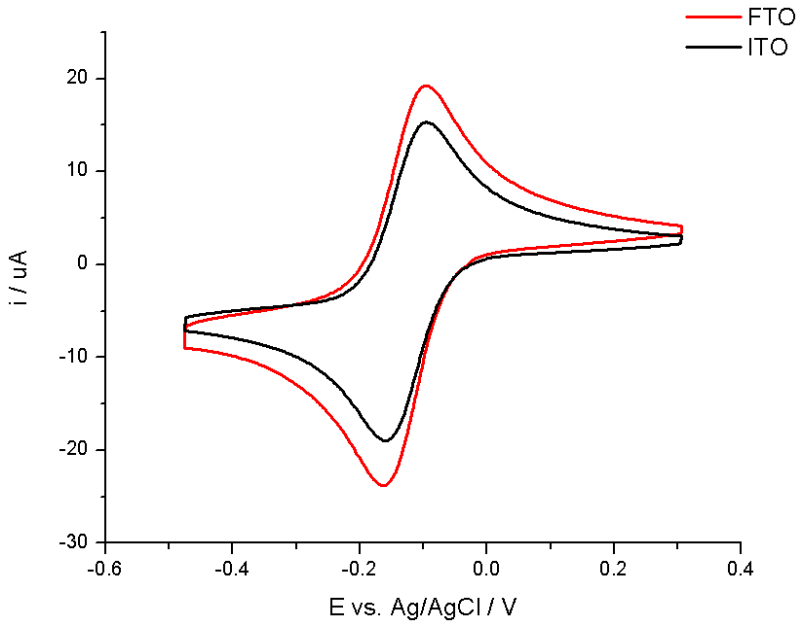

Figure 4.12. Cyclic voltammograms of 0.10 mM $Ru(NH_3)_6^{3+}$ in 50 mM $NaClO_4$ electrolyte solution, with Pt coil counter electrode and Ag/AgCl reference electrode.

Table 4.2. Formal potential of $Ru(NH_3)_6^{3+}$ in 50 mM $NaClO_4$ as per conductive glass electrode:

Electrode	Oxidation Potential (V)	Reduction Potential (V)	Formal Potential (V)
Conductive Glass			
Fluorine-doped Tin Oxide	-0.09567	-0.1627	-0.1292
Indium-doped Tin Oxide	-0.09519	-0.1595	-0.1273

* CE = Platinum coil, REF = Ag/AgCl

The excellent quality of these CVs of $Ru(NH_3)_6Cl_3$ in $NaClO_4$ ensured that the both FTO and ITO were both electrochemically active and a suitable substrate for electrical stimulation of neuronal cell experiments.

4.3.2 Substrate issue

Surveying literature, there is less literature using FTO[13-14] than ITO[1-2,15-17] as a substrate for electrical simulation experiments: Schmidt *et al.* attempted to use ITO as a substrate for their electric stimulation experiments with PC12 cells and reported it lead to an unfavourable interaction with the cells.[1] However, other researchers such as Kimura *et al.* reported success using ITO as a substrate as well as inducing PC12 cell differentiation on this substrate upon electrical stimulation.[2] Lakard *et al.* who used FTO as a substrate for culturing rat neuronal cell line 13S124, reported similar adhesion rates for FTO compared to glass, however a slightly faster proliferation rate was observed.[13] While there were reported successes with both FTO and ITO as substrate, these transparent conductive electrodes became ideal candidates for the electrical stimulation of PC12 cells due to the optical limitations of gold and the adherence issue with IDA of electrodes.

While attempting to culture PC12 cells onto the laminin-coated FTO substrate, it was found that while PC12 cells could adhere and grow, these samples were easily prone to contamination. This contamination was later identified to be traceable to the Millipore water source. The contamination of FTO and ITO was much more problematic even when the IDA of electrodes, gold, glass and plastic substrates were cultured simultaneously. The initial cleaning method was brought into questioned: sonication in 20 mM SDS followed by deionized water

(18.2Ω, milliQ), and 70% ethanol, with intensive rinsing (deionized water) in between. There are also many different cleaning methods of FTO/ITO reported in literature, such as Guo *et al.* who cleaned their ITO slides in detergent, followed by ultrasonication in acetone, ethanol and ultrapure water while Pluk *et al.* cleaned their ITO slides with isopropanol and water.[15-16] After trying various cleaning methods, it was found that cleaning the FTO in Piranha solution (3:1 H_2SO_4:HOOH) followed by autoclaving led to the least amount of contamination. After each cleaning step, a FTO electrode was tested to ensure no loss of conductivity.

While conducting other experiments with the gold electrodes it was eventually determined that the source of contamination was due to the source of deionized water. Replacing the deionized water source and employment of the vigorous cleaning method lead to successful experiments with FTO samples. The contaminated source of deionized water was also removed from all subsequent cell culturing processes. To ensure no contamination, the cleaned and sterilized FTO glass slides were placed in a BD Falcon tissue culture plate (6 cm diameter) and allowed to incubate for 3 days prior to seeding. Prior to electrical stimulation, the adhered PC12 cells on the FTO were incubated for another 3 days to ensure no contamination. If signs of contamination were visible, the FTO was re-cleaned and sterilized.

4.3.3 Electrochemical stimulation experiments

Differentiation induced through the electrical stimulation of the PC12 cells grown on FTO were attempted using the following scheme:

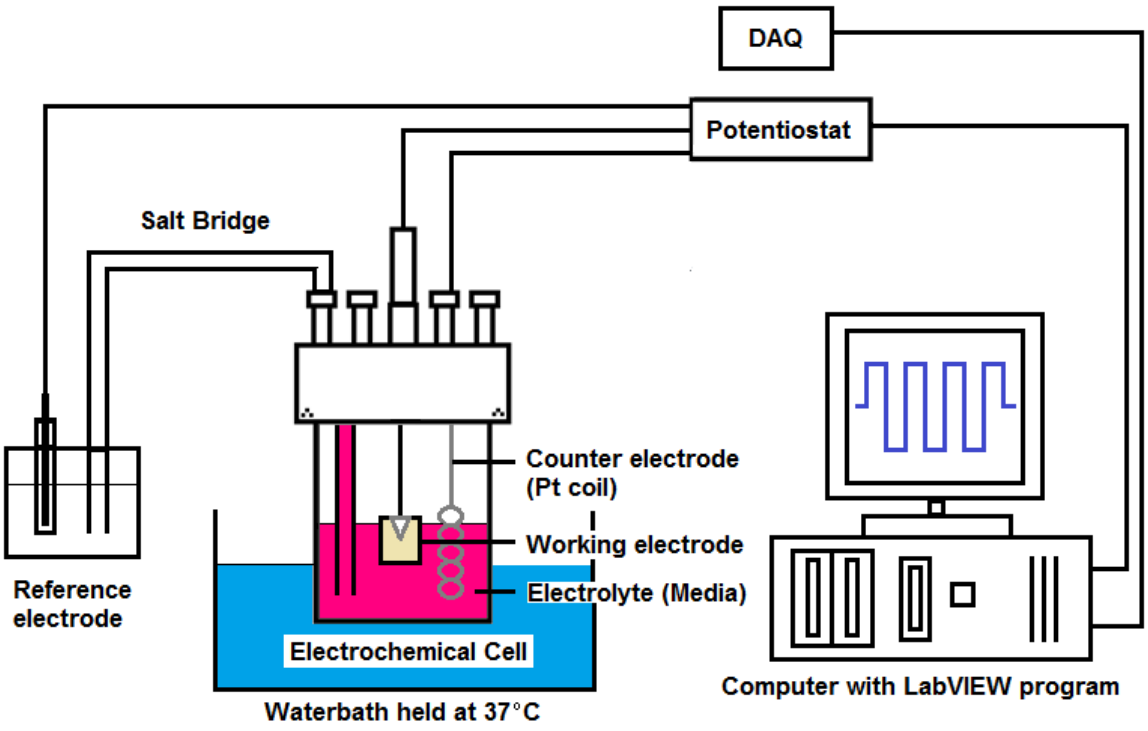

Figure 4.13. The schematic diagram of the electrical stimulation experiments.

Unlike the IDA of electrodes which has its own internal counter and reference electrodes, an external Pt coil counter electrode and a Ag/AgCl reference electrode were used to complete the three electrode system. The electrochemical cell was housed in a water bath set at 37°C and 25 mM HEPES was used (in RPMI 1640 media supply).

4.3.4 Pulsing parameters

Using the pulsing program, a constant potential of -100, -50, +50 and +100 mV was applied for the duration of 1 hour. In these experiments, the PC12 cells remained adhered, although some loss was noted. However, there were no immediate signs of PC12 cell differentiation observed after electrical stimulation. Even after incubating these electrically stimulated PC12 cells for 24 hours, the cells remained undifferentiated.

A square pulsing sequence of ±50 and ±100 mV from the OCP (at a frequency of 0.50 Hz) for the same duration was also employed. In these experiments, immediate signs of differentiation were observed, i.e. flattening of the cell body and small neurite extension from the cellular body, with longer neurite extension observed for ±100 mV. For the samples exposed to ±50 mV pulsing sequence, the cells were placed in the incubator for 24 hours and were fixed and imaged (see **Figure 4.14**). **Figure 4.14** shows some PC12 cells which display initial signs of differentiation, where the flattening of the cell body is observed and neurite extension can be seen from some PC12 cell bodies (encircled in **Figures 4.14 a** and **b**). The sample exposed to ±100 mV pulsing sequence was allowed to incubate longer to determine if neurite growth would continue or cease. After 48 hours of incubation, it was observed there were less signs of differentiation and by 96 hours all cells on the FTO coverglass displayed no signs of differentiation, potentially indicating electrically induced PC12 cell differentiation is reversible similar to the removal of NGF in the chemical differentiation process.

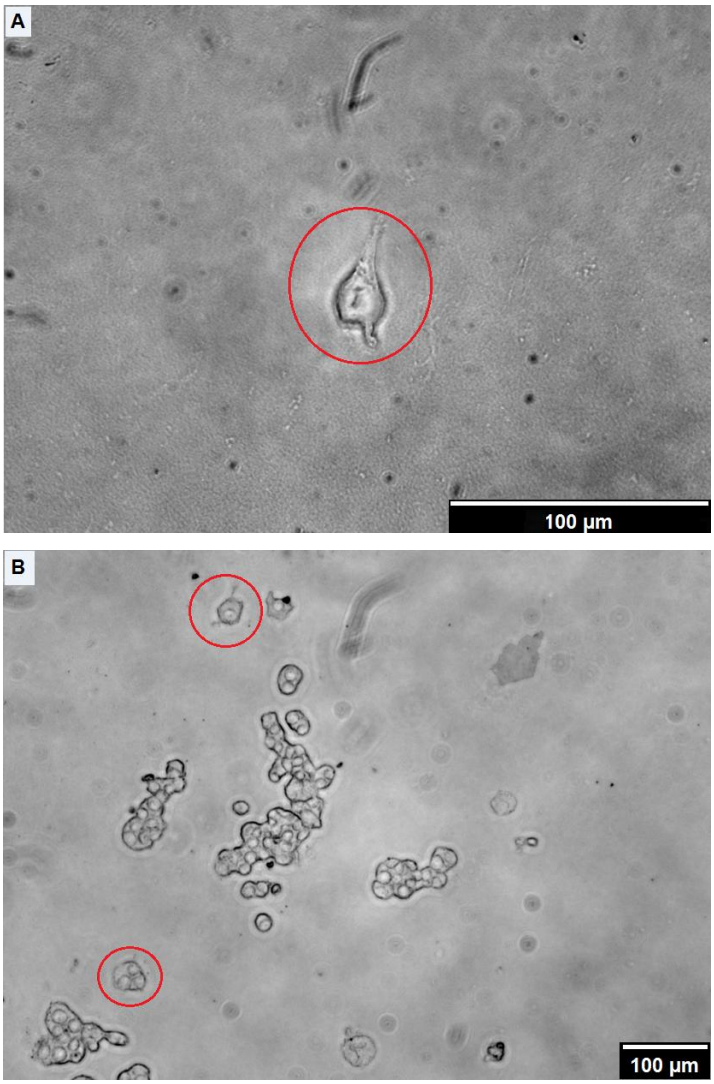

Figure 4.14. Optical images of PC12 cells exposed to 1 hour of ± 50 mV pulsing followed by 24 hours of incubation: **a**) 40x objective and **b)** 20x objective

After fixing in cold methanol, optical images for analysis were obtained (**Figures 4.14 a** and **b**). Optical images which contained easily identifiable individual cells were analyzed to determine the average length of neurite extension due to electrical stimulation. In this analysis, any neurite outgrowth from the cell body was included.

