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Substrate Stiffness and Adhesivity Influence Neuron Axonal Growth

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SUBSTRATE STIFFNESS AND ADHESIVITY INFLUENCE NEURON AXONAL GROWTH

by

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Abstract

The nervous system is a complex organ system that coordinates all of the actions of an animal. The transmission of signals between different body parts by this organ system allows the body to function as a whole and interact with the external environment. It is clear then that the nervous system is integral to the function and survival of those organisms in which it has developed; and yet much is lacking in our understanding of the system's key constituents: neuronal cells or, *neurons*. In satisfaction of the Honors Thesis, this research investigates how certain conditions of the extracellular environment affect neuron growth. Growth substrates were developed to present two such conditions – substrate stiffness and adhesive ligand coating density – to cultured neurons at quantities reflecting *in vivo* values that are hypothesized to result in optimal growth. Once working culture methods were established for the growth of neurons on these substrates, imaging and data collection techniques were developed to characterize the growth of these cells and produce a qualitative and quantitative understanding of how neuron growth is influenced by the environmental conditions of interest. While specialized growth substrates were successfully developed and methods of data collection were validated with some preliminary data confirming the basic hypothesis, further refinement of culturing techniques will be required to grow sufficient densities of neurons to provide a more complete picture of the interplay of influence between the substrate properties of stiffness and adhesivity.

Acknowledgements

This written thesis, along with the countless hours in the lab put into the research, is dedicated to **Dr. Alireza Sarvestani** for allowing me the opportunity to perform said research in his lab and for providing wise and thoughtful mentoring throughout my undergraduate career.

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Introduction

It is understood that the development and function of a cell intrinsically depend in part on the stiffness of the substrate on which it grows, as well as the nature of the adhesive interaction between molecular ligands on the substrate surface and the surface of the cell [1]. These conditions of the extracellular environment hereto referred to as *stiffness* (or rigidity) and *adhesivity* respectively are the main focus of this research project. In general, it is hypothesized that a cell grown in culture will function most optimally when conditions can be created *in vitro* that closely mirror the natural conditions inside the organism. This makes sense within the framework of the evolution of biological species: the conditions present in a particular tissue are as such because they provide the most optimal environment for the cells of the tissue to perform their required functions. This research project aims to validate this conclusion and evaluate a wide range of the extracellular conditions of substrate stiffness and adhesivity in order to identify the optimal *in vitro* environment for neuron growth – knowledge that has promising implications for future attempts to engineer functioning neural tissue for therapeutic applications.

In the case of substrate stiffness it has been found that neurons, as they are found in the relatively soft tissue of the nervous system, thrive on similarly soft materials *in vitro* [2,3]. In terms of chemical adhesivity, it has been shown that neuronal development in the brain is dependent upon gradients of chemo-attractant and chemo-repellent molecules bound within the extracellular environment [3]. More specifically, extracellular signals such as ligands bound to the substrate can stimulate the rapid outgrowth of an axon,

thereby directing the development of the polarity and functionality of the cell [4]. This phenomenon has been demonstrated with substrate-bound molecules common in the nervous system and known to be adhesive to neurons, foremost the extracellular proteins fibronectin and laminin. For instance, it has been shown that axon specification can be triggered when one of the developing processes of a neuron crosses onto a region of substrate coated with laminin [5]. It is apparