Manipulating cellular growth responses to patterning, apoptotic, and environmental cues

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MANIPULATING CELLULAR GROWTH
RESPONSES TO
PATTERNING, APOPTOTIC, AND
ENVIRONMENTAL CUES

by
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We hereby recommend that the dissertation prepared under our supervision by James N. McNamara entitled Manipulating Cellular Growth Responses to Patterning, Apoptotic, and Environmental Cues be accepted in partial fulfillment of the requirements for the Degree of

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ABSTRACT

In this work, various methods of controlling cell growth are examined. Cell-cell interaction, apoptotic cues, three dimensional scaffolds, and non-adherent environments are evaluated for their ability to affect the differentiation, morphology, and growth rate of different cell types. Previous work has shown that cell growth and cell morphology can be influenced by patterns of polymers coated on surfaces in two dimensions, designated here as the $x$ and $y$ dimensions of a standard Cartesian coordinate system. Tissue engineering and regenerative medicine studies have shown limited success in modifying growth in the third, $z$ dimension. This work considers not only the $x$, $y$, and $z$ dimensions, but also the fourth dimension, time. The time dimension is explored via apoptosis. The $x$ and $y$ dimensions are inspected through surface patterning, and the three physical dimensions are scrutinized together by means of scaffolding–cultured cells and cytophobic surfaces for non–adherent aspects of cell patterning. The project goals accomplished here are: (1) to develop a simple model of apoptosis that can be used to determine visually the temporal progression of apoptosis and to determine whether staurosporine-induced apoptosis can be delayed by pre-treatment of cells with glutamate, (2) to determine the viability of a cellulose/gelatin biologically-derived 3D cellular scaffolding construct as a platform for tissue engineering, (3) to develop a novel cell-adherence blocking strategy that will improve the localization of cell adherence to patterns deposited by the NanoEnabler® system.
Measures of apoptotic activity based on digital images showing changes in cell area, cell shape, nuclear area and nuclear shape were used to develop the Cell Area Factor and Nuclear Area Factor model. Biochemical assays for mitochondrial activity and for caspase 3 (casp3) activity showed a delay in staurosporine induced apoptosis. Digital images of the scaffold materials demonstrated the scaffold’s ability to encourage cell invasion, growth and differentiation. The image observations were supported by MTT assays showing increased metabolic activity of the cells indicating proliferative culture. The adherence blocking strategy discussed resulted in 3D growth of cancerous brain tumor cells tracked via digital imaging and tumor area analysis. Calcein vital dye staining supported the evidence for a growing tumor colony.

The three approaches for cell growth modification, apoptotic stimuli, scaffolding directional cues, and negative adhesive cues (cytophobic surfaces), are considered as building blocks that can be combined in a broader tissue engineering strategy to control the adhesion, morphology, and differentiation of cells. The apoptotic modulator chronicled in this work can be used to modify biological pathways in vitro and provide a more biomimetic environment that can be used to engineer tissues and to formulate and test new experimental hypotheses. The success of the biologically derived cellulose/gelatin material indicates that further work is warranted to develop it as a scaffold to support and cultivate 3D engineered, spatially defined tissues. The cytophobic surface has lead not only to a new blocking strategy, but also to the unexpected result of leading to a novel 3D model of cancer progression in vitro that closely resembles the in vivo situation.
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Author

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DEDICATION

Without Mike and Sheila Hancock, I would not be here. Steve and Eric, Ronnie, and Jim, you pulled me through when I needed a hand – every time I reached. Leah, Billy, Patty, my adopted siblings, you carried me here with your love and friendship. For Danny, and Nancy, my best friends from way back when, you two have always been there when I needed you. Yoko and Rach, Morgan and Amy, Jim and Krystal I have missed y’all dearly

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CHAPTER 1

INTRODUCTION

1.1 Overview of Cells and Biology

1.1.1 Cells

Cells are the most basic unit of life, according to Schleiden and Schwann, circa 1839 [1]. In the 1700s, the microscopist Anton von Leeuwenhoek called the single celled organisms he found “animalcules,” and it took almost one hundred years to get to Schleiden and Schwann’s cell theory from there. The term “cell” is still in use, and the theory, though evolved somewhat, stands, even today. As knowledge advances, ever more is found about the amazing diversity and similarities that cells display. The word “cell” comes from the Latin cella meaning small room, and was used first by Robert Hooke. In his 1655 tome Micrographia [2], Hooke called them cells, and the name stuck. Hooke is also known for his work in physics, with “Hooke’s Law” being taught in all current physics classes.

Humans are multi-cellular organisms, with many specialized groups of cells that function in specific capacities. Groups of cells with similar functions are known as tissues. Groups of tissues form organs, and organs form systems. As humans age, tissues, organs and systems become less and less effective at their specific tasks, leading to symptoms such as dementia, decreased mobility, increased blood pressure, and gray hair, among other things. As humans, aging and injury require medical treatments to restore lost functionality, and enhance the quality of life, especially as the current level of
healthcare brings longer life spans. As technology advances, there come more complex treatment strategies, in an effort to address the issue of aged, diseased, or traumatized tissues and organs. These scenarios require treatments with increasingly narrow specificities that may be administered to genetically specific individuals, meaning some treatments such as cartilage tissue replacement involves external growth of the patient’s own tissue. This reduces immunological factors for tissue transplant, but these increasingly stringent requirements bring us to the idea of growing tissues and even entire organs or tissue structures as direct replacements for damaged or diseased tissue. There have been attempts at mammalian tissue culture since the 1870s, when the French physiologist Claude Bernard outlined the concept that the tissue environment is somewhat of a function of the tissue’s own metabolic processes, and that these processes shape the environment, then the environment shapes the tissue in reciprocity [3]. Tissue growth must be controlled at the cellular level.

1.1.2 Why Pattern Cells?

This project of manipulating surfaces and morphology responds to a basic need in many disciplines for controlled cell growth. Applications exist in such fields as tissue engineering, cell-based drug screening, cell-based drug delivery for new drugs, and basic biological studies of cell-cell interactions and morphology [4], [5], [6]. Using varied techniques to achieve patterning of the different cell types leads to many procedures, all with similar goals.

All of these methods fall under the field of regenerative medicine. Regenerative medicine is defined by the National Institutes of Health (NIH) as “the process of creating living functional tissue to repair or replace tissue or organ function lost due to age,
disease, damage, or congenital defects” [7]. The aforementioned goals toward cellular control, apply to a large pool of research that is growing larger every day. The NIH states approximately 500,000 transplants are performed per year, in United States alone, while as of 2010, 108,000 people were waiting for organ donors [7]. All of the research being done now on the cellular level will not be able to help those 108,000 people, but may one day eliminate the waiting list, as technology builds further on the cellular level to create whole organs in precisely defined, three dimensional (3D) structures. And they must all start from individual cells, at the level which is now being explored.

1.2 Financial Justification for Targeting Controlled Cell Growth Patterns

1.2.1 Nervous System Injuries

The hierarchical organization of life is defined as cells forming tissues, tissues forming organs, organs forming systems, systems forming an organism. The existent tissue and organ structures that wear out due to every day human life, or are injured from our many endeavors, form the basis of our designs to regenerate tissues, and even organs. With age, or injury, the result is often tissue damage that does not heal to a functionally equivalent set–point in comparison to the younger or undamaged tissue. This incomplete or ineffective healing is the starting point for our goals of manipulating cell growth. After many injuries, such as to the spine, the results of tissue damage can be debilitating. The National Spinal Cord Injury Association states that there are approximately eight thousand new spinal cord injuries per year in the U.S. This is a small percentage of the total population of over three hundred million in the U.S. Obviously, these are costly injuries [8]. These costs can be upwards of three million dollars per individual, as shown by the National Spinal Cord Injury Statistical Center in Birmingham, Alabama [9]. These
are health care costs that can possibly be done away with, assuming the advent of a tissue regeneration paradigm allowing engineered tissue to repair or replace injured/damaged/aged tissue.

Spinal cord injury is only a small part of the regenerative medicine picture. Two other important pathologies are traumatic brain injuries and, degenerative diseases such as amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig’s disease. These are all targets for biomedical engineering solutions that could involve patterned cell response. Again, finances should not be an issue when one begins to calculate the costs associated with not repairing these disorders.

1.2.2 Cancer and Cell Patterning

Turning attention to cancer, one would recognize immediately that cancer is an obvious target for research, but not so obvious a target for patterned cells. The current project aims at two dimensional patterning (2D), the $x$ and $y$ planes. Growing cancer cells in 2D and testing drugs and delivery systems on said culture is the current “state of the art.” With the recent advent of 3D culture systems, cancer cells could be grown in an environment more similar to the in vivo environment. This would lead to more effective initial trials, and possibly cull the less effective drugs prior to animal or pre-clinical studies [10]. Taking this 3D culture further, and patterning the matrix to mimic the extracellular matrix would give an unprecedented level of parity to in vivo experimentation. These 3D efforts are relatively new, but show great promise in mimicking the intercellular milieu to successfully pattern cells as they would be found in their native tissue formations [11]. By engineering tumors that occur and progress more like the in vivo counterparts, as will be seen later in this research, cancer research would
advanced, giving new perspective with in vitro models that allow more accuracy when modeling the in vivo scenario. The National Cancer Institute alone spends approximately five billion dollars per year on research and treatment development [12], illustrating the financial imperative for furthering cancer research.

1.3 Theoretical Basis for Cell Patterning

1.3.1 Cell Culture in the Laboratory

This document has already covered, albeit incompletely, some of the logic used in choosing to pursue patterned cell cultures. The goals have been set, by the scientific community at large, to regenerate damaged or diseased tissue, and improve the quality of life for many. This line of research begs the question “how can cells be coaxed grow in three dimensions, as they do during the developmental stages?” As stated earlier, there have been attempts at mammalian tissue culture since the 1870s, when the French physiologist Claude Bernard outlined the concept that the tissue environment is somewhat a function of the tissue’s own metabolic processes, and as these processes shape the environment, the environment then shapes the tissue [3]. He also stated that for proper determination of tissue properties, the tissue must be grown in an artificial system. This sparked the use of primary cell culture, cells taken directly from a living animal, well over one hundred years ago. Scientists have been trying to grow tissue that matches native tissue ever since. The first simple cultures were problematic, due to the limitations of aseptic technique; rendering the idea that tissue culture was an intricate, complex endeavor.

By 1912, the technology to grow tissue outside the body had reached a new height. Dr. Alexis Carrel, a French surgeon, cultured chick embryo heart muscle cells in a
flask, and was able to observe them pulsating [13]. This captured the imagination of the scientific community, and Dr. Carrel proposed the cells could live indefinitely. The flask remained active for 20 years, but no one was ever able to reproduce the experiment. In 1961, Leonard Hayflick published a limitation of cultured cells at about 50 subcultivations, or about one year in culture. This is known today as the Hayflick limit, and is often cited as the current limit today, although the DeCoster lab uses even more stringent passaging guidelines [14].

1.3.2 Cell Culture Surfaces

All laboratory work was carried out in glass, at one time. Petri dishes of various sizes, flasks of different shapes—serving varied purposes—and other lab ware are all created from glass. Glass has the correct optical properties, and it is conducive to microscopy. The drawbacks were washing requirements, often acid wash followed by base washes to rid the surface of protein deposits, and one had to be quite careful not to scratch the growth surface [15]. Glass has worked for most cell culture needs, as the hydrophilic surface seems to carry the correct charge for cell attachment, but due to the drawbacks, some looked for easier methods with disposable lab ware [15].

Polystyrene is a clear polymer used in CD and DVD cases, insulation, and inexpensive, disposable coolers at the grocery store. It is a clear polymer that has been used extensively in disposable lab ware. It is highly hydrophobic [16], which is perfect for non-adherent cultures. For tissues that require adherence for differentiation and reproduction, there needs to be a treatment of the surface. Typical treatments include protein adsorption or adsorption of amino acids such as poly L-Lysine. Indeed, poly-L-Lysine, the polymeric version of the amino acid Lysine, is the standard treatment to
increase adhesion [17]. Meanwhile, many companies offer treated polystyrene, alongside untreated for adherent and non-adherent cell growth respectively. These treated polystyrene culture vessels use a variety of biological coatings, such as extracellular matrix (ECM), collagen, laminin, or polysaccharides found in the ECM [18]. Chemical methods can be used to modify a surface. For example, treatment with sulfuric acid produces a surface layer with many hydroxyl groups, which increases wettability, and adhesion for many cell lines [19], [20]. These various coatings and treatments make polystyrene lab ware quite suitable for most tissue culture activities, and very popular. Dishes for experimentation made of polystyrene, are available coated and uncoated, and are similar to those seen in Figure 1.

Figure 1. Polystyrene dishes available from the Sarstedt company [15].

Many other types of polystyrene and glass, dishes, slides, and flasks, are available commercially. These disposables can be helpful in the lab, and are the standard lab ware at this time, but the other surfaces are of interest, especially to neuroscientists. Silicon, the material of computer chips, and a percentage of most glasses, is also desirable for growth of cells, as silicon can be machined with great precision. Because gold surfaces
are inert and highly conductive, there is much interest in coaxing cells to grow on gold. As seen in Figure 2, cells can be grown on a nanometers thin layer of gold sputtered onto a glass cover slip. The functional applications here include biosensors and biological "chips" with embedded circuitry [21].

![Figure 2. CRL2020 human carcinoma cells on gold sputtered glass cover slips.](image)

Other metals, such as titanium, are of great interest to tissue engineers in the context of joint replacement. These surfaces will be reviewed in the literature review portion, but the focus of this project is on glass and polystyrene.

**1.4 Project Overview**

1.4.1 Three Tiered Approach to Controlling Cellular Growth

This project examines cellular patterning from three distinct conceptual perspectives: (1) use of the cellular process of apoptosis as a prompt for directing cellular architecture (i.e. glutamate modulating staurosporine induced apoptosis), (2) use of cellulose materials to act as scaffolding material which encourages cell invasion, growth and differentiation, thereby affecting cell patterns of growth, rate of growth and
differentiation of the cells in culture, and (3) use of cytophobic, or negative adhesive cues to increase the fidelity of patterned cells in culture by preventing local adhesion to surfaces, thus controlling pattern and cell shape, while possibly affecting differentiation.

1.4.2 General Hypothesis

The project was conceived based on the hypothesis that cellular mechanisms govern adhesion, growth, and differentiation could be controlled by specifically engineered surfaces and environs for cells in culture.

1.4.3 Experimental Hypotheses

The first portion of the project presents measurements of apoptosis-induced cellular changes and tests the hypothesis that staurosporine-induced apoptosis can be delayed by treatment of the cells with glutamate.

The second portion of the project was conducted to determine whether cellulosic fibers could be a suitable biologically derived cell culture scaffold to be used as a tissue engineering construct. Specifically, the experiments tested the hypothesis that cellulose/gelatin would support cell growth, allow penetration of the cells, and alter cell morphology.

The third portion of the project postulates a quantifiably better strategy for blocking non-specific binding of cells than the current method of using bovine serum albumin. The tested hypothesis was that cell growth could be locally prevented by a cytophobic surface treatment.
CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 Common Patterning Methods and Goals

2.1.1 Introduction to Patterning Methods

There are many common methods for modifying our chosen surfaces. Current and past literature will be reviewed here that details various strategies for chemical modification of glass and polystyrene, along with mechanical methods, such as micromachining of the surface. The chemical approach, starting with bulk surface modification, and proceeding through multi-step, complex procedures for achieving patterns that guide and coax various cell types to grow will be discussed.

Lithographic methods use lasers with various masks and steps to add and remove layers of chemicals. These procedures are complex, and require a great deal of time to manufacture, making them less attractive as technology marches forward.

Variations of the chemical modification method use stamps manufactured from polymers or use special machines to apply small amounts of chemicals to the chosen surfaces to achieve either an adhesive, or a non-adhesive area for the cells, are also used. These methods are all useful in their way, and as the reader will find, by drawing on a broad knowledge base of patterning techniques, one may choose a method or methods that best suit the situation and accommodations available. Many researchers choose methods that dovetail with each other, or they manipulate the methods to create a
completely different protocol by designing the steps of the protocol in an order that suits their particular purpose.

2.1.1.1 Lithography

Lithography, as defined by the Merriam-Webster dictionary has two meanings. The first is “the process of printing from a plane surface (as a smooth stone or metal plate) on which the image to be printed is ink-receptive, and the blank area ink-repellent.” The other definition is “the process of producing patterns on semiconductor crystals for use as integrated circuits.” The latter definition is the one which is addressed here. Semiconductors such as silicon are of great interest in patterning of cells. Patterning cells on the silicon after fabricating electrodes or other devices, fabricated from glass (or other silicon based substrates) would be of great interest [22], [23], [24].

The “critical feature size” is the smallest feature of the pattern that is desired. With this feature in mind, one chooses the substrate and coating for said substrate. The procedure for micromachining often uses photosensitive chemicals to make the patterns, called photo resist, or more simply, resist. This is the basis of optical lithography, using resist that is sensitive to light in the optical spectrum of wavelengths. This resist is usually a liquid, of varying viscosities, dependent upon the chosen application and feature size. The resist applied to circular silicon wafers, using a machine of precision design called the spin-coater. The spin-coater uses precision rotation speeds to achieve specific surface thicknesses of resist, based on manufacturer stated viscosity. There are frequently tables supplied with the resist from the manufacturer, providing information pertaining to the quantity of resist to use, rate, and duration of the spin coating. Once the resist is coated on the wafer, there may be further processing, such as heating. These steps are
again based on the type of resist and thickness of the layer, which may be as small as 130 nanometers thick [25].

With the pattern in mind, and resist chosen, the wafer must be prepped for coating. They preparation method is usually cleaning with isopropyl alcohol and acetone, then baking it to remove any water. The wafer is then coated with resist, and heated to cure the resist.

Next a photo mask is applied. The mask is basically a blocking agent, such as an adhesive plastic, that may be patterned in the design of the device or cell pattern that is desired. This pattern is cut out of the plastic by complex plotting software, on a special printer. With the desired features cut into the mask, it is then applied to the wafer. Once the mask is in place, the wafer is exposed. Once exposed, the resist polymerizes and changes characteristics. After exposure, the wafer is put into a developer solution, and depending upon the solution used, the exposed portion or the unexposed portion may be washed away. As in Figure 3, the exposed portion is washed away.

Figure 3. Example of negative lithographic patterning.
While there is no problem with developer and cleaning solutions for electronic devices, the combination of electronic devices and cell culture require different standards of chemistry.

The overall schematic for the process is shown in Figure 4, which has been excerpted from MEMSnet [26].

![Lithography schematic](image)

**Figure 4.** Lithography schematic [26].

Other methods of lithography, the so-called “soft lithography” of dip pen methods and similar methods of deposition of various reagents are also employed [27], [28], [29], [30].
2.1.1.2 Chemical Methods of Surface Modification for Adhesion

Many of these methods of chemical modification are the basis for further linking of adherent molecules to a glass surface. Glass is a hydrophilic surface, owing to its many hydroxyl surface groups [16]. The surface chemistry of glass is such that it is suitable for many types of cells to grow on, allowing for protein adsorption to which the cells can then anchor [31]. Many cell lines have an affinity for growth on glass, and not other surfaces. This affinity is the impetus for chemical treatments and surface modification, as many lines adhere, while many lines and types do not, or their morphology is quantitatively different when using an untreated surface.

One of the most common methods of surface modification is the coating with poly-L-lysine. This is a synthetic polymer of lysine, an essential amino acid that has basic properties, and carries a net positive charge at physiologic pH. The lysine molecule is shown in Figure 5.

![Figure 5. Amino acid lysine.](image)
The Lysine molecule above shows the different active sites of the amino acid, and these are important when discussing the differences between poly-L-lysine and peptides. The poly-L-Lysine polymer differs from a peptide. Peptides, and longer strands of peptides called proteins are formed from polymerization as well, but the reaction is the condensation of the alpha (α) amino of one moiety combining with the alpha (α) carboxyl of another moiety. The result is a covalent bond between the α-nitrogen and the α-carboxyl carbon. Poly-L-lysine uses the amine group on the end of the amino side chain, the epsilon (ε) amine group for polymerization with the α-carboxyl group, creating slightly different chemistries than a peptide bond. These differences owe to the relative distance of the ε-amine from the α-carboxyl group. The relative proximity of the α-amine yields a peptide bond that shows polarity of a partial double bond, stabilizing the structure. It is this particular trait that gives rise to the secondary structures of proteins, such as α-helices, when the dipole moments align. The main difference, the so called ε-bond for the condensation, yields a polymer with monomers as shown in Figure 6.

Figure 6. Illustration of ε-bonded lysine residues.
As seen previously, the poly-L-lysine with the ε-amide bond has a compact structure, with amine groups carrying a slightly positive charge alternating along the backbone. A poly-lysine peptide would look like Figure 7.

![Figure 7. Peptide bonded lysine residues.](image)

The two figures illustrate clearly the difference between the different types of “poly-L-lysine,” synthetic polymer, and peptide form. When discussing poly-L-lysine throughout the rest of this work, the synthetic polymer form will be the implied meaning unless otherwise stated. This small point is important though, as the cell membrane that encompasses the cell is composed of a phospholipid bilayer. This phosphate-ion-based molecular layer has the “polar head,” the phosphate group, situated on the inside and the outside of the membrane, as seen in Figure 8. This highly negatively charged
phosphate group has a great affinity for the positively charged polycation PLL. Of course, the inclusion of proteins in the membrane, such as receptors and transporters may drastically change the membrane net charge, but this is the basis for the attraction for most cells.

The next method of chemical surface modification that is common for lab ware is silane treatment [32]. Borosilicate glass is the most common type of glassware found in the laboratory, and the trade name for this is Pyrex® [33], [34], which is often used in cell culture. Using silane, the reduced form of the silicon molecule, is common for patterning [35]. The silicon molecule with four hydrogen molecules bound to it, shown in Figure 9, is the basis for the many silane derivatives used for surface coating.
Silane treatments can be aqueous, organically solvated, or applied by vapor deposition [36], [37], [38], [39]. Some of these methods require heat, some acid bath treatment prior to the application of the silane treatment, and some require even harsher chemistries to etch the surface of the glass to expose more of the hydroxyl groups present in the glass. Alkoxy- derivatives of silane are also used, as the oxidized form of the silane can bind covalently to the surface of the glass [40]. Glass surfaces display hydroxyl (OH) groups that are the sites of adsorption [41], [42], and the sites of interaction with siloxanes such as 3-aminopropyltriethoxysilane (APTES) [43], as shown in Figure 10.
When the compound is applied, the result is a coordinated bond between the silane oxygen groups and the surface. This orientation then leaves a monolayer [32], [39] of the silanes bound covalently to the glass surface, with an active amine group tethered to the surface. This group is used to react with proteins for immobilization on top of the polymer layer. The cartoon, illustrating the functional groups left on the surface, is shown in Figure 11.

![Figure 11. Cartoon illustrating APTES coordination with glass surface.](image)

This bond can then be heat cured, and is not easily broken. Before heat curing the silane layer can be easily hydrolyzed by rinsing with water. After curing the bond is permanent, and allows easy protein immobilization by reacting with the free amine group shown circled in red. Once the proteins are on the surface, they then become covalently bound [44].

This APTES method is a variation that is frequently used, but there are other silane compounds available. APTES, as a coating itself makes the surface of glass hydrophobic [44], but the presence of the amine group allows for protein binding, and cell adhesion. The so-called chlorosilanes, and fluorosilanes, are used to create highly hydrophobic surfaces that do not allow cell attachment or protein adsorption [45], [46].
This allows us to choose either adhesive or non-adhesive surface coatings, and using the chemistry of the end group of the silane to then bind selective adherent proteins or polymers to the silane coating, giving a true bottom-up nanofabrication method for cellular patterning [47].

Another method of chemical modification to cell culture surfaces is the “engineered polyelectrolyte layering” approach [48]. Surfaces are modified en masse by the addition of alternating charge polyelectrolyte layers. This approach is called the “Layer by Layer” method by many or just LbL [23], [49], [50]. This method uses the attraction of oppositely charged poly ions to create surface coatings with Angstrom level resolution [50], and is really quite simple to achieve.

With a chosen substrate, determining the surface charge is the first step. Next, one would choose a poly ion, such as polystyrene sulfonate (PSS), as in Figure 12, if the surface has a positive charge. In the case of glass, the accepted beginning layer

![Polystyrene sulfonate ionic molecule](image)

Figure 12. Polystyrene sulfonate ionic molecule [51].

would be a polycation such as polyallylamine hydrochloride (PAH), shown in Figure 13, to complement the surface charge of the target substrate.
By immersing the substrate in a solution of the polymer, it will automatically assemble a monolayer. Since the polymer still carries its charge, after washing, the substrate with oppositely charged layer is then immersed in a solution of a polyelectrolyte with the same charge as the substrate surface. This arranges a layer of that polymer on top of the previous level. The process is repeated, and various poly ions are able to be included. There are also modifications used to trap proteins between the layers [23], [52]. Dr. Yuri Lvov, here at Louisiana Tech University has shown that enzymes layered onto surfaces in this manner retain their catalytic activity, and has demonstrated such [53], with schematic shown in Figure 14, excerpted from that work.
To prove these layers do indeed exist, as they are unable to be seen without the use of electron microscopy, mass buildup is measured. In order to measure mass buildup, the quartz crystal microbalance (QCM) is employed. This machine uses a quartz crystal and gold electrodes on each side of the thin crystal. The unit is attached to a high frequency resonator, and the changes in the crystal resonance can be used to calculate the amount of mass deposited on the electrodes [54]. An example of a QCM electrode setup is shown in Figure 15. As the layers are deposited, the frequency shifts lower, and the change from layer to layer is then used to calculate the mass of substance in the layer.

With the addition of measuring the surface charge via zeta potential, the supporting nature of both measurements reveals successful layering.

These approaches also yield great flexibility when designing experiments. By choosing the molecules or poly ions carefully, one can simultaneously influence binding and morphology of the target cells, using one or many of these techniques.

![Figure 15. QCM crystal [54].](image)
2.1.1.3 Surface Machining

Surface modification at a structural level, by removing surface material is the focus of this section. This aspect of controlling cell growth and morphology is not a large part of the current work, but will be addressed here toward a more complete understanding of the available technologies.

Laser ablation is a method for making micron and sub micron level patterns in various materials. Using a UV wavelength laser, and short, defined pulses, reports show that this method can produce both non-destructive patterns [55], [56], [57], and complex trench structures [58]. These two methods offer flexible alternatives to traditional lithographic patterning, in that no harsh chemicals are used in the process. The details of lithography were discussed previously, so there is no need to cover that process here.

These two methods offer an alternative to lithography, allows custom designs of surface patterns similar to lithography, with a faster turnaround time, and less investment. As mentioned previously, using the method described by Cheng, et.al, a major advantage of their approach is the opportunity to manufacture asymmetric features [58]. While their research focuses on the manufacture of micro fluidic systems, these features are important for cell patterning as well [25], [41], [59], [60], [61]. By allowing the design of asymmetric features, there is the promise of experimental data that can quantify surface topography influence on cell shape and morphology, as adhesion is considered the basis of tissue architecture [62]. By varying features with precise control, as shown in Figure 16, quantitative measurements of morphological changes may be made with respect...
to pattern variations. This precision and variability in 3D patterns at the submicron scale are what give the primary advantages over lithographic methods with similar critical dimension resolution.

These are standard micromachining processes now, but older technologies such as ion beam, etc. are used in conjunction with lithographic methods to be discussed elsewhere.

2.1.1.4 Microcontact Printing

The term “microcontact printing” was coined in the mid- to late- nineteen-nineties as polymer technology advanced to make patterns on the micron scale without using the biologically unsuitable chemistries of classic lithographic patterning [63], [64], [65], [66]. The field was dominated by Harvard scientist George Whitesides, who is believed to have first used the term, referring to his new method of precise control for self assembled monolayers or SAMs. This new technology was primarily an advancement of non-lithographic patterning, as there were no harsh chemicals required. Initially, the surfaces were gold, silicon dioxide (SiO₂) surfaces. These were areas of interest for such activities as manufacturing electrodes with cells patterned on them, or silicon wafers with an oxide layer that was then patterned for biochip production [21], [65]. As discussed earlier, though, glass surfaces prepared correctly display similar characteristics to the SiO₂
surfaces. This allows microcontact printing on glass, or by using such reactants as APTES, also mentioned earlier, the pattern can be determined, and printed with the silane, and then bulk treated with whatever protein is desired to adhere to the pattern, thus forming a micron-scale pattern of proteins for selective cell adherence [67].

The process begins, with lithographic patterning of silicon wafers to create the master stamp profile in relief, or negative. Once this is achieved, a polymer mold is made, in many cases of polydimethoxysilane (PDMS), and elastomeric polymer. Once the polymer is cured, one surface is inscribed with the master pattern, and can then be brought into contact with liquid suspension of the pattern target. This is much the same as “inking” an old fashioned rubber stamp. The PDMS face with pattern and target molecule is then brought into contact with the prepared surface, and removed, leaving a thin film of patterned target molecule, as shown in Figure 17.

![Figure 17. Cartoon of microcontact printing method [66].](image)

As diagrammed in the cartoon, the method does seem quite simple, after the lithographic process to make the master stamp. But the advantages are real, in that none
of the lithographic chemistry required ever comes into contact with the biological materials. There is no danger of residual chemistry issues, or mistakes that could damage or destroy expensive cell cultures, or those that require painstaking cultivation, such as neurons. By using this simple method many have had great success patterning various cell types by tailoring the target molecule to the specific cell type, and forming patterns to prove they could indeed, coax cells to grow in defined shapes.

2.2 Cellular Response to the Native Environment: Development, Differentiation, Growth, and Apoptosis

2.2.1 The Cell Cycle and Differentiation Cues

2.2.1.1 The Cell Cycle

Almost all cells follow a growth and replication pattern referred to as the cell cycle. The cell cycle is the chain of events leading to replication of the cells. The phases of the cell cycle are broken into four distinct portions: G₁, S, G₂, and M. The first growth phase, or “gap” is begun directly after division that results in two identical daughter cells. The G₁ and G₂ became known as “gap” phases because there was no discernable activity [68], [69] seen in the microscope between the synthesis of new DNA and the mitosis portion (where S and M come from). During G₁, size is increased, and transcription and translation take place, building many proteins for the new cell. The S phase, synthesis, is where the DNA is replicated. Once the DNA replication is complete, the cell goes into G₂. The chromosome pairs begin to align along the centerline of the cell, which then marks entry into the M phase. The entire cycle is displayed below in Figure 18, adapted from Wikipedia [70]. This cycle is simply divided into where action can be seen, things are happening. As scientists, to study these aspects of the cell cycle on a large scale is nearly impossible. These divisions named so easily are accompanied by thousands of
activities inside the cell that we cannot see, such as protein manufacturing, and cellular differentiation.

Figure 18. Cell cycle graphic adapted [70].

The initial cell of the fertilized egg in animals is capable of becoming all of the various cell types in the body. The entire genetic code is there, and as the cell grows, it expresses various proteins which are the hallmark of a specific cell type, but deeper changes are also taking place. Changes in the RNA metabolism, along with the levels of gene activation for various genes not correlating to proteins expressed on the cell surface, those that can be seen, and levels of varying substrates [71]. All of these things are shaped in part by the environment of the cells. Not just the physical environment, but the social order, the types of cells that surround the developing cell [72], [73]. These all play a role in said development, influencing the cell, and helping it along its differentiation and growth path.

2.2.1.2 Contact Inhibition

Contact with other cells, and the extracellular milieu itself plays an important role in the development of organisms. Developing animal cells interact in a complex manner
with each other. This entire phase of development of the growing organism, the individual cells are responding to stimuli such as the surfaces of other cells they are in contact with, the extracellular matrix, temperature, etc. They also are susceptible to soluble chemicals, proteins and the various hormones, growth factors, and cytokines in the extracellular milieu. All of these changes not only are affected by the processes of differentiation, but they have an effect on the differentiation process itself in a feedback loop mechanism of control. All of the aforementioned growth factors, implied transcription factors, cytokines and physical stimuli should represent targets for experimental control of morphogenesis. With this thought in mind, the discussion will now focus on the selected avenues of approach currently in use to control cell morphology, adhesion, and patterning.

With the early cell culture experiments, cells were noted to not grow exactly as they had in the original tissue, but could survive for many days was a scientific feat in the early nineteen hundreds [13]. Cells taken directly from an organism, and placed in growth medium in a dish or flask, are known as primary cells. It is common knowledge that primary cells grow only in two dimensions, the x and y directions, using the growth surface as a planar reference to the Cartesian system. They will grow until all of the cells touch boundaries all around. Once there is contact with the wall of the dish or flask, or other cells, growth and spreading is then down regulated. This is phenomenon has been termed “contact inhibition” [73], [74]. As the cells grow and spread out, they stop once they make contact with other cells, and once they fill the available growth area, their metabolic rate slows [75], [76], [77]. This growth inhibition is not exhibited in cancer cells, one of the primary symptoms of disease, unchecked growth and replication. This fact alone should make surface patterning and contact-based guidance strategies a
valuable tool in the anti-cancer arsenal. By ascertaining the mechanism of this contact inhibition, that cancer cells do not normally exhibit, the knowledge could well translate into effective treatment strategies aimed at returning cancer cells to a state of exhibiting contact inhibition. There is experimental evidence of the ability to return the contact inhibition to cancer cells by treatment with a plant-based protein of the carbohydrate binding lectin family concanavalin A [78]. Lectins are a family of proteins that function as simple carbohydrate recognition and binding moieties. The lectins are heavily relied upon in the human immune system, which also hints at possible immune-activation against cancer, given the appropriate stimulus. Plant-lectin-binding implies the presence of abnormal surface glycoproteins, which are obviously recognized by the human immune system as self antigenic, and therefore non-inflammatory, or non-response-generating. Further evaluation would be merited, and could produce a vaccine of sorts, against cancer.

2.2.1.3 The Extracellular Milieu

Examining the physical context of the cell culture environment, and the effect that physical contact with varying surface features such as channels, protrusions and barriers of various scale, can hold the answers to many questions about contact inhibition, growth guidance and characteristics of both normal and cancerous cells. Results will now be examined from many studies that will enlighten as to the suitability of the various techniques previously discussed to not only affect cell morphologies, and growth patterns, but to also use these techniques to predetermine cell growth patterns and morphologies. Can this be done successfully? Can it be repeated? What level of precision might be expected? The challenge will be to define mechanisms for the observations, and
in doing so, can progress be made toward designing and implementing man-made, bottom-up tissues? These tissues could be used as test beds for *in vitro* experimentation that more closely resemble the *in vivo* reality. Can these tissues be refined to the point of using them for implants?

### 2.2.2 Apoptosis Shapes the Differentiation Process

Apoptosis has been termed “programmed cell death” and is a normal process in the development and continuing life of complex, multicellular organisms [79], [80]. It is crucial to homeostasis, and is a highly complex process, regulated on many levels, through many pathways [79]. Aberrations in the apoptotic machinery result in diseases like cancer, arthritis (autoimmune) and perhaps even degenerative disorders [81].

Scientists have observed the phenomena for many years, and are always discovering new pathways leading to the same end: the demise of the cell [82], [83]. Three of the common, well characterized pathways include intrinsic and extrinsic, and the perforin/granzyme pathway. These three pathways have many different triggers or initiators, but all are what is known as caspase dependent. This simply means the pathways converge at the final step toward death, caspase-3 activation. Caspase-3 is the main effector caspase, one of many proteins of a family of tightly regulated enzymes that perform roles in the apoptotic cell death cycle. Many of these enzymes exist as a pro-enzyme, and activation of one pro-enzyme, or pro-caspase, activates more copies of the enzyme, which then activates other caspases. This is the classical enzyme cascade, and amplifies the reaction at each step, until, at the caspase-3 portion, there is no turning back, and the cell has reached a certain commitment point. At that point the negative physiological changes begin to be insurmountable, and the cell dies.
The physiological changes seen the earliest are cell shrinkage and pyknosis, or the irreversible condensation of the chromatin DNA material. These changes are visible within a few hours in culture. The pyknosis and shrinking of the nucleus can be used to quantify the extent of apoptosis in culture, via image analysis [84]. This Nuclear Area Factor, introduced by Dr. Mark DeCoster, can be very useful for quantification of apoptotic cells in biocompatibility testing. Pyknosis, and nuclear shrinkage is said to be the classic feature that is characteristic of apoptosis, but other features occurring later can be easily identified as well. “Blebbing” is the appearance of rounded, detached cells in the area of apoptosis, which is a singular cell-, or small cluster of cells-type of process, and does not usually spread to large areas of cells. Blebbing and overall shrinkage can be seen easily during cytoplasmic condensation, as the cells prepare to be phagocytosed by macrophages or parenchymal cells. All of the processes of apoptosis can be summed up as simple packaging for disposal.

The packaging for disposal analogy is a key for differentiating apoptosis from necrosis, another form of cell death. Necrosis is a process where the cell actually explodes and dumps it contents into the interstitial space. Much of the cellular contents are not normally exposed, especially reactive proteins such as the cytochromes, contained in the mitochondria. When these are turned loose in to the extracellular milieu, they can cause inflammation and surrounding cells to become necrotic. Therein lies the most visible difference: apoptosis is a process of shrinking, such as when one packs up and moves to a new home, where all of the items from the old home actually fit in a much smaller truck, with necrosis, everything is sent out of the cell in disarray, as if a tornado hit the home form the moving analogy. This difference is exemplified when a virus invades a cell and hijacks the translational machinery to produce many more virus
particles. At a certain point, the cell membrane fails, and the rupture lets the new virus particles out into the extracellular milieu, causing inflammation and spread of infection, a growing radius of cell destruction. This is a stark contrast to the processes involved in apoptosis which do not release cellular contents, become phagocytosed (which prevents inflammation and necrosis in the surrounding tissue). An interesting note to differentiate is that the phagocytic cells do not produce inflammatory cytokines upon ingestion of apoptotic cells, which is contrary to the virus example, in that upon phagocytosing necrotic tissue elements, inflammatory cytokines are produced [80].

The mechanism or mechanisms of apoptosis require a great deal of energy, as does packing to move. This particular trait can confuse some when testing for biocompatibility, as the MTT test is often used. This test measures metabolic activity, and can be hard to interpret, based on the fact that it works by chemical processes involved in the mitochondrial metabolic chain. As stated, a cell can be dying, and show an increased mitochondrial metabolic rate, while a cell that is not yet ruptured, but is in the process of necrosis, may show reduced activity due to mitochondrial injury. This is a point to consider when designing experimental procedures, but it can still be used effectively as a test, if the hypothesis is arranged correctly, and supporting tests are made, so as to verify and be certain to differentiate between the two processes.

As mentioned earlier, all of the pathways of apoptosis converge at caspase-3, but their initiations are different. In the extrinsic pathway, initiation is accomplished by receptor/ligand binding. The receptors are from the tumor necrosis factor (TNF) receptor family [79], [80], and receive external cell signals that can lead the cell to apoptosis. Again this process converges at the caspase-3 executioner protein, with the intrinsic pathway, and the perforin/granzyme pathway.
The intrinsic pathway is initiated by many things, but is not specifically receptor mediated responses, such as the way removal of nerve growth factor results in death of the nerve. Other factors include radiation damage to DNA, toxins that have no specific receptor, hypoxia, hyperthermic shock, etc. These things result in a change in the mitochondria, which then releases proteins that start an apoptotic cascade, and later in the process, release endonucleases and other proteins that seal the deal, accelerating the apoptotic process. For this reason the intrinsic pathway is sometimes labeled the mitochondrial pathway. While it is possible for the events to take place in such an order that the mitochondrial changes are initially bypassed, cascading caspases will result in the late phase changes that accelerate the intrinsic pathway.

The perforin/granzyme pathway is in some instances the same as the extrinsic pathway, as cytotoxic T cells displaying the Fas ligand (FasL) interact with the Fas receptor (FasR) on cells, initiating apoptosis. In other cases, such as viral infection with virile protein expression on the cell surface, the T cell adheres to the infected cell and secretes perforin. The protein perforin makes a hole in the cell membrane (perforates it), and then the T cell puts granzyme A and granzyme B containing granules through the pore to initiate caspase cascades and thereby initiating apoptotic destruction of the infected cell before the virus can propagate. Figure 19 shows a diagram of the cascade.
2.3 Cellular Responses to Patterning In Vitro

2.3.1 Responses to Mechanical Surface Modifications

2.3.1.1 Knife-Cut Patterns

The first approach to be discussed will be the simpler mechanical methods to surface modification. These methods date back to the nineteen seventies, when scientists at Duke University Medical Center attempted growth of heart muscle columns from dissociated chick heart muscle cells [85]. The patterning methods included coating tissue culture ware with rat tail collagen, then blocked cell binding with a coating of agar film. To demonstrate preferential binding, they used exacto knives to cut slits in the agar film, exposing the collagen underneath. Cells were then seed on the prepared surface, revealing the differential binding.

The Duke scientists were able to get cells to align in the slits in the agar, and produce column of aligned, synchronous beating heart cells. These were tested and used
for electrophysiology experiments. The authors also contend that due to the relative similarity of the columns to the in vivo orientation of heart muscle cells, their results of the electrophysiology experiments would be closer to reality than similar results of tests performed on monolayer cultures [85]. These results show success, repeatability, and offer a modest level of precision. The resultant tissue was received as an improvement on the test model, but there is a long way to go before looking toward implantation.

2.3.1.2 Laser Ablation

A slightly less simple method of surface mechanical modification is the removal of surface material to form contact guidance patterns for cell adhesion. The laser ablation method is included here as it is much less physically complicated, than the acid or base etching encountered in photolithography. As precision control of the laser is required to modulate pulse length to femtoseconds, manipulate the beam trajectory on the micrometer and nanometer scale, control beam angle to extremely tight tolerances, and penetration depth of the beam on the nanometer scale, this process does not truly qualify as “simple”. By current standards, the requisite computing capacity for these precise controls can be supplied by a personal computer that can be purchased for about five hundred dollars. Thissen, et al. described a method of burning off surface coatings and etching into the surface itself to create 3D surface architecture [55]. Figure 20 shown next is a digitally enhanced photo of their cellular results.
Figure 20. Selective binding of Bovine corneal epithelial cells. Stained with pico green [55].

Most of the laser-based processes do take advantage of chemically modified surfaces [86], [87], [88], but it is valuable to know as much about the applicable technologies as possible to implement the materials and equipment available to the current research question being explored.

2.3.1.3 Simple Surface Scratches

Other simple methods include simply scratching the surface of a glass cover slip, and then determining how the cells react. In the case of the Polish scientists at Jagiellonian University studied contact guidance of chick embryo neurons with that model. By placing a 0.2um scratch (width, depth), they found the growing neurites would follow along the edge of the scratch [89]. There is plenty of current evidence to support the contact guidance conclusion, as many reports have been made about the effect of surface topography on cell morphology and adhesion [56], [90], [91], [92]. Many of these studies have shed light on the exact nature of what makes a cell adhere to a given surface, and the preferential factors from the cell perspective [62], [66], [90], [91], [93], [94], [95]. Cell morphological polarity, for example, is regulated by the cadherin family of
adhesion proteins, and cell polarity plays a key role in determining the fate of the cell [96]. The cadherins not only mediate focal adhesion attachment to substrata, but are also vital to cell-cell contacts [97]. Chen and Öbrink [97] showed in nineteen-ninety-one that the cadherins were necessary for cell contact inhibition by transfecting cancerous cells with cadherin expression vectors. The transfected cells exhibited contact inhibition. Baker and Humphrey’s observation that cancer cells can exhibit contact inhibition when treated with the correct glycoprotein recognition protein came before the identification of the cadherin family, and the detailed mechanistic explanation of cell-cell interactions [78]. These results, when regarded side by side point to the conclusion that mechanical methods of surface modification to manipulate cells may be used with what is known about contact guidance and contact inhibition.

2.3.2 Lithography Induces Guided Cellular Responses

2.3.2.1 Early Lithography Successes

The history of lithographic methods of patterning for cell guidance dates back to the nineteen sixties and seventies, when early experiments to enhance the adhesion of cells in culture and control their growth patterns were first taking place [98], [99], [100]. These early attempts used simple grid patterns with palladium [98], [100], [101]. This mode of cell confinement was first reported in 1967, and was subsequently repeated by many [98], [100], [101]. This method enjoyed success, with notable results of defined patterns being observed, and successful confinement to said patterns. These successes were expensive, as palladium is a rare metal, and precious, and the application process needed special vapor deposition equipment. As was discussed earlier, surface topography is critical in cell attachment, hence it is critical in guidance. Letourneau’s work showed
cellular preference for the biological coating of the amino acid ornithine over the palladium coating, but palladium was preferred over bare polystyrene [100]. As is known of polystyrene, cell adhesion on bare polystyrene precipitated the move to various surface treatments to decrease the cost of tissue culture wares, leading to the conclusion that the cells were not choosing the palladium in as much as they were choosing a micro- or nanoscale architectural change over the less hospitable surface of the polystyrene. The others showed similar results.

2.3.2.2 Complex Photolithographic Methods

Here, begins the more complex arena of true engineering lithography. These processes, as discussed earlier, require specific skill sets, training, advanced knowledge, and expensive equipment. Given the nature of lithography and its use in highly advanced micromachining for the electronics industry, with the current applications at the nanometer scale, the usual state of affairs is that if one knows biology, one probably does not know much about lithography, and vice versa. This often brings interdisciplinary collaborations in play to achieve significant results [25]. Such is the case with many studies, such as those occurring here at Louisiana Tech in the early years of the two thousands. Dr. Mike McShane, an engineer practiced in lithographic micromachining partnered with a Dr. Mark DeCoster, a neuroscientist at the Neuroscience Center of the LSU Health Sciences Center in New Orleans. The team worked together to produce patterns on glass which were a hybrid of lithography technique and layer-by-layer assembly (LbL). By designing the pattern protocol, they were able to create differential patterns, fluorescently labeled, that could be used to study neuronal cell attachment. This work was very successful and it showed high precision of patterning, at a scale down to
ten microns [23]. The patterns also enabled the neuronal cells to exhibit specificity for the pattern with great fidelity. Preference for one type of nanofilm as opposed to another was also on display, as the cells preferentially chose the film composed of secreted phospholipase A$_2$ (sPLA$_2$) over PLL, a previously discussed, well characterized cell adhesive coating.

Some groups start the process with silane treatments similar to those discussed previously, and create blocked areas on top of the silanes to then discourage cell adhesion [102]. This method also shows great fidelity and precision, and the lithographic patterning uses a polymer film photo resist that is easy to apply, and requires no special machinery. Indeed, the results obtained by Lorn, et al. were achieved using no special equipment, save for common tissue culture hoods, and a chemical fume hood. This method, or rather, combination of methods is clever, and cost efficient. It also happens to work.

Others have used lithography to create wide ranging 3D surface topography, which includes both surface microgrooves and microsteps to test the ability of neurons to react to the topographical changes [103]. Hirono et al. conclude, based on the results of their study, that the nerve cells cannot surmount much of a vertical challenge. Their tests included multiple variations of the microstep size, as well as multiple depths of the grooves, and after a certain point, the cells could not or would not overcome the barrier. This has been noted in the author’s four years of cell culture experience as well, that cells will grow up to, but not on, the vertical surfaces of the containers in which they are grown. In the case of flasks, often the flask has an angled surface to elevate the neck opening as seen in Figure 21. This angled neck will support cells, but is meant to help
maintain sterility by limiting the exposure of the opening to liquid media, which can spill.

This researcher has observed the more aggressive cell lines such as the rat glioma CRL 2199 grow up the angled surface. Primary cells, non-cancerous cells, and the transformed "immortal" cell lines will not attack the slope, which is of great interest, and suggests another avenue of study for surface contact guidance. Similar experiments concerning micro channels and neurons were performed by Mohoney et al., finding that the channels reduced the architectural complexity of the neuronal cells [104]. This suggests a need for diversity in the guidance cues to improve cellular response.

During development, the growing nervous system extends neurites to the outer reaches of the body. This growth takes place with amazing precision, and the neurites are guided by diffusible signals and by surfaces of the cells they are growing [68], [105]. Nagato et al. focused on this aspect of guidance and took an approach similar to the Hirono group, but chose to create patterns of higher precision to more closely resemble the dimensions of a small nerve bundle, which may be used to guide growing neurites. The group determined that contact guidance along micromachining aspects parallel to the polarity of the cell was common, but that perpendicular contact guidance was exhibited exclusively in the central nervous system. This is an interesting result, and could be
important in the design of regenerative constructs for spinal injury using many of the
techniques outlined here [106].

These various approaches using similar methodology show excellent
reproducibility and exhibit impressive levels of precision, owing to the advanced state of
the art lithographic process. These processes are the heart of the computer industry,
where critical dimensions are in the tens of nanometers. The benefit is that biological
sciences are now able to incorporate these technologies with relative ease when compared
to twenty years ago [107]. There are obvious advantages to these methodologies, but
current results are rudimentary, and are not close to tissue level organizational control.
This study chose not to use any of the complex photolithography methods, but used a
variation of soft lithography that will be discussed in the next section.

2.3.3 Chemical Surface Modifications and Microcontact
Printing Induce Adhesion and Guided Growth
Patterns

2.3.3.1 Soft Lithography Builds on Chemical Surface
Modification

Chemical surface modification that has already been discussed method-wise
includes layers of adherent peptides, proteins, and other biopolymers on tissue culture
surfaces. There are also the self assembled monolayers of various molecules, including
the electrostatically bound poly ions of the layer by layer assembly method. Surface
silanization is to be included here as well, along with the so called “soft lithography”
techniques of microcontact printing. Moreover, micropatterning results will be discussed
which were obtained not through soft lithography, but by variations of the “dip pen”
nanolithography process.
All of the aforementioned procedures have the common feature in that they all add some chemical structure to the surface, with application method being the greatest difference. They all use different surface bonding techniques as well, but the results seen show very similar successes, and all the methods together promise additional levels of control of cellular patterning.

2.3.3.2 Poly-L-Lysine (PLL)

The first adhesive polymer to be examined is PLL, one that has been used in DeCoster's lab for years. PLL adhesion is commonly accepted, and is in widespread use [17], [24], [95]. The results of PLL treatment are actually variable. Simply coating surfaces to cover with a layer of 0.005% PLL in deionized (DI) water, and letting it sit for 1 hour or more, will achieve cell adhesion results. With too high a concentration, approaching 0.05%, the treatment becomes cytotoxic [108]. This approach only offers adhesion control, unless applied after patterning, as many have done [23], [24], [49], [52], [95], and have been discussed already. PLL monolayer application is much more consistent than coatings of large biomolecules such as proteins, due to their tertiary and quaternary shape characteristics. The cells prefer a more adhesive surface, and tend to achieve a level of differentiation and gene expression that correlates more closely to the \textit{in vivo} situation in regards to shape, and production of various proteins that can be measured [62], [109].

2.3.3.3 Surface Silanization

Using a silane surface treatment can improve the durability of surface coatings layered on top of the silane layer [4], [36], [46], [110]. This gives the layers the added stability of non-hydrolysable bond to the surface. With this surface pre-coating, non-
adhesive regions are easily patterned as well, giving more precise control over cell patterns and shapes [110].

This siloxane derivitization lends itself especially well to microcontact printing. The use of elastic polymer stamps that are dipped into a patterning substrate, and then brought into contact with a surface, benefits from having a reactive group available on the surface as discussed previously [111]. Siloxane treatment is similar to the LbL method, but is not based on electrostatic attraction. The bond is covalent, but the end result is similar: microcontact printing invariably requires a means of adhesion to the surface be it electrostatic, covalent, or undefined as the attraction for thiols to gold that was the basis for some of the first self assembled monolayer (SAM) research [65], [112].

There has been a great deal of success using all of these methods, and research into new applications continues. The challenge now is to characterize the binding and morphology of the surfaces. There are methods available to quantify mRNA production to determine the developmental state of different cell types that can be used to ascertain each application’s appropriateness for each cell type [95], [113], [114].

As progress is made these methods will become more reproducible, increase fidelity, and can be the first steps toward tissue level organization, once the characteristics of each surface treatment is determined and how to apply them toward 3D patterns.

2.4 Literature Review Wrap Up

The apoptosis information was vital to the first goal stated for this project of developing a simple model of apoptosis. While reviewing apoptosis in-depth, the notion
occurred that this process could be used in conjunction with scaffolding and patterns to create a more biomimetic in vitro scenario for 3D cell growth.

All of this information in the review is helpful for the second goal pertaining to the scaffolding. Evaluation of the scaffold material in light of this knowledge makes the scaffolding an attractive platform for use with applications of patterning and apoptotic sculpting, as discussed elsewhere in this work.

Much was learned about these various techniques of surface modifications and patterning, in order to choose what methods would be best suited to the chosen goals of each portion of the project. All options were discussed with various faculty members, searching for alternatives to the current adherence blocking strategy, in an effort to identify suitable methods that could be used or adapted to use in cell culture applications. As the reader will see, it is this literature review for the third stated goal of this project-increase the fidelity of cell adherence to patterns deposited by the NanoEnabler® system by developing a novel cell–adherence–blocking strategy which yielded and developed the concept for the 3D cell model of tumor progression discussed later.
CHAPTER 3

MATERIALS AND METHODS

3.1 Apoptosis Testing

3.1.1 Glutamate Delay of Staurosporine Induced Apoptosis

3.1.1.1 Staurosporine, an Apoptotic Stimulus

Staurosporine (STS) is a bacterial product that is chemically a multicyclic indole alkaloid, C_{28}H_{26}N_{4}O_{3}. It is a non-specific protein kinase inhibitor which acts as a potent apoptotic stimulus in many cell lines. An observation in the DeCoster lab of staurosporine–treated cells showing morphological difference in similarly treated cultures pre-treated with glutamate brought a great deal of attention. The hypothesis was formed that apoptosis could be delayed by pre-treatment of glutamate, which is the major excitatory neurotransmitter in the central nervous system (CNS). A goal was set to determine cell area changes over the course of the apoptotic process. This information would be used to create a model of apoptosis that could be used to determine the apoptotic state of a culture based on its comparison to the model. The cells used for this project include rat cancerous cells, CRL 2199, and CRL 2303, normal rat astrocytes, and human Jurkat lymphocytes.

3.1.1.2 Calcein Fluorescence Area Measurement Procedures

Experiments were designed to test the hypothesis. The experiments included control groups of astrocytes obtained from Lonza, Jurkat cells CRL 2570 and CRL 2199
rat glioma cells. The Jurkat cells and the CRL 2199 line, both obtained from American Type Culture Collection (ATCC), were to be compared to apoptotic groups treated with 20 μM STS and 20 μM STS groups pre-treated with 1mM glutamate (GLU) solution. These experimental groups were plated in twelve-well tissue culture plates from Greiner, at similar cell densities at each iteration of the experiment. To quantify the differences between the cultures, the live (healthy, or apoptosing) cultures were fluorescently stained with Calcein AM®. This staining was achieved by incubating cells in a Locke’s solution with 0.2% Calcein AM® and 0.1% pluronic acid for 1 hour at 37°C and 5% CO₂. Fluorescent images were recorded with Leica DMI6000 inverted fluorescence microscope, and Leica DFC 290 black and white three megapixel digital cameras. Imaging proceeded over multiple fields of standard wells of twelve-well tissue culture plates. The multiple field images were then analyzed using Image Pro® software to determine the calcein positive, or fluorescing, cell area. Cell areas for each treatment condition were compared at multiple hourly time points. For each imaging session the “reverse time point method” was used, a time consuming and difficult organizational method requiring addition of metered reagents at times such that when the experiment was ready for staining, different time points were all ready to be stained with fluorochrome at the same time. Experiments were repeated at least in triplicate, creating multiple data sets of cell areas to be graphed for comparison at varying time points for each group: control (treated only with Locke’s solution, a sterile sugar and salt solution), STS treated, and GLU pretreated STS.
3.1.1.3 DAPI Nuclear Area Measurement Protocols

The same treatment protocols were then followed, again using all three cell types (in separate vessels) to determine effects on nuclear material, as apoptosis presents a specific endpoint scenario in regards to DNA: there is fragmented DNA that shows specific patterns when electrophoretically separated. This pattern is called DNA “laddering,” as the fluorescent bands on the gel are evenly spaced and resemble a ladder. Dr. DeCoster previously introduced the Nuclear Area Factor as an apoptosis diagnostic measure, and has shown a decrease in area correlating to progression of apoptotic processes. With this in mind, the treatment protocols were run, but this time the fluorochrome used was 4, 6-diamidino-2-phenyindole or DAPI. This molecule intercalates between the DNA double helix molecules and fluorescent intensity of the molecule increases when bound to DNA. Cells were fixed at the time point specified, using ice cold absolute methanol, rinsed and covered with PBS until ready for staining. DAPI solution was prepared by adding 0.1% dye to Locke’s solution and vortexing. Plates were incubated overnight in refrigerated storage. Stained cells were imaged on the Leica DMI6000 as before, with image analysis providing data on nuclear area. Multiple time points for each treatment condition were recorded as with the Calcein experiments. The areas for each time point and condition are then compared with graphical analysis.

3.1.1.4 MTT Assay Correlation

The previously discussed experiments were mirrored with the MTT assay. MTT or dimethylthiazol diphenyltetrazolium bromide is yellow in color, but when added to living cells is broken down to a purple colored product in the mitochondria of the cells. The purple product is measured in the Thermo Scientific Multiskan Spectrum plate
reader, for high throughput. A complete description of the assay can be found in Appendix A. The absorbance is quantified, and in relation to the number of cells in the culture, gives a qualitative measure of culture health, i.e. healthy cell cultures or controls will produce a high reading, where apoptotic cultures will produce a lower reading. This data can be charted and compared at specific time points.

3.1.1.5 Caspase 3 Measurement

As previously mentioned, caspase 3 (casp3) is a major effector enzyme in the apoptotic process. In order to test whether the observations were indeed delay of the apoptotic machinery, a molecular biology assay was used to determine casp3 enzyme levels at similar time points with similar treatment protocols to the previous experiments. The CASP3C kit from Sigma-Aldrich was used to determine the casp3 levels of astrocytes and CRL 2199, or Jurkat T Lymphocyte cultures treated again with STS, or pretreatment with glutamate and STS, and compared to controls treated with Locke’s solution at similar time points to the previous experiments.

Cells are plated as previously discussed. Again, treatment protocols are similar to earlier experiments. The protein extraction of the cells must be accomplished to measure casp3. Upon completion of the time points specified by earlier experiments, cells are lysed (broken apart) with a lysis buffer which will then be collected and centrifuged. The lysis buffer chemistry is such that enzymes and similar proteins stay in solution, while structural proteins and membrane bound proteins do not. After this extraction, the enzyme containing lysis buffer is diluted with the assay buffer and the casp3 synthetic substrate in a ninety-six well plate for use in the Thermo Scientific Multiskan Spectrum plate reader. The solutions are incubated for ten to twelve hours (the calculation takes
into account the time of incubation). The assay uses p-nitro aniline (pNA), a molecule which can be quantified by spectrophotometry at 405 nm using Beer’s law, in Equation 1.

\[ A = \epsilon L c \]  

In Equation 1, the absorbance \( A \), is equal to the molar extinction coefficient \( \epsilon \) multiplied by the path length \( L \) and the concentration of the solution. Knowing the absorbance, and the molar extinction coefficient, and a path length of the spectrophotometer, we can then calculate concentration. One prepares standard curve by measuring absorbance of solutions prepared at specific concentrations. The values are plotted and a best fit line is produced which will be used to determine the concentration of the experimental protein extraction. In Figure 22, an example of a standard curve for one of the casp3 experiments is presented.

Figure 22. Example of pNA standard curve performed at each iteration of casp3 experimentation.