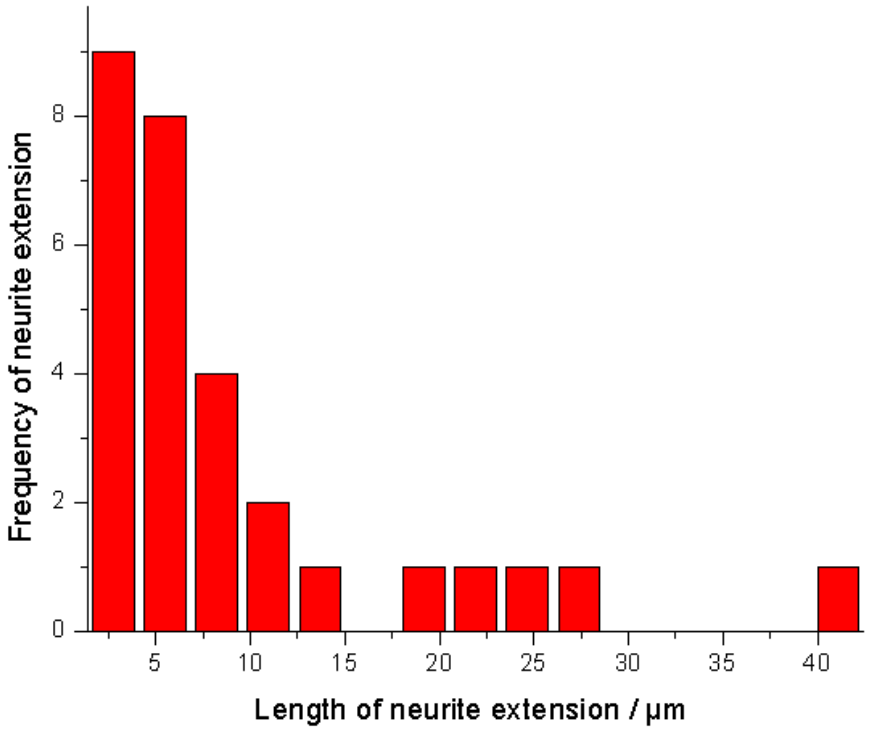

Figure 4.15. The frequency of the neurite extension of electrically stimulated PC12 cells (±50 mV from the OCP at 0.50 Hz for 1 hour followed by 24 hours of incubation).

For the conditions of this experiment, 13.8% of electrically stimulated PC12 cells displayed neurite outgrowths (n=210). The neurite outgrowths were in the range of 2.74-41.1 μm in length, with a mean ± s.d. neurite outgrowth of 9.35 ± 9.19 μm. However, under the definition of differentiation, where the neurite extension is ≥ 2x longer than the cell body, only one cell would be considered differentiated (n=210).

Comparing the electrically stimulated neurite extension to the NGF induced differentiation, the mean ± s.d. neurite extension was 9.35 ± 9.19 μm after 1 hour of pulsing at ±50 mV from the OCP followed by 24 hours incubation. This extent of differentiation is comparable to 24 hours of exposure to 10 ng/mL NGF which had an average neurite length of 10.0 ± 9.93 μm. These results suggest that 1 hour of electrical stimulation of ±50 mV from the OCP at a frequency of 0.50 Hz followed by 24 hours of incubation is similar to that of a PC12 cells exposed to 10 ng/mL of NGF for 24 hours. These results are consistent with Schmidt *et al.* who reported in 1997, faster (almost double) neurite extension to those PC12 cells grown and electrically stimulated on polypyrrole films than those not subjected to electrical stimulation (film and tissue culture polystyrene controls).[1] In this study, Schmidt *et al.* applied a constant potential of 100 mV for 2 hours and had pre-exposed and maintained their PC12 cells in media containing NGF for the duration of their experiment.[1] Whereas in my electrical stimulation experiments, a pulsing sequence of ±50 mV from the OCP was used and the PC12 cells were never exposed to NGF. Any signs of PC12 cell differentiation was believed to be contributed directly due to the electrical stimulation and not spontaneous differentiation. This experiment contained PC12 cells with a passage number of 12 and no signs of differentiation was observed prior to electrical stimulation.

In these electrical stimulation experiments, the average population of differentiated PC12 cells was 0% whereas in the NGF control experiment, after 24 hours of 10 ng/mL NGF exposure an average of 1% of the total cells were differentiated. This could be due to the longer incubation time the PC12 cells on FTO had prior to the electrical stimulation experiments to ensure no contamination was observed prior to electrical stimulation. Having a longer incubation time for the cells on the FTO substrates allowed for the PC12 cells to undergo proliferation, potentially causing higher populations of cell aggregates. In both electrically

stimulated and NGF-driven differentiation, the majority of the PC12 cells found in aggregates remained undifferentiated. In both cases, only several of the cells found in the parameter of the aggregates displayed the differentiated phenotype. For this reason, only the neurite length was compared between the electrically-driven and NGF-driven differentiation and not the population of differentiated cells.

Kimura *et al.* also showed successful differentiation with PC12 cells grown on ITO which were electrically stimulated for 5, 30 and 60 minutes using a rectangular pulsing sequence of 100 mV with a frequency of 100 Hz, followed by incubation for 24 hours repeated for 3 days and followed by 2 days incubation.[2] Unlike Schmidt *et al.*[1], Kimura *et al.*'s electrical stimulation experiments were completed in the absence of NGF.[2] It was reported by Kimura *et al.* that increasing their stimulation time to 60 minutes from 30 minutes, almost doubled their differentiated rate but increasing beyond 60 minutes led to damaging the PC12 cells.[2] With successful signs of PC12 cells beginning differentiation, another attempt was completed using the same pulsing parameters (±50 and ±100 mV at 0.50 Hz) changing only the duration of the electrical stimulation. Using a longer duration (2 hours) did not result in any PC12 cells showing initial signs of differentiation (neurite outgrowth or flattening of the cell body). It was observed in these longer experiments that there was a loss of cells on the substrate (e.g. the cells were detaching). More experiments need to be conducted to determine if these results are consistent with Kimura *et al.*'s observation of cellular death at longer durations of electrical stimulations.[2]

While initial signs of differentiation were shown with a pulsing sequence of ±50 and ±100 mV around the OCP in the absence of NGF, more experiments must be conducted to determine the ideal parameters to induce differentiation. In some of the electrical stimulation experiments where PC12 cells immediately showed initial signs of differentiation (small neurite extension or flattening of the cell body), however after incubating the sample for 48+ hours, the neurite outgrowths retracted back into the cell body and the cell body remained flattened. This effect is proposed to be similar to that of Greene and Tischler's observation of neurite retraction with the removal of NGF from their PC12 cultures.[8] Based on the results of this study and those of Kimura *et al.*, repeated electrical stimulation may be the key to successful differentiation in the absence of NGF.[2] However, with the difficulties observed with FTO contamination, attempts

at repeated electrical stimulation were limited. Having identified and rectified the likely source of the contamination (i.e. the water source), future experiments should be done to optimize the electrical pulsing conditions that lead to PC12 differentiation.

After observing some successful initial signs of differentiation, the effect of pulse amplitude was investigated. Analogous experiments to those described above were performed using ±25 ±50, ±100, ±250, and ±500 mV pulses from the OCP. Similar to previous observations ±50 and ±100 mV showed the beginning signs of differentiation, whereas ±25, ±100, and ±500 mV from the OCP showed no signs of differentiation. For the sample that was exposed to ±250 mV no cells remained adhered. After incubating for 24 hours the PC12 cells remained undifferentiated.

In Kimura *et al.*'s electrical stimulation of PC12 cells study, they also looked into the mechanism behind the electrically induced differentiation.[2] They found that while electrically stimulating the PC12 cells, the c-fos gene expression levels increased within the cell. C-fos is a protein which can indicate active neurons and functions by altering gene transcription in response to cell surface signals.[18] It has also been reported that c-fos in some systems can induce differentiation.[19-21] In a study by Morgan and Curran, the role of ion flux and c-fos expression was evaluated. They noted that in PC12 cells, c-fos has a role in early events associated with receptor occupation which lead to long term changes in gene expression.[22] Inducing c-fos expression in neuronal cells has also been reported to lead to physical changes which can be very minimal or drastic, such as inducing differentiation.[22] Morgan and Curran also noted that elevated potassium concentrations can induce c-fos generation only when extracellular calcium is present.[22]

In Kimura *et al.*'s electrical stimulation experiments, it was found that c-fos production could be inhibited by nifedipine, an L-type calcium ion blocker, which indicates calcium and c-fos play roles in the electrical induced differentiation process.[2] Their work suggested that electrical stimulation activates or increases c-fos expression through an L-type calcium ion channel, a voltage-dependent calcium channel.[2] Kimura *et al.*'s work suggested the electrical stimulation did not cause depolarization and that Ca^{2+} only gradually enters the cell as a result of

the electrical stimulation.[2] The majority of Ca^{2+} influx into the cell was believed to be due to the electrical stimulation and a high K^+ concentration, allowing immediate influx of Ca^{2+}. An extracellular source of Ca^{2+} is required (such as in the biological media) for K^+ to induce c-fos expression.[2,22-23]

The electrical stimulation experiments which used a pulsing sequence with a lower amplitude (25 mV) from the OCP was not sufficient enough to induce the differentiation of the PC12 cells. Using this pulsing sequence may not be sufficient enough to activate L-gated calcium channels, preventing the influx of Ca^{2+} into the cell. As it has already been shown by Kimura *et al.* the activation of these Ca^{2+} channels are important in initiating differentiation.

Higher pulsing sequences with amplitudes of 250 and 500 mV were also unsuccessful in the induction of PC12 differentiation. However, these unsuccessful differentiation attempts are believed to be due to electroporation. Electroporation is the process of short, repeated high-voltage pulses used to overcome the cell membrane barrier.[11,24] The transmembrane potential of a cell which has been stimulated by an external field is affected by several parameters: a form factor describing the impact of the cell on the extracellular field distribution, the applied electric field, the cell radius, and the polar angle with respect to the external field.[24] Electroporation occurs when the external electric field applied is larger than a threshold or capacitance of the cell membrane.[11] For eukaryotic cells, the threshold is similar for various cell types and is commonly reported to be approximately 1 V.[24-25] Since prokaryotic cells are typically smaller than eukaryotic cells, the electric fields required to achieve electroporation is lower in eukaryotic cells.