Then measurement of the unknown protein solutions proceeds, and is plotted on the standard curve to give a concentration of pNA produced from reaction of the synthetic substrate analog for the casp3 enzyme. The appearance of a yellow color and
increased readings from the spectrophotometer indicate increased casp3 activity. This increased activity is known to correlate with escalating apoptotic death outcomes. Activity level is determined by equation, as well. Shown next in Equation 2, is the example calculation of casp3 activity.

\[
\text{casp3 activity} = \frac{\text{\(\mu\)mols pNA produced} \times \text{dilution factor}}{\text{reaction time in minutes} \times \text{volume of sample in mL}}
\]

(2)

This test shows great specificity and is supplied with purified casp3 enzyme as a control, and along with measurement of control groups is repeatable. The data from multiple time points and treatment conditions can then be charted and compared.

3.1.1.6 Image Analysis of STS Treated Cultures with Image Pro Analyzer®

Images were collected as stated previously, and analyzed with Image Pro Software version 6.0 developed by Media Cybernetics. All images were assessed for cell area or nuclear area, as appropriate for the staining condition (Calcein AM® stains the whole, live cell, whereas DAPI stains only the nuclear material). The software also calculated the roundness of the bright structure, with the unit circle reference being a roundness value of one. With the extremely high signal to noise ratio of fluorescence microscopy, the software can, with little operator input, determine the number of bright fluorescing objects, which are the cell bodies or the nuclei. The illumination pattern enables the number, roundness, and cross-sectional areas of these objects to be quantified.

Cells that have grown together can be difficult to distinguish. The software can, in many cases, use an average size threshold, or another parameter of chosen by the user to distinguish abutting or overlapping cells, but user input is sometimes needed to ensure
accuracy. Multiple frames for each experimental condition were processed. In addition, each condition had multiple time points. Each time point was repeated in triplicate for each condition, at each repetition of the experiment. Data were exported in Excel® format and used to chart and compare differences between conditions and time points.

3.2 Scaffolding Tests

3.2.1 CRL 2020 Cell Culture Testing

3.2.1.1 Cellulose Scaffolding for Tissue Engineering Applications

Cellulose scaffolds produced by the lab of Dr. Yuri Lvov were tested as a tissue engineering platform. Experiments were designed to visually and quantitatively assess the scaffolds ability to support and encourage cell invasion, growth and differentiation.

3.2.1.2 Scaffolds for Microscopy

CRL 2020 human brain cancer cells were obtained from ATCC. Cells were cultured as recommended in RPMI 1640 plus 10% Fetal Bovine Serum (FBS) as growth medium. Both gelatin and cellulose fibers/gelatin micro scaffolds (with 75 wt% fibers) were cut into thin slices approximately 1.5 mm thick, sterilized using 70% ethanol, and washed with phosphate buffered saline (PBS). Two additional PBS washes completed preparation. Cells were trypsinized to suspend the cells, and the suspension centrifuged at 250 X g to pellet. Pelleted cells were resuspended in fresh media, counted and diluted to deliver a 1 ml cell suspension with $2 \times 10^6$ cells to seed onto the matrix. Matrix materials were seeded in single wells of 24-well tissue culture plate. After seeding, the media was changed every other day and the cultures were maintained for 16 days at 37 °C and 5% CO₂.
At 17 days *in vitro* (DIV) culture, the brain cancer cells were stained using Calcein AM® as described previously. Cells were imaged using a LEICA DMI RE2 Confocal Laser Scanning Microscope with multiphoton source or Nikon Ti 2000 fluorescence microscope. Specific images are labeled as to origin.

3.2.1.2 MTT Testing

MTT testing was chosen to compare the progress of cell cultures with the fiber/gelatin scaffold, gelatin scaffold, plain copier paper, and no scaffolding (control group). CRL 2020 cells were seeded at similar density to the imaging experiment, proportional to the smaller construct size used, approximately 1/8 of the disc-shaped scaffold. Time points were defined at 1, 3, 9, and 17 days, with the 17 day terminal matching previous work. The scaffold portion was removed from the culture well and assayed with the MTT method as stated earlier. The culture well used for each scaffold sample was also assayed as a normalizing factor to account for any cells that may have grown in the dish, and not the scaffold construct. Experiments were repeated in triplicate, and data were charted for comparison.

3.2.2 CRL 2303 Cell Culture Testing

3.2.2.1 Scaffolds for Imaging

CRL 2303 cells are clonal lines similar to the CRL 2199 cell line discussed previously. The CRL 2303 variety expresses a certain transfected gene more actively than the CRL 2199 variant. This makes them well suited for visual assessments, as the gene expressed is an enzyme which will produce a blue–colored product trapped within the cell when exposed to the X-gal substrate, a synthetic galactose molecule.
Cells were seeded as before and visualized at 17 days with the Leica DMI6000 microscope. Phase contrast images were collected for appraisal.

3.2.2.2 MTT Assay of CRL 2303 Cultures with Scaffolds

MTT was carried out as described previously with the scaffolding, and time points again of 1 day, 3, 9, and 17 days. Assays were repeated in triplicate. As before, data were charted for comparison.

3.3 Fabricating Selective Non-Adhesive Cell Culture Surfaces in Conjunction with Patterned Adhesive Surfaces

3.3.1 Evaluation of Standard BSA Blocking vs. Polymer Non-Adhesive Coating

3.3.1.1 BSA Blocking and Goals for Increased Efficiency

In the DeCoster lab, there have been many previous publications dealing with patterned cellular responses; dating back to before DeCoster was even at Louisiana Tech. The most recent publications on patterning, by Dustin Green in DeCoster’s Lab used an APTES coating with gluteraldehyde cross linking agent on glass cover slips followed by patterning of PLL with the NanoEnabler® system [115]. This system deposits liquid on surfaces via a cantilever on a silicon chip. The cantilever is hollow, and attached to a machined reservoir for the liquid to be applied. After liquid is loaded in the reservoir, the machine relies on microprocessor controlled servos to achieve sub-micron precision patterns in the x and y dimensions, delivering droplets of as small as femtoliters, depending on dwell time, and cantilever bore. This method achieved excellent results over the short term with Bovine Serum Albumen, or BSA, a blood protein found in the plasma portion of cow blood, applied to the surface after patterning to bring about blockage of non-specific cell binding. The process is simplified in Figure 23.
As the days go by for cells in culture, they modify their surroundings with secreted proteins that can be adsorbed to the BSA coated surface. Also, proteins from the serum-containing media can displace the BSA on the surface. These processes lead to decrease in the fidelity of the cells adherence to the pattern over the course of days.

The actual methodology of the printing with the NanoEnabler® (NeN) system requires a great deal of preparation, which can be done in large batches, to reduce the overall start to finish time of each experiment. 25 mm square common glass cover slips are washed thoroughly with two sonication baths in absolute ethanol at thirty minutes each, alternated with deionized (DI) water, for thirty minutes each also. This is followed by drying with compressed nitrogen stream. The slips are then oxidized in the UV/ozone cleaner supplied with the system for one hour. The slips are placed in a covered vessel with 50μL of APTES solution, and heated at 60° C for two hours. This is the APTES vapor deposition stage. This is followed by curing of the silane layer at 100° C for sixteen hours. Upon cooling the slips are incubated in a 6% gluteraldehyde solution for two hours at room temperature. Rinsing with filtered DI water, followed by drying in compressed nitrogen stream completes the preparation. The slips are now ready for printing with the NeN system.
Patterns are designed based on Green’s work suggesting that larger cells such as astrocytes prefer a larger adhesive spot, in the range of fifteen to twenty microns in diameter, while smaller cell types such as the CRL-2303 line prefer a ten micron spot diameter. These characteristic diameters show better capture characteristics in Green’s work, as the cells needed to be seeded in very low concentrations [115], and reflect preceding discussions in regards to surface topography and cell adhesion. Patterns are entered into the NeN computer, and the cantilever reservoir is filled with one μL poly-l-lysine labeled with the fluorescent molecule FITC. After a process to coordinate the x, y, and z plane controls, location of the surface to be patterned is confirmed. Printing of the pattern at this point takes only a few minutes, of course depending on how many spots are patterned.

Once the patterns are complete, BSA solution at one milligram per milliliter is placed on the slip for one hour. Upon removal of the blocking solution sterilization of the patterned slips is completed by UV irradiation for fifteen minutes on each side of the slip, using the sterilization UV source in the laminar flow hoods in the tissue culture facility.

Sterile slips can be stored in sterile conditions for many weeks. They can then be used whenever the cell cultures are ready. Cells are trypsinized, pelleted, resuspended, and counted as described previously. Cells are seeded on the patterned slip in a sterile polystyrene tissue culture dish with only 100 microliters of cell suspension. This suspension is placed in the area of the pattern, and allowed to incubate for 15 minutes. The number of cells can be adjusted, according to the area of patterned surface. Growth media containing serum is then carefully added to fill the culture dish. Cells will adhere to the patterns and show pattern fidelity for three to four days when seeded at low density (hundreds of cells). These constraints prompted a search for a blocking agent that would
be amenable to higher seeding densities, and longer resistance to non-specific binding. These characteristics are needed in the design and implementation of tissue engineering constructs.

3.3.1.2 Polymer Blocking for Increased Fidelity

The polymer is referenced in the Louisiana Tech University Report of Invention, but the methods of its use will be described here.

Polymer is prepared in aqueous solution at prescribed concentration using Sterile DI water, and polymer, with vortexing, and sonication if necessary. NeN cover slips are prepared as before with the exception of the gluteraldehyde treatment. After trial and error experimentation, it was determined that the gluteraldehyde cross linking of the APTES layer changed the hydrophobic characteristic of the surface to a less hydrophobic condition. With the omission, the other changes are substituting the polymer solution for BSA solution, and seeding the cells at higher densities (thousands of cells).

3.3.1.3 Cytophobic Surface Treatment for 3D Cell Cultures

During the empirical experimentation portion of the polymer/patterning project, there was doubt as to the efficacy of the treatment. Dr. DeCoster suggested a different approach for testing the polymer properties in culture. A protocol was devised to test the polymer polystyrene, known for its hydrophobicity. Simple external circular markings on polystyrene dishes were contrived, and the inner, cell culture surface was treated with the polymer confined by miniscule volume to the circle. Incubation period was for one hour. DI water rinse followed, and cells were immediately trypsinized and processed as previously described to place a prescribed number of cells in the treated dish.
Further experiments were conducted with entire inner surface of the vessels coated with polymer solution. Experiments were completed comparing static polymer treatment with dynamic polymer treatment using a shaker assembly.

3.4 Additional Information

Additional information pertaining to the methods discussed herein can be found in Appendix A.
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Apoptosis Project

4.1.1 Apoptosis and the Cell Area Factor

4.1.1.1 The Cell Area Factor

The DeCoster lab has recently promulgated a simple qualitative model of the apoptotic process. The Cell Area Factor (CAF) is a numerical reference to the decreasing of measured roundness of the cell. The CAF is the product of the measured roundness and the cell area as measured by image analysis. This factor decreases as apoptosis progresses, until the cells are compacted and ready to be phagocytosed, at which point the factor levels off. The basic features of the hypothesis are demonstrated in Figure 24.
Figure 24. Hypothesis of simple qualitative model of apoptosis based on cell shape and size.

This model can be useful for categorizing the apoptotic state of a cell or culture based solely on microscopic observations. The data collected for this work seems to support the hypothesis. Roundness values were enumerated by the image processing software, along with area values have been charted for the various cell types. Figure 25, shows the roundness of Jurkats and astrocytes under experimental conditions.
Figure 25. Roundness of Jurkat cells and astrocytes 5 hours post treatment with STS.

The figure shows nuclear roundness decreasing in both cell types (DAPI), along with overall cell roundness decreasing (Calcein) from the control case to the STS treatment. Control cells have no treatment. This is significant in opposition to the necrotic case of cell death, where the cells expand. Here it is seen clearly the nuclear material and the cell itself is shrinking due to apoptosis.

The CAF shows dramatic change in the morphologically polarized astrocytes as opposed to the spherical Jurkat blood cells. Referring to Figure 24, the expected response of Jurkat cells would be to shrink, but not necessarily become more round, as their silhouette is round already, whereas the astrocytes would change drastically to a more round morphology. This is exactly the case, as shown in Figure 26.
Figure 26. CAF shown for Jurkats compared to astrocytes, demonstrating dramatic change during apoptosis.

This dramatic change in astrocytes morphology is plainly visible under the microscopic inspection, but with fluorescence microscopy, accurate measurements can be taken. Apoptotic changes are evident in astrocytes early on, and two hours after apoptotic stimulus, the changes are drastic. Examples of these changes can be seen in Figure 27, which is of control (untreated) and apoptotic astrocytes two hours post treatment.
4.1.2 Apoptosis and Cell-Cell Interactions

During the course of the apoptosis project, the observation of a change in cell-cell interaction of the Jurkat cells was made. The apoptosis model explained previously would not predict any cell-cell interactions amongst the Jurkat cells, a free floating spherical cancerous cell line. Normally these cells do not interact, but after STS treatment, there is a change. This change results in aggregation during the apoptotic process, and then separation of the aggregates with shrinkage of cell area occurs. The Calcein stained cells shown in Figure 28 highlight this activity.

Figure 27. Astrocytes untreated (a), and 2 hours post STS treatment (b).

Figure 28. Panel a) Calcein stained Jurkats, no treatment, b) Calcein stained Jurkats at 2 hours post STS, and c) Calcein stained Jurkats at 6 hours post STS treatment. Clumping is circled in RED.
This clumping process may have a basis in the apoptotic stimulus, i.e. staurosporine may cause this action, or the apoptotic process itself may. There are known membrane changes in cells undergoing apoptosis, including Annexin V being displayed on the cell surface, as opposed to its usual location on the inner cell membrane. This protein is an effective marker for apoptosis in many cell types, as there are antibody kits to identify apoptosing cells with this application. Some of the changes must alter cell surface proteins in such a way to cause the agglomeration. This activity is charted in Figure 29, showing the number of bright objects for Calcein dropping off and then increasing again, to match the bright objects for DAPI, which continue to rise linearly. This effect seen in the DAPI stained DNA is due to the DNA being chopped up by endonuclease caspases. This increases the number of bright objects, and they are not clumping inside the cells, the nuclear material is being digested and packaged for recycling.
Figure 29 Bright objects as counted by Image Pro Plus® software. Red series shows Calcein objects (whole cells or clumps) and blue series shows DAPI objects (DNA with increasing fragmentation as apoptosis progresses.

This clumping activity is interesting and should warrant further study, to examine the mechanism of the cell-cell interaction. Elucidation of this mechanism could be of great value to patterning in three dimensions.

4.1.3 Apoptotic Modulation and Biochemical Assessment

4.1.3.1 Glutamate Delays Apoptosis in Normal Brain Cells and Cancerous Glial Cells

The glutamate pre-treatment discussed in the earlier section was shown to delay apoptosis morphological progression. The delay factor is difficult to quantify with simple
microscopy, but with advanced image processing techniques, cell area can be measured, along with cell roundness. These can help quantify the stage of apoptosis, and when compared to other cultures treated similarly, the net delay can be seen. Physical variations can be seen in Figure 30. These variations are slight, but quantifiable.