When electroporation occurs the electrical current causes temporary pores or holes to be created in the transmembrane of the cell. These pores or holes allows osmotic transfection of ions or molecules present to enter the cell through diffusion or electrophoretically driven processes allowing passages through the destabilized membrane.[24] The electroporation effect caused by using large amplitudes of 250 and 500 mV from the OCP prevents the activation of the L-type calcium ion channel could act similar to Kimura *et al.*'s use of nifedipine (L-type calcium ion blocker), hindering differentiation.[2] It was previously reported by Mark *et al.* that increases in intracellular Ca^{2+} does not contribute to neurite extension.[26] With no c-fos

expression being activated or increased through these ion channels, the cascading effect resulting in differentiation may not occur.

4.4 Summary to the electrical stimulation experiments

Although the initial preferred choice of electrodes was an interdigitated array of gold microelectrodes, it was found to be an unsuitable substrate to electrically induce PC12 differentiation. However, using these IDA of electrodes did lead to key insights in the initial electrochemical preparation. It was determined that a short bubbler should be used to feed the 5% CO_2 mixture into the headspace of the electrochemical cell to maintain biological pH. A stronger chemical buffer, such as HEPES is also beneficial in maintaining the pH of the media more readily than bicarbonate buffer present in the media. A pre-bubbler degasser was also necessary to prevent evaporation of media electrolyte. Using the interdigitated array of microelectrodes also led to the realization that although PC12 cells could be cultured in suspension, when the PC12 cells are subcultured on laminin-coated tissue culture plates a stronger adherence of the PC12 cells to the substrate is observed.

While there is fewer literature using FTO as a substrate and Schmidt *et al.* reported unfavourable PC12 cells - ITO interactions, it was found in this study that the FTO conductive glass was a viable substrate for electrical stimulation experiments.[1] It was also found that applying an anodic or cathodic potential alone to the PC12 cells led to no immediate initial signs of differentiation. However, using ±50 and ±100 mV pulsing sequence around the OCP (at a frequency of 0.50 Hz) led to initial signs of differentiation being observed immediately after only an hour of electrical pulsing. These observations included the flattening of the cell body and small neurite extension (>2.79 μm). The average neurite extensions from the cell body after only 1 hour of a 50 mV pulsing electrical stimulation sequence was comparable to 24 hours of 10 ng/mL NGF exposure. This observation is in agreement with Schmidt *et al.*'s observation that electrical stimulation is faster at initiating differentiation than normal chemical inducing means.[1]

Applying pulsing sequences of ±25, ±250 and ±500 mV from the OCP did not lead to inducing PC12 differentiation. This could be due to two different causes: 1) for ±25 mV from

the OCP, the activation of the L-gated calcium channels may not have been achieved with such a small potential from the OCP and 2) for ± 250 and ±500 mV from the OCP, electroporation could have been occurring. Since the influx of ions and molecules are through these pores created by electroporation, the influx of Ca^{2+} through the L-gated calcium channels, triggering c-fos expression and differentiation could not occurring.

4.5 References

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5. Conclusions

The choice of substrate and chemical adhesion layer is vitally important to the growth and viability of the adhered cells. In 2009, Jans *et al.* reported that peptide immobilization was required to obtain viable hippocampal neuronals on SAM-functionalized gold.[1] Jans *et al.* immobilized a peptide molecule, PA22-2, onto different SAMs.[1] PA22-2, a laminin-derived synthetic peptide is known to sustain cell adhesion and promote neurite outgrowth. It was also reported that without this peptide molecule, their hippocampal neuronal cells would die within 1 or 2 days when seeded.[1] These researchers also reported that SAMs alone are not enough to sustain cell adhesion and neurite outgrowth.[1]

In this work, varying levels of cellular adhesion and preference to different chemical modification layers has been demonstrated for all 3 cell lines (3T3, RN46A and PC12 cells). It has been previously demonstrated that it is possible grow cells onto extracellular matrix proteins, such as laminin and poly-L-lysine coated on conductive substrates.[2-3] Unexpectedly, it was shown herein that cell lines can be grown directly on amino terminated self-assembling alkane thiol monolayers. In several of the experiments described in this thesis where the cells were seeded onto AUT-modified gold, perinuclear blebbing was observed. The direct effect AUT-modified gold may have on the cells is currently unknown, however this could modify the cell's response through changes in its biological processes. For the PC12 cells, there was less preference for the AUT-modified gold and those cells that were observed were qualitatively deformed as determined by AFM measurements. Adherent NIH/3T3 and RN46A cell lines were shown to be successfully grown and maintained when cultured directly onto gold surfaces for up to 4 days. This is a very significant result which could aid the advancement of coupling cell growth with spectroelectrochemical techniques. Bio-adhesion layers can often provide chemical interferences that complicate the interpretation of spectroscopic data. For example, a peptide such as laminin will provide amide I and and amide II infrared signatures that will overlap those of biological cells. Furthermore, intimate contact between the cell and the metallic surface allows for more precise control of the electric field applied to the cell body.

Using the PC12 cell line, the undifferentiated cells were able to differentiate upon the addition of NGF, which was confirmed using optical microscopy and immunocytochemistry. When PC12 cells differentiated, the circular cell body became flattened and neurite outgrowth extended from these cell bodies. Comparing varying concentrations, from 0-30 ng/mL of NGF, it was found that 10 ng/mL NGF provided an optimal dose. Using higher concentrations of NGF (30 ng/mL) led to difficulties determining which neurite belonged to which cell body, however longer neurite outgrowth was observed at these higher concentrations. Lower NGF concentrations led to little neurite growth. Using the optimal 10 ng/mL of NGF, it was possible to determine the length of neurite (μm) per day of exposure to NGF and use this as a comparison for any successful electrically induced differentiation. These cells also show more sensitivity than the NIH/3T3 or RN46A cells where mutation (spontaneous differentiation) was observed within >18 passages of culturing.

Through initial electrical stimulation experiments with an interdigitated array of microelectrodes, it was determined that while PC12 cells can be cultured in suspension, when cultured on laminin-coated tissue culture plates, PC12 cells show stronger adherence thereafter (4x after two laminin passages). This mutation is extremely advantageous to electrical stimulation experiments where constant manipulation of the electrode will occur. With these nonadherent cells, physical or mechanical perturbation of the electrode or substrate caused adhered cells to fall off the laminin coated substrate more easily than the adherent 3T3/RN46A cells.

Through the completion of this MSc thesis, an electrochemical set up required for future electrochemical and FTIR coupled experiments was developed. A 5% CO_2 gas mixture should be fed into the air portion of the electrochemical cell through a pre-bubbler degasser to prevent the media electrolyte from evaporating. While the gas mixture is not sufficient to maintain biological pH, the addition of 20-25 mM HEPES stabilizes the pH for the duration of the experiment.

In a report by Schmidt *et al.*, it was reported that ITO was unsuccessful as a growth substrate.[2] If this is true than similar conductive oxide glasses such as FTO should exhibit

similar difficulties. Initial experiments with FTO electrodes led to conflicting results in this thesis. While culturing of PC12 was possible, the FTO substrates were more easily prone to contamination. After various cleaning methods were tested, it was determined that Piranha solution (30% H_2O_2:70% H_2SO_4) followed by autoclaving was the best for preventing contamination. This rigorous cleaning procedure allowed for these conductive glass substrates to be used as a suitable support for cell culture and electrical stimulation.

The most important result in this body of work is the successful demonstration that PC12 cells could be differentiated through electrical stimulation means. It was found that using one hour of stimulation through the application of a rectangular pulsing sequence of ±50 and ±100 mV from the open circuit potential led to noticeable acceleration of PC12 cell differentiation on FTO electrodes. Electrically stimulated PC12 cells (±50 mV), provided an mean ± s.d neurite extension of 9.35 ± 9.19 μm (n=21). This average neurite length is comparable to the NGF induced differentiation (control), where the mean ± s.d. neurite length was 10.0 ± 9.9 μm when exposed to 10 ng/mL of NGF for 24 hours.

While I have shown some successful signs of differentiation both through chemical and electrical stimulation of the PC12 cells, more trials need to be completed to obtain a stronger statistical analysis. While two trials of ±50 and ±100 mV were completed, and each trial showed positive initial signs of differentiation, a comparison between each pulsing amplitude was not completed due to time restraints. It was observed that ±100 mV pulsing did lead to longer neurite outgrowth however these trials were not imaged and analyzed for comparison. Although these preliminary electrical stimulation results are promising: the PC12 cells exposed to 1 hour of pulsing ±50 mV from the OCP is comparable to exposure to 24 hours of NGF (9.35 ± 9.19 μm vs.
10.0 ± 9.9 μm), more trials need to be completed to obtain the relative rate of differentiation through electrical stimulation means and allow for comparisons with the NGF induced differentiation.

More experiments also need to be conducted to determine the ideal pulsing parameters. In the completion of this thesis, the only parameter that I changed within my experiments was

the amplitude of the pulse sequence. Having seen positive initial signs of differentiation using a pulsing sequence of ±50 and ±100 mV from the OCP but not ±25, ±250 or ±500 mV, suggested ± (50-100) mV could be the required range of amplitudes to induce differentiation. An attempt at inducing differentiation using a constant cathodic or anodic stimulation at these potentials were also applied, both of which did not induce differentiation. Not only is the amplitude of the pulse an important parameter to the induction of differentiation, but the frequency of pulse is can also an important role in the success of the electrical stimulation. As I have appeared to have found two amplitudes from OCP which can induce differentiation, the frequency of the pulse should also be explored next. Using a relatively slow frequency (0.5 Hz), a higher frequency could perhaps induce differentiation faster and more successfully. Repeated electrical stimulation should also explored to maintain and further neurite outgrowth, as some of the electrically induced differentiated PC12 cells reverted to the non-differentiation form when incubated for 2 days post stimulation (similar to NGF reversibility).