![Figure 30. Astrocytes. Panel a) with STS at 4 hours, b) glutamate pre-treatment then STS, 4 hours, c) control. The first two images in each panel are time lapse images of the same spot; the third image is a Calcein stained sample of same treatment condition and time point.]

Comparing the control cells in panel c, Figure 30, the morphology seen is the accepted norm. In panel b, there is obvious damage, but in comparison to the fluorescent image for panel a, the damage is less. These fluorescent images are examples of the images used to quantify the steps of apoptosis through cell and nuclear area, and cell and nuclear roundness, with each frame yielding multiple data points. With the calcein
images there may be dozens of cells, and each experimental condition had multiple time points, with four frames per time point yielding a high number of data points for increased statistical significance. In the case of the DAPI photos, a lower magnification was used to increase the field size, and therefore include more data points.

4.1.3.2 Caspase 3 Assay Results Show Delay in Appearance of Activated Caspase 3

The morphological changes during apoptosis have been discussed and demonstrated, as have the apparent delay of the apoptotic process. The question remained whether this was an artifact, a result of staurosporine induced apoptosis only, or if it affected the actual mechanisms of apoptosis. With the caspase 3 assay, the question is answered, fully. Many proteins are produced as needed throughout the life of the cell, but the caspase exist in the cytosol as pro-enzymes. This is an inactive form of the enzyme. Once a portion of the protein is cleaved away, enzymatic activity of the molecule is enhanced, and the apoptotic cascade commences. The data shows a quantitative delay shown in caspase 3 activation. The activity levels recorded by the assay imply the amount of active caspase 3, hence as activity increases, amount of active caspase 3 increases. This is shown in Figure 31, where the most dramatic feature is the activity of the small amount of human recombinant caspase 3 tested.
Figure 31. Normalized casp3 activity of astrocytes, showing slightly less activity at 2 hours for the glutamate pretreatment, then significantly less at the 6 hour point. Human caspase 3 positive control shows the assay is functioning.

As seen in the figure, the human caspase 3 shows the assay is working properly, and the glutamate pretreatment group active caspase 3 level never gets above the level of the STS only treatment at the six hour mark. Recall the apoptotic damage in the four hour time point photos showing damage. Shown in Figure 32 is an example of the difference between zero hour and six hour STS treatment. The cells on the left in (a) are the same as the cells on the right in (b), photographed before commencement of protocol. A physical mark was made in the external surface of the vessel to facilitate alignment to the same cellular region at various times, enabling tracking of the same population of cells for measurement and comparison purposes over the course of the apoptotic process.
The morphological differences are more pronounced at the six hour point than at the four hour point. This is also based on the idea of the enzyme cascade, a physiological feature found in many control systems throughout the body, such as the cascade of the blood clotting mechanism. As more enzymes are activated, the overall effect becomes more pronounced, as each molecule of enzyme produces a large net effect, with the cascade effects becoming similar to geometric growth. This proceeds until a tipping point is reached, and the cell cannot be recalled from the apoptotic pathway. The evidence seems to support that apoptosis can be modulated by glutamate to a certain point, possibly pushing the effects back only an hour or two. Ensuing experiments showed promise for continued delay of the apoptotic process with successive additions of glutamate. This result is shown in Figure 33, which shows a similar trend with the STS and G+S treatments as the earlier work.
Figure 33. Comparison of two glutamate additions, one before and one after STS treatment (green), with STS (blue), and glutamate pretreated STS protocol (red).

There is also a slight peak of the caspase activity for the glutamate pre-treatment protocol in the earlier work, and can be seen in Figure 31, showing similar trending. The repeated glutamate addition protocol was limited in repetitions and the activity levels seem to warrant closer examination.

**4.1.3.3 MTT Evaluation**

The MTT assay has been discussed previously, with results for this arena of testing being inconclusive. The graph in Figure 34 shows varying levels of mitochondrial activity at varying time points. Correlation with the other methods is inconclusive.
Figure 34. Astrocyte MTT data reflecting varying mitochondrial activities for each treatment condition.

At time point 0, the mean absorbance values are slightly lower for STS and G+S than for the control. However, the lower values are not caused by the treatments because treatment was not started until after the imaging. At time point 2, the STS may have acted over a long enough time to cause some metabolic depression. Time points 4 and 6 demonstrate increased activity over control groups, however. Because apoptosis is an energetically demanding process, the increase in cellular metabolism is consistent with an apoptotic process.

4.2 Scaffolding Results and Discussion

4.2.1 Microscopic Evaluation

This portion will focus largely on the work done in collaboration with Dr. Yuri Lvov in 2010. The result of the collaboration was a publication in the journal *Acta Biomateriala* [116].
Digital microscopic images were recorded to visibly present evidence of cellular invasion, growth, and differentiation. The following image, Figure 35, shows a confocal microscopy phase contrast image and the subsequent fluorescent image. The two images were then merged with image analysis software to provide the third image in each panel showing the overlay of the fluorescence on top of the phase contrast image. The gelatin/fiber scaffold (lower panel, arrows) is showing organization of the cells along the fiber surfaces, allowing a place for the cells to adhere. The arrows in the upper panel indicate the leading edge of the gelatin with cells seeded. Notice the difference in morphology between the two, both seeded at the same density, and grown in the same incubator for the same amount of time. The fiber/gelatin scaffold shows the cell are
elongated and assuming their normal morphology. The gelatin alone in the upper panel shows evidence of undifferentiated growth. The cells have enough support to grow, but the target is to achieve growth and differentiation. This is a positive result, nonetheless, as it demonstrates the gelatin’s ability to support the cells. Taken together, the two results suggest there is a better ratio of fiber to gelatin that may encourage greater growth while still allowing for differentiation.

The fluorescence images, examples shown in Figure 36, demonstrate similar morphologies to the confocal images, with cell–fiber alignment obvious.

Figure 36. Fluorescence imaging of CRL-2020 cells with gelatin (upper panel) and 75% cellulose fiber and gelatin (lower panel). Panel: a) phase contrast; b) epifluorescence; c) merged image [116].

The fluorescence images show similar alignment of the cells with the fibers, though not to the extent shown on the confocal images. Confocal microscopy produces much more precise images with its laser illumination of a very fine section of the sample, termed a z-section. This is a reference to the z-plane of the coordinate system, as the laser
illuminates on one plane for each image, with user-defined settings for the thickness of the plane.

4.2.2 MTT Data

The MTT assay was used to measure the cell growth with the scaffold over the course of the experiment. These data, shown in Figure 37, shows an increase in the metabolic activity of cells growing in culture with the scaffold.

![Figure 37. CRL 2020 MTT data for cell grown with and without various scaffolding.](image)

The cells are slightly behind the control group at all points until the last. Fiber and gelatin together (blue, labeled “fiber”), and the gelatin alone rebound nicely by day 17 and are ahead of the control groups then. The paper group, samples of common printer paper show some cell invasion, but the density of the fibers of paper may impede too much. A photo of the whole plate in Figure 38 shows purple formazan crystals, the product of the MTT reaction with active mitochondria.
The picture shows many pieces of information. In “a,” the fiber construct has shifted, revealing an outline of its previous position. For this reason, the wells were tested as well, after scaffolding was removed. A similar circumstance has arisen in the wells labeled “c,” with the printer paper. Wells labeled “b” and “d” are gelatin and control. These appear to be no different, but with the assay, differences were revealed, and are seen in the preceding chart.

4.2.3 Scaffolding Implications

To model physiological tissues in vitro with the same properties and differentiation aspects as the in vivo situation, one must address the three dimensional nature of native tissue, discussed in many other scenarios in this document. To account for these differences, there must be a construct of three dimensions to which the cells can attach, grow and proliferate in. Many methods of tissue engineering exist today for approaches to 3D cellular growth, but none so far has stood out as the best for any particular application, in pertinence to cell type, location in the native tissue, etc. Issues
for choice of material for the constructs required by the application have yet to be characterized fully, but the obvious credentials are easily identified. The material must not be cytotoxic. It must harbor the cells, and promote growth and differentiation, especially in the circumstance when one would choose a stem cell for the particular application being studied.

Cellulose fibers are naturally occurring, abundant resource, and are present already in many plants and bacteria. Cellulose also offers great promise in cell culture and as a biomedical material, due to many factors including its biocompatibility. As mentioned, it is already abundant in living things on earth, and is biocompatible with human tissue, as well. The surfaces of cellulose fibers have reactive groups that can manipulated for protein adsorption and binding. It is also attractive from an engineering standpoint, in that its mechanical strength makes it a god choice for scaffolding, and its resistance to hydrolysis would make it ideal for applications requiring permanent structural support.

Cellulose is a glucose polymer with a bond structure that humans and most animals do not possess the enzymatic capacity to degrade or digest. Buildup of large quantities of cellulose would be detrimental, of course, but used as a platform for ex vivo growth of tissue with subsequent re-implantation, the material has been shown to be safe for use with granulation tissue and bone formation [117], [118]. It has also been shown that this starting material can promote cardiac cell growth, while enhancing the connectivity of the cardiac cells, thereby increasing the electrical functionality of the excitable cells [119]. Cartilaginous tissue can exhibit enhanced development on cellulose fiber scaffolds when the scaffold is pre-treated with calcium phosphate [120], and the high density of reactive hydroxyl groups makes protein immobilization with fibronectin,
and other cell adhesive proteins, a simple method for further enhancing cartilage growth [121]. The densities of the glucan chain structures in the cellulose fibers impart the necessary strength to support cell aggregates [122], and the fibers are very stable under dynamic stress [123].

While the fibrillar structure provides good mechanical properties for tissue culture applications, it does not naturally degrade in the body. If this is desired, the cellulose material can be easily degraded with cellulase, an enzyme that digests cellulose. This enzyme naturally occurs in termites, giving them the ability to digest wood fibers. Similarly use of the enzyme would provide removal of the scaffold without harm to the tissue-level organization resultant of the construct [122].

There is little research available demonstrating use of cellulose fibers as a 3D scaffold, due to the lack of three dimensional architecture in its native form. Addition of gelatin, a collagen derivative that is biodegradable, non-immunogenic, and inexpensive, may add to the 3d characteristics of cellulose based tissue culture scaffolds. Collagen and gelatin have been shown to have effective applications in tissue engineering scaffolds, already [124], [125], [126]. By using these two methods in a combinatorial approach, the goal was to synergistically increase the effectiveness of the resulting construct.

The ideal qualities of a scaffold material would include not only mechanical strength, but a high porosity that would allow sufficient oxygen diffusion to sustain a growing cell aggregate. Mechanical testing was also performed by the Lvov group to elucidate the properties of the construct in comparison to the stated ideals. Mercury porosity testing put the porosity of the 75% fiber/gelatin construct at approximately seventy percent, with pores large enough for nutrient transport as well. These same scaffolds showed a Young’s modulus of roughly eight times the gelatin alone, indicating
a much higher mechanical tensile strength, and exhibited four times the peak stress as the gelatin alone.

The fluorescence images revealed a greater propensity for alignment of cells and fibers in the fiber/gelatin construct than the gelatin alone. While the alignment aspect is important, it is also worthy of note that both scaffold conditions were seeded with the same number of cells, and appeared to have greater fluorescence in the gelatin alone, suggesting better cell survival and proliferation with the human glioblastoma line CRL-2020. It is also suggestive of a possible alteration to the approach, using less fiber and more gelatin, as no other conditions were studied.

Despite the suggestion that there may be greater survival in the gelatin alone, cell invasion to the inner portion of the matrix was confirmed with confocal microscopy. Cells can grow throughout the construct, and are less clustered in the fiber/gelatin matrix than gelatin alone; leading us to believe the fibers may provide better guidance to effect cell growth. In the light of previous discussions about contact guidance, and surface chemistry, along with topography, the assumption would seem to be supported.

Cellulosic materials have demonstrated good biocompatibility and mechanical strength to be used as a biomedical engineering material. It has been shown that different cellulosic materials can be used for bone [118], cardiac [119], and cartilage growth [120]. In the experimental scaffolds, the gelatin coating rendered the fiber surface suitable for cell culture, as well as providing spacing for the cellulose fibers. The surface and structure of scaffolds can significantly influence the interaction between scaffold and cells as well as cell growth, migration and differentiation [124]. The rough structure of cellulose fiber/gelatin scaffold may also play an important role to promote cell adhesion and interaction with materials. It was reported that the interconnections between
microfibers facilitate cells to cover the micro-fiber scaffold [127], and this work has discussed the importance of micro- and nanotopography at length. In the cellulose fiber/gelatin scaffold, the interconnection was not limited to between fibers, but also included between gelatin film and fibers. In this way the scaffold may provide better support and guidance to the cells.

The gelatin scaffolds tended to dissolve quickly in cell culture conditions, making it impossible for us to remove the scaffold intact. However, MTT analysis did show that for both scaffold types, cell viability/metabolism increased 5-fold by 17 DIV compared to values at 2 DIV. This increase is not reflected in the chart, as all values are normalized to the control for that time point, meaning the value of the control cells becomes one, with the other treatment conditions a ratio of the raw value for that condition to the raw value of the control at the time point.

The gelatin/cellulose fiber scaffold provided two important aspects for tissue engineering: three dimensional structures within which cells could grow, and sufficient porosity and liquid uptake so that cell culture media containing nutrients could penetrate into the matrix. At the microscopic level, cellulose fibers within the gelatin matrix also appear to provide sufficient pattern structure to allow for some alignment of cells. In fact, the fibers were chosen in part due to their intrinsic width, sufficient to allow for single cell alignment, such as is shown in. Cell alignment is beneficial for neural tissue engineering where cellular orientation is important. Additionally, cell alignment may influence the cell morphology, cytoskeleton organization and differentiation, which has been suggested many times in this work.
4.3 Non-Adhesive Cell Culture Surface Fabrication

4.3.1 Non Adhesive Surfaces in Conjunction with Patterned Adhesive Areas for Cell Culture

4.3.1.1 BSA Blocking and Polymer Non-Adhesive Coating

The NeN system offers quick, precise patterning that is simple to reproduce, and is flexible to the needs of the project. The surfaces patterned on are readily available cover slips, or other suitable surfaces for tissue culture. With glass, the method of linking the biological liquids to the glass surface was silanization of the cover slips. While our group achieved levels of success, our patterns had been limited in size, and the cell densities required to seed small patterns and maintain fidelity in binding were very low. Hence, a blocking agent was sought that would perform at a level higher than the bovine serum albumin (BSA) in use. The patterns look like that in Figure 39.

Figure 39. FITC conjugated Poly-L-lysine patterned on APTES coated glass cover slip.

Once the pattern is applied to the cover slip, then an agent to block non-specific binding is applied over the entire surface. As the results were good with BSA for small areas, we wanted to increase pattern size and complexity, which would require higher density and larger volumes of cells for seeding. Experiments showed we would need
better blocking of the non-binding area. Patterned cells seeded at lower densities, such as referenced earlier, can be seen follow the patterns during growth for the first two to four days of growth, after which, the non specific blocking seems to deteriorate. Successful patterning with PLL is shown at 200x magnification in Figure 40, at two days on the pattern, the astrocytes shown are expanding away from the pattern. These results led to questioning the suitability of BSA blocking for more complex designs that would require more time for the cells to grow, or higher seeding densities, as previously mentioned.

![Figure 40. Astrocytes on PLL pattern. Left panel is fluorescent image while right panel is phase contrast through the filter set for fluorescence, giving it green tinge.](image)

The search for a blocking agent led to polymer chemistry, and an inexpensive block copolymer was brought in to experiment with. The initial cytotoxicity experiments involved simply putting the dissolved polymer on small spots on polystyrene tissue culture ware. The spots were removed and rinsed with water. When cells were added, it was realized immediately that there was potential in this treatment. Figure 41 shows the first results in an interesting image that is a mosaic of twenty separate images of CRL 2303 cells seeded in a dish with the circular outline treated with only a few microliters of
the polymer solution. These images were captured at 100x magnification and stitched together with Photoshop® to create on large image.

Figure 41. Large mosaic image showing high level of cell exclusion from treated area, even at 2 DIV despite high seeding density of CRL 2303 brain cancer cells

This was the point where research on the patterning aspect shifted to more of the capabilities of the polymer.

4.3.2 Polymer Treatment on Large Scale for 3D Tissue Cultures

4.3.2.1 Initial Results of Large Scale Treatment

As discussed earlier, most tissue culture polystyrene is treated to make the surface more amenable to cell culture. This treatment allowed the glioma cells to adhere after only a few days. Astrocytes seeded on the polymer were not as quick to adhere, but would nonetheless adhere, if left long enough. Untreated tissue culture polystyrene was used with markedly improved results. Astrocytes grown in the untreated dishes adhered, but assumed a spindly, highly polarized shape instead of their normal morphology. CRL-2303 cells were slightly thinner to the eye, as well. The most noticeable difference was
that the coating now lasted longer, and there was little encroachment of the cells into the treated area. Astrocytes are shown in Figure 42 at 1DIV, and in the control group with no treatment are spread all over the plate, while the treated circle showed exclusion.

![Figure 42. Astrocytes on treated circle, left. The right panel is untreated control.](image)

The glioma cells showed similar responses, with heavy seeding, showing little to no encroachment on the treated areas, as seen in Figure 43 at 1 DIV. Heavy seeding of glioma cells would usually lead to confluent monolayers of cells.

![Figure 43. Glioma cells treated circle and un-treated circle side by side.](image)

The coating remains cytophobic for many days, with minimal invasion of even the aggressive CRL-2303 line. The results were promising and suggested additional uses for the coating. Once it was established that the inhibition of cell adhesion persisted for
days, based on the seeding density, as Figure 44 shows after 2DIV, the choice was made to pursue larger scale experiments with the coating.

![Image](image001.png)

Figure 44. CRL 2303 at 2DIV with no encroachment on treated surface.

The next discovery was that if the entire surface was coated, then protein adsorption and cell adhesion dwindled to almost nil, as brain cancer cultures were maintained for over a month in non-tissue-culture-treated, polymer coated dishes. Further experiments were done that led to the filing of a report of invention to the university. Those experiments will be discussed in the next section.

4.3.2.2 Neurospheres, Cytophobic Surfaces, and Tumorgenesis

This brings the discussion to cytophobic coatings. With the primary cells derived from neonatal rats bread at the Louisiana Tech University Biomedical Engineering animal handling facility, this tissue source was used to produce large quantities of
neurospheres via the cytophobic coating. As shown in Figure 45, the spheres are large and contain a large number of neural stem cells.

![Figure 45. Confocal slice of hollow neurosphere stained with calcein A.M.](image)

The application of the polymer coating in respect to the glioma cell line became interesting, as well. There are reports of gliomas forming neurosphere like structures, which normally adherent cell lines are not supposed to do. Once grown in vessels treated with the polymer coating for non-adherence, the CRL-2303 cells began to reproduce rapidly, in a non-adherent spherical culture. This type of 3D culture is not unlike the natural progression of tumors in vivo. With the cells free to grow in all dimensions, the colonies will continue growing until they become diffusion limited, and necrotic areas can be seen within the sphere. This progression is similar to tumors in the body that do not possess the factors required for angiogenesis. The non-angiogenic tumors have what is called a low "proliferating fraction." The progression of these CRL-2303 cells is startling, and can be seen in a 7 day montage in Figure 46. Their ability to grow
Figure 46. CRL 2303 tumor progression.

to over three times the original area in 7 days shows tremendous growth. The growth
calculated by image analysis software is in two dimensions, with the normalized growth
at over three hundred percent, as represented in Figure 47.

Figure 47. Graphical representation of tumor growth (red)
vs. astrocyte growth in non-adherent environment.
This method of cancer cell growth can shed new light on cancer biology, as this may be one of the first, if not the very first system to accurately model tumor progression in vitro, in three dimensions. Computational biologists have long been rewriting code to model tumor growth, and robust algorithms are available, but none are based on laboratory results under fixed conditions, there are variations due to age, time of onset, etc. that increase the variability of the data. This method has the capacity to be set for high throughput, with high throughput image analysis as well.

Non-adherent tissue culture vessels are commercially available, as the interest in stem cell technology is high. The need of such vessels arises in cancer research and stem cell research. Cancer is known for its ability to continue to grow, and reproduce inexorably, and this is a similar trait of stem cells, although stem cells only do so once isolated from their native tissue. Otherwise, stem cells are quiescent, and await the call of injury or disease before they set off on their reproduction and differentiation path. Perhaps there are answers for each field in the other: cancer may hold the key to igniting the natural healing power we already possess, and stem cells can show the way to turn off the unruly cancer cells that will not cease growth.

4.3.2.3 Significance of Spheroid Modeling and Neurosphere Cultures

Neural stem cells were only just identified in the nineteen nineties, making them a relatively new realm of research. Neurospheres are also a relatively new area of research, as they were only reported approximately ten years ago. Until the identification of a neural stem cell, the prevailing belief was that after a certain point, neurons do not regenerate, and no new neurons are produced. Since no one ever saw a new neuron, they did not exist. Since the discovery, and subsequent verification of the neural stem cells,
there has been a great deal of interest in these types of cells. The regenerative medicine field of course looks to stem cells to heal injuries and disease with various administrations of stem cell technologies.

To demonstrate what stem cells may be capable of doing, a definition of a stem cell is in order. The basic definition of the embryonic stem cell is an undifferentiated cell capable of mitotic division through many cycles, to produce a clonal, or identical, population of cells. This capacity is what separates stem cells from differentiated adult cells. Due to some function of differentiation, the repeated replication of the cell leads it farther and farther away from its original genetic signature. As mentioned, neural stem cells are progenitor cells, as well, but they differentiate, only into neural cell types, such as astrocytes, neurons, or microglia. Embryonic stem cells are labeled as “pluripotent,” as they may differentiate into any type of tissue, depending upon the stimulus. With limited differentiation the neural stem cells are only “multipotent.”

With this ability to differentiate into any type of neural tissue, the neural stem cell is targeted as a “magic bullet” to fix all the mental ailments. Stem cell treatments have been proposed to treat depression, seizures, Parkinson’s disease, and many others. This clinical application requires robust protocols for cell handling and use. A reliable way to produce these cells is needed. Currently, there have been reports of teams around the country that have been able to “reprogram” certain types of cell into a stem-cell like state, where it can become more than one cell type. Reprogrammed, multipotent or pluripotent, these are interesting cells from a biological standpoint. They are just as interesting in the biomedical perspective, and generating stem cells could be beneficial to cell patterning experiments. This is where stem cells and neurospheres merge. Sphere forming is a phenomenon that only happens with neural tissue with a large amount of undifferentiated
cells. As the animal matures, the production of neurospheres is less implying a level of differentiation from which there is no return to stem-cell-like states.

With this portion of the research recently completed, a grant was won allowing for LONI computing time, and a computational biologist to further the work started here. The tumor spheroids and the novel polymeric coating that allows their growth will be evaluated in much greater detail.
CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

Throughout the various experiments of the three distinct, but related projects examined here, the continuous thread has always been a question. “How can this apply to an experimental setup and exhibit control of another facet of the cells in question?” Apoptotic cues affect cell shape and growth rates. These cues are not easily applied to individual cells or even whole cultures, as some of the other aspects of controlling cell growth and morphology.

Glutamate was shown to delay the caspase-3 response to staurosporine stimulus, and this effect can be used to modulate apoptosis. Future exploration seems warranted.

The scaffold material was biocompatible, and showed excellent long term cellular activity, as indicated the MTT data. This behavior suggests the scaffolding should be evaluated further.

The cytophobic cell surface coating can be used as part of a multifaceted bionanocomposite approach to cellular architecture. Applications for this coating in medicine, and biology can be pursued based on evidence discussed here. There is also the aspect of the stem cell biology that this coating can induce, simply by growing cells in non-adherent culture conditions, which would lend itself well to ensuing research. As
Figure 48 shows, there are many factors within the bounds of this dissertation that could still be explored.

![Diagram](image)

**Figure 48. Applications of results discussed throughout this dissertation to future avenues of enquiry.**

All of these aspects have brought a fuller understanding of the complexities that govern life at the cellular level, while many questions remain to be explored.

### 5.2 Future Work

These projects all hold great promise for future work. There is room to continue working with Dr. DeCoster on many ideas for multiplexing all of the levels of control exhibited here. There are LbL approaches that can add nuances to the fiber/gel structure, along with localized hydrophobic coatings, and adsorbed protein growth factors that could be used to stimulate growth without being consumed and degraded, due to the chemistry of the situation. There are soluble cytokines that would be interesting to pattern using the NanoEnabler, a system that could be used to a much fuller extent.
There exists a need for development of a beginner’s level microscopy class, as the Biomedical Engineering students here at Louisiana Tech University are by and large unfamiliar with microscopy, and the resources here are phenomenal. By raising the comfort level of the students with the equipment, and by encouraging them to spend some quality time with the machines and the image processing software, the results, and quality of work would increase. Just in helping with some of the other students, the author has seen great improvements in the work of students who sought help. There was also evidence of advanced theoretical planning of the work, when they begin to understand how to use the microscopes.
APPENDIX A

MATERIALS AND METHODS
**A.1 Locke’s Solution**

Reagents for 250 ml solution

- 2250 mg of sodium chloride (NaCl) (154 mM)
- 104.4 mg of potassium chloride (KCl) (5.6 mM)
- 75.6 mg of sodium bicarbonate (NaHCO₃) (3.6 mM)
- 84.5 mg of calcium chloride (CaCl₂·2H₂O) (2.3 mM)
- 252.3 mg of glucose (5.6 mM)
- 1.25 ml of 1M stock 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) (5 mM)
- Water (Purified) 248.75 ml

**Preparation of Locke’s’ solution**

Add the components together in the following manner:

1. Dissolve the components in 100 ml of purified water and add to the vacuum filtration unit.
2. Add 100 ml of purified water to the vacuum filtration unit.
3. Add water with dissolved components.
4. Add 1.25 ml of 1M stock Hepes.
5. Add remaining amount of purified water.
6. Place cap on unit. Carefully turn on the vacuum.
7. Allow all the liquid to pass through the filter. Turn off the vacuum before bubbles form.
8. 
9. Twist the top of the vacuum unit off carefully. Screw the sterile cap onto the container of the media.

10. Label as Locke’s solution with date and lab name. Store in refrigerator at 4 °C.

A.2 Thawing and Culturing of Cells

Thawing cells

1. Remove vial of cells from liquid nitrogen and place in water bath preheated to 37 °C.

2. Thaw the vial containing the cells (approximately 5 minutes).

3. Remove the vial, and wipe the vial with ethanol and kim wipe under laminar hood.

4. Gently transfer the cell suspension into 15 ml centrifuge tube and add 6 ml of prewarmed rat brain endothelial cell growth medium drop wise onto the cells.

5. Mix the cell suspension by aspirating using a pipette and transfer the cell suspension to appropriate flask coated with attachment factor solution.

6. Label the flask with cell type, date and passage number.

7. Incubate cells for 24 hours at 37 °C in 5% CO₂ incubator.

8. Remove media from cells to remove traces of dimethylsulfoxide (DMSO) and add fresh, pre-warmed cell culture media.

Feeding cells

1. Remove 3 ml of media from 25 cm² flask into a waste beaker.

2. Add 3 ml of prewarmed cell culture media to the cells.

Label the flask with the date of feeding.
A.3 Isolation of primary rat brain cortical cells

1. Put a pair of gloves on.

2. Place 8-10 ml basal media in centered dish with transfer pipette.

3. Place 10 ml basal media in 50 ml centrifuge tube in the holder and recap.

4. Remove one pup from the box by pinching the skin above the spine near the lower back and set on a paper towel.

5. Spray the rat (pup) with alcohol and pick the pup up once more by the pinching method.

6. Quickly set cervical scissors behind the ear being cautious of the paws. Once the pup straightens its neck, make one very quick and decisive cut. Dip scissors in alcohol container without utensils and place scissors on a clean paper towel.

7. Blot head at point of disarticulation on paper towel and wash the entire surface with alcohol. Place body in the discard dish.

8. Pinch the skin underneath the head by its neck. Place micro-scissors underneath the skin at the top middle of the head at the disarticulation point. Cut the skin by pulling it up and away from the surface. Dip the scissors in alcohol to clean.

9. This should expose the skull (transparent appearance). While using the micro-scissors, cut the skull with the same motion as the skin. Dip scissors in alcohol to clean and place on diaper with cervical scissors.

10. The skull should separate. With the large forceps, curl back both sides of the skull to expose the brain. Dip forceps in alcohol to clean.

11. Locate olfactory bulbs and place the small spatula between the bulbs and the brain. Scoop the brain out and into the basal media. Dip the spatula in alcohol to clean.
12. Take the spatula and remove the cerebellum at the demarcation line and place the cerebellum in the discard dish. Dip the spatula in alcohol to clean and place on diaper.

13. Pierce the brain with the large forceps (to keep in place) and begin removing meninges and blood vessels with the small tip forceps. Discard waste to discard dish. Remember to turn brain to remove meninges and blood vessels from both sides.

14. Once the blood vessels and meninges are removed, uncap 50 ml tube containing basal media and place all brain tissue into the media. Recap.

15. Remove brain tissue from the 50 ml pipette by aspirating with 10 ml pipette and a pipette aid.

16. Blow out tissue in the 15 ml tube.

17. Add 8 ml complete media to the brain tissue and titrate with a 5 ml pipette. Aspirate until the cells are suspended in the media.

18. To let the brain tissue settle, place the 15 ml tube in large beaker of ice for 5 minutes.

19. The supernatant will contain the neuronal cells. Remove the supernatant, place it in a 15 ml tube, and place it on ice.

20. Repeat step 19 twice.

21. Centrifuge the neuronal cells in the three 15 ml tubes and follow the rat brain cell culturing protocol as usual to count and plate cells.