My results appear to be consistent with literature: electrical stimulation is faster at inducing differentiation than normal chemical means. It appears that stimulating PC12 cells for 1 hour at ±50 mV from the OCP followed by 24 hours of incubation, led to a mean ± s.d. neurite length (9.35 ± 9.19 μm) consistent to 24 hours of exposure to
10 ng/mL NGF (10.0 ± 9.9 μm). These results are somewhat consistent with Schmidt *et al.* who compared their PC12 which was previously exposed to NGF for 24 hours, followed by electrical stimulation and another 24 hours of incubation to PC12 cells exposed to 48 hours of NGF.[2] They reported the PC12 cells exposed to electrical stimulation resulted in at least 2x the neurite length of those only exposed to NGF.[2] My results are also consistent with Kimura *et al.* who also achieved successful differentiation of PC12 cells in the absence of NGF.[3]

My results also show that when too small an amplitude is used for the pulsing sequence, the PC12 cells show no signs of differentiation which is potentially due to non-activation of the L-type calcium channels. However, when too large of an amplitude is used, electroporation occurs, pores or holes are created in the cell membrane allowing ions and molecules to transfer through which bypasses of the activation of the L-gated calcium channels, preventing differentiation. Thus to complete our long term objectives of employing FTIR to study

electrically induced differentiation, it is important to determine the ideal electrical stimulation parameters to induce differentiation.

Although much of my results have already been reported in literature, as a long term objective of the Burgess laboratory is to study electrically stimulated neuronal cell differentiation while using Fourier Transform infrared spectromicroscopy, the biological techniques and methodology required to complete this objective must be introduced to this physical chemistry lab. As the work presented in thesis is largely consistent with literature, development of methodologies and techniques have been established and preliminary attempts at combining FTIR with electrically induced different can be initiated at this time. However, more progress at this time such as determining the ideal electrical stimulation parameters is required towards achieving our long term objective of using FTIR spectromicroscopy to study the process of electrically inducing differentiation.

5.1 Future Work

While initial electrochemical stimulation experiments with PC12 cells show promise for inducing differentiation, more experiments need to be conducted to determine the ideal electrochemical parameters. However, preliminary experiments can be conducted at the mid-IR beamline at the Canadian Light Source to couple FTIR with electrochemically induced differentiation, using the 1 hour pulsing sequence of ±50 mV at 0.50 Hz from the OCP. A major advantage to synchrotron-based FTIR is the ability to use a smaller spot size of higher, localized brightness, allowing better signal to nose ratio were various biological molecules consumed or produced in the differentiation process can be detected unavailable to tradition FTIR instruments. According to Gunning *et al.*, the RNA concentrations to increase within 1 day of exposure to NGF while DNA synthesis decreases after differentiation.[4] The monitoring of these DNA/RNA bands could be of interest while monitoring in-situ FTIR and electrically inducing differentiation.

The current success with electrically induced differentiation was completed in the absence of NGF; a comparison study can be completed using PC12 cells initiated by NGF-driven

differentiation followed by electrical stimulation using various parameters (e.g. constant cathodic/anodic potential or pulsing). Completing protein expression analysis would also be of interest in an effort to better understand the electrically induced mechanism.

It also of interest to compare the rates of differentiation using different electrodes and electrically conducting polymers. Not only can IR spectroscopy be used, but other analytical techniques, such as scanning electrochemical microscopy could also be used to study the effects of electrically induced differentiation. Progression towards electrical stimulation of other neuronal cell lines, such as the RN46A cell line or primary neuronal cultures can also be completed.

Although the NIH/3T3 and RN46A cell lines were not the main focus of this thesis, more trials need to be completed in order to determine the preferred chemical modification layer for each cell line. Repeated attempts were made throughout the completion of this thesis to increase the number of trials for each cell line but difficulties had arisen preventing the success of these experiments. More chemical modification layer/substrate tests need to be completed to get a better comparison between chemical modification layer preferences.

In this body of work no attempts were made to determine which biological process is affected by the presence of 11-amino-1-undecanethiol (AUT) on the gold substrates. The AUT led to perinuclear blebbing or swelling and was observed for the NIH/3T3 and RN46A cell lines. Due to the common chemical modification layer in almost every observed unhealthy looking cell, it is believed that AUT is the direct cause of the perinuclear swelling. The biological process (or processes) which is affected could influence the cell's behaviour or response to electrical stimulation. It is important to determine what protein or process is affected by the AUT monolayer and potentially how these cells are affected. Surface patterning with various chemical modification layers and self-assembled monolayers can also be examined, especially with AUT to determine if there is a preference for the SAM which may cause morphological defects.

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