**A.4 Astrocyte New Media**

For 250 ml total media, use the following amounts:
- 12.5 ml Horse Serum (5.0 %)
- 12.5 ml Fetal Bovine Serum (5.0 %)
- 1.25 ml Penicillin/Streptomycin (0.5 %)
- 223.75 ml Ham’s F-12K media with L-Glutamine

Add the components together in the following manner:

1. Add 100 ml of Ham’s F-12K media to sterile vacuum filtration unit.
2. Add Horse Serum, Fetal Bovine Serum, and P/S to vacuum filtration unit.
3. Add 123.75 ml of Ham’s F-12K media to unit.
4. Place cap on unit. Carefully turn on vacuum.
5. Allow all the liquid to pass through the filter. Turn off vacuum before bubbles form.
6. Twist top of vacuum unit off carefully. Screw sterile cap onto container of media.
7. Label media as Astrocyte New Media with date and lab name. Store in refrigerator.

**A.5 CRL-2020 Cell Protocols**

A.5.1 Thawing Cells:
1. Remove vial of cells from liquid nitrogen and immediately place in water bath preheated to 37°C.
2. Thaw quickly (approx. 3 minutes).
3. Remove vial, wipe vial with ethanol and kimwipe under hood.
4. Transfer the contents of the vial to a 75 cm² flask containing pre-warmed CRL-2020 medium. Gently mix contents of vial without creating bubbles and check for pellet at bottom. Wash vial with warmed media. It is not necessary to remove the cryoprotective agent from the cells.

5. Label flask with cell name, date, and passage number.

6. Incubate culture at 37°C in 5% CO₂ incubator.

A.5.2 Feeding Cells

1. Remove 10 ml media from 75 cm² flask (5 ml from 25 cm² flask) into a waste beaker.

2. Add 10 ml pre-warmed CRL-2020 media (5 ml for 25 cm² flask) to the cells.

3. Label flask with date of feeding.

A.5.3 Splitting Cells or Replating Cells

1. Remove media from cells into waste beaker.

2. Wash flask once with 10 ml 1x PBS (1 ml 10x PBS + 9 ml sterile water). Pipet PBS gently into flask. Lay flask down once. Lift flask and remove PBS.

3. Add 6 ml thawed Trypsin/EDTA to flask. Cap the flask and gently shake to help cells detach. This may take about 5 minutes. Check flask under microscope to ensure cells are detaching.

4. Remove Trypsin/EDTA and cells from flask into 15 ml centrifuge tube. Wash flask once with ~6ml cold CRL-2020 media. Remove CRL-2020 media from
flask and add to centrifuge tube to deactivate Trypsin/EDTA. **Important—Mix tube by inversion (don’t vortex) before Centrifuging.**

5. Centrifuge cells in tube using a counterbalance in pre-cooled centrifuge (10°C) at 150x rcf for 7 minutes.

6. After spinning, remove supernatant liquid into waste beaker.

7. Resuspend pellet in desired amount of cold CRL-2020 media. (3 to 4 mLs depending on size of pellet) Resuspend pellet from bottom of tube, then vortex until cells are evenly distributed throughout media.

8. Optional: before plating count cells if needed, see cell counting protocol.

9. Plate cells onto new flasks or dishes, adding the appropriate amounts of cold CRL-2020 media.

10. Label flasks and dishes with cell name, date, and passage number (passage number increases when splitting cells).

**A.5.4 Freezing Cells**

1. Remove media from cells into waste beaker.

2. Wash flask once with 10 ml 1x PBS (1 ml 10x PBS + 9 ml sterile water). Pipet PBS gently into flask. Lay flask down once. Lift flask and remove PBS.

3. Add 6 ml thawed Trypsin/EDTA to flask. Cap the flask and gently shake to help cells detach. This may take about 5 minutes. Check flask under microscope to ensure cells are detaching.
4. Remove Trypsin/EDTA and cells from flask into **pre-cooled** 15 ml centrifuge tube. Wash flask once with ~6ml cold CRL-2020 media. Remove CRL-2020 media from flask and add to centrifuge tube to deactivate Trypsin/EDTA.

5. Centrifuge cells in tube using a counterbalance in pre-cooled centrifuge (10°C) at 150x rcf for 7 minutes.

6. After spinning, remove supernatant liquid into waste beaker.

7. Resuspend pellet in desired amount of cold CRL-2020 media (1.3 ml if freezing down entire flask to 1 cryovial). Remove pellet from bottom of tube, then vortex until cells are evenly distributed throughout media.

8. Add 5% DMSO (70 µl for 1 cryovial to make 1.4 ml total volume) drop wise to cells. Mix by inverting centrifuge tube.

9. Transfer cell mixture to **pre-cooled** cryovial.

10. Label cryovial with cell name, date, and passage number (passage number is same as on flask).

11. Put cells on ice in refrigerator for 30 minutes.

12. Put cells in freezer for 30 minutes.


14. Transfer cells into liquid nitrogen. Record cane number.

A.5.5 CRL-2020 Complete Media

   a. For 250 ml total media, use the following amounts:
203.75 ml RPMI-1640 media

2. 25.0 ml Fetal Bovine Serum (10%) (located in -80° freezer)

3. 1.25 ml Penicillin/Streptomycin (200 fold dilution)

4. 2.5 mg Adenine

5. 0.25 mg ATP (located in -80° freezer)

6. 8.75 mg Cystine (use 1N NaOH, drop wise to aid dissolution. 40μL per add, 120μL max)

7. 891 mg HEPES

8. 3.75 mg Hypoxanthine

9. 7.5 mg Proline

10. 0.25 mg Thymidine

Add the components together in the following manner:

1. Put 20 ml RPMI-1640 media in 2 15 ml centrifuge tubes (10 ml in each tube).

2. Add measured components to these tubes and mix until dissolved.

3. Add 100 ml of RPMI-1640 media to sterile vacuum filtration unit.

4. Add Fetal Bovine Serum, P/S, and 20 ml RPMI-1640 media containing components to vacuum filtration unit.

5. Add 103.75 ml of RPMI-1640 media to unit.
6. Place cap on unit. Carefully turn on vacuum.

7. Allow all the liquid to pass through the filter. Turn off vacuum before bubbles form.

8. Twist top of vacuum unit off carefully. Screw sterile cap onto container of media.

9. Label media as CRL-2020 media with date and lab name. Store in refrigerator.

A.5.6 CRL–2303 Media

A.5.6.1 Media Components

221.3mL Delbecco’s Modified Eagle’s Medium (DMEM)

1.25mL Penn/Strep (PS)

25mL Fetal Bovine Serum (FBS)

2.5mL Amino Acid Solution

A.5.6.2 Making the Media

1. Add 110.65mL DMEM to filtration unit.

2. Add 1.25mL PS to filtration unit.

3. Add 25mL FBS to filtration unit.

4. Add 2.5mL Amino Acid Solution to filtration unit.

5. Add 110.65mL DMEM to filtration unit.

Connect sterile filtration tubing to filtration unit and vacuum nozzle.

-make sure to hold filtration unit while performing this task, so the unit does not turn over

While holding unit slowly turn on vacuum to a medium drip.

Remove vacuum seal before media has been completely filtered.
A.6 DAPI Protocol for Living Cells

Notes:

- Stock solution labeled “D,” located in -80°C freezer, 14.27 mM in DI water
- Locke’s located in 4°C fridge in IFM
- Will be performing 2 dilutions:
  - First is a 1 to 100 dilution with your stock. Take 3 μl of your stock and dilute with 297 μl of Locke’s.
  - You will then use the dilution performed above and do another dilution, a 1 to 10 dilution. Most likely you will be adding this to a dish with 3 ml of media. Therefore a 1 to 10 dilution would be 300 μl from your first dilution in the 3 ml dish.
  - Once DAPI has been added place dish in 37°C incubator for 10 minutes (depending on the health of the cells you may need a longer time to load due to the fact that healthy cells take longer to load DAPI than damaged or dead cells).

A.7 CRL 2570 (Jurkat T-cells) Media

A.7.1 Requirements

1) RPMI-1640
2) Fetal Bovine Serum: 25 ml.
3) Penicillin/Streptomycin: 1.25 ml.

A.7.2 Procedure

1) Adjust the filtration unit and add about 112.5 ml. of RPMI-1640 to it.
2) Then add about 25ml. of Fetal Bovine Serum (FBS) on it. (Thaw FBS in hot water bath for 10-15 min.)
3) Add 1.25 ml of Penicillin/Streptomycin solution on it.
4) Then again add 112.5ml. RPMI-1640 on it and start the air suction to filter media.

5) After completing cap the media bottle and mark it as CRL 2570 media and store at 2-8 °C.

A.7.3 Thawing

1. Pre-warm flask with media for 15 minutes in CO₂ incubator
2. Thaw cryo-vial, always clean the cryo vial with kim wipes and add cells to flask
3. No need to wash cryo-preservation medium

Subculturing:

1. Remove sufficient cells from plate or flask (example 8 mls from T75 flask)
2. Make sure to replace an equal amount of media (warm) to the plate or flask you took from.
3. Centrifuge at 150 RCF (refrigerated) for 5 minutes to form pellet
4. Resuspend pellet and count if needed.
5. Plate resuspended cell on plate or flask and increase passage # by 1.

**Note:** These cells do not attach to the surface of the dish or the flask.

**NOTE:** Centrifugation after DMSO cryopreservation damages cells, therefore, passaging after thawing should be done without centrifugation.

Later passaging may be accomplished with centrifugation.

**A.8 Lonza Astrocyte Protocols**

A.8.1 Thawing Cells

6. Remove vial of cells from liquid nitrogen and immediately place in water bath preheated to 37°C.
7. Thaw quickly (approx. 3 minutes).

8. Remove vial, wipe vial with ethanol and kimwipe under hood.

9. Gently transfer 1 ml cells into 15 ml centrifuge tube and immediately add 6ml 
   **pre-warmed AGM** (Astrocyte Growth Media) dropwise onto cells. Takes approx. 2 minutes.

10. Mix cell suspension by inverting the tube carefully twice. DO NOT VORTEX CELLS.

11. Transfer cell suspension to appropriate flask or dish with **pre-warmed AGM**.

12. Label flask with cell type, date, and passage number (passage number increases when thawing cells).

13. Incubate cells for 4 hours at 37°C in 5% CO₂ incubator.

14. Remove medium from cells; leave small volume so cells don’t dry out. Add fresh, **pre-warmed AGM**.

15. Every 4-5 days, change 50% of the media to fresh, pre-warmed AGM.

   **A.8.2 Feeding Cells:**

1. Remove 10 ml media from 75 cm² flask (5 ml from 25 cm² flask) into a waste beaker.

2. Add **10 ml pre-warmed AGM** (5 ml for 25 cm² flask) to the cells.

3. Label flask with date of feeding.

   **A.8.3 Splitting Cells:**

4. Remove media from cells into waste beaker.

5. Wash flask once with 10 ml 1x PBS (1 ml 10x PBS + 9 ml sterile water). Pipet PBS gently into flask. Lay flask down once. Lift flask and remove PBS.
6. Add 6 ml thawed Trypsin/EDTA to flask. Cap the flask and gently shake to help cells detach. This may take about 5 minutes. Check flask under microscope to ensure cells are detaching.

7. Remove Trypsin/EDTA and cells from flask into 15 ml centrifuge tube. Wash flask once with ~6 ml cold AGM. Remove AGM from flask and add to centrifuge tube to deactivate Trypsin/EDTA.

8. Centrifuge cells in tube using a counterbalance in pre-cooled centrifuge (10°C) at 150x rcf for 7 minutes.

9. After spinning, remove supernatant liquid into waste beaker.

10. Resuspend pellet in desired amount of cold AGM. Remove pellet from bottom of tube, then vortex until cells are evenly distributed throughout media.

11. Plate cells onto new flasks or dishes, adding the appropriate amounts of cold AGM.

12. Label flasks and dishes with cell name, date, and passage number (passage number increases when splitting cells).

A.8.4 Freezing Cells:

1. Remove media from cells into waste beaker.

2. Wash flask once with 10 ml 1x PBS (1 ml 10x PBS + 9 ml sterile water). Pipet PBS gently into flask. Lay flask down once. Lift flask and remove PBS.
3. Add 6 ml thawed Trypsin/EDTA to flask. Cap the flask and gently shake to help cells detach. This may take about 5 minutes. Check flask under microscope to ensure cells are detaching.

4. Remove Trypsin/EDTA and cells from flask into pre-cooled 15 ml centrifuge tube. Wash flask once with ~6ml cold AGM. Remove AGM from flask and add to centrifuge tube to deactivate Trypsin/EDTA.

5. Centrifuge cells in tube using a counterbalance in pre-cooled centrifuge (10°C) at 150x rcf for 7 minutes.

6. After spinning, remove supernatant liquid into waste beaker.

7. Resuspend pellet in desired amount of cold AGM (1.3 ml if freezing down entire flask to 1 cryovial). Remove pellet from bottom of tube, then vortex until cells are evenly distributed throughout media.

8. Add 5% DMSO (70 µl for 1 cryovial to make 1.4 ml total volume) drop wise to cells. Mix by inverting centrifuge tube.

9. Transfer cell mixture to pre-cooled cryovial.

10. Label cryovial with cell name, date, and passage number (passage number is same as on flask).

11. Put cells on ice in refrigerator for 30 minutes.

12. Put cells in freezer for 30 minutes.

14. Transfer cells into liquid nitrogen. Record cane number.

**A.8.5 Lonza Astrocyte Growth Media**

**ALL components must be sterile. Do NOT sterile filter using vacuum filtration.**

For 500 ml total media, use the following amounts:

- 15 ml Fetal Bovine Serum
- 0.5 ml Ascorbic Acid
- 0.5 ml G/A-1000
- 1.25 ml Insulin
- 5.0 ml L-Glutamine
- 477.75 ml Astrocyte Basal Medium (ABM)

Add the components together in the following manner:

1. Add the components to the bottle of ABM.
3. Label bottle as AGM with date and lab name. Store in refrigerator.

**A.9 MTT Assay (Jurkat-CRL 2570)**

Procedure for a 24 well plate

It takes 400µL of a solution to completely cover the bottom of a well in a 24 well plate. This being considered, 9.6mL of MTT would be needed to treat an entire plate. 11mL will be prepared to allow a safety buffer volume.
1. Dilute MTT to 5.0mg/mL in Locke’s solution then follow a 4x dilution for a final concentration of 1.25mg/mL for this 13.75mg of MTT powder needs to be added to 11mL of Locke’s solution.

2. Media is removed from cells. Do not wash.

3. Centrifuge (at 200 RCF for 7 minutes) the cells to form pellet, remove the supernatant, discard then add 400μL of MTT solution to each tube (Make sure the MTT solution is warm).

4. Let MTT incubate for at least one hour. In viable cells the yellow solution will yield blue crystals.

5. After 1 hour remove the MTT and cells together, put in Eppendorf tubes spin at 200 RCF for 9 minutes. At this point remove supernatant and discard.

6. Add 250μL of 91% Isopropanol to the pellet and mix.

7. Transfer 200μL of the Isopropanol with dissolved crystals into wells of a 96 well plate. Dispose of the remaining liquid.

8. Cells should be covered with 400μL of PBS to preserve them.

9. Measure the plate reader to a wavelength of 570.
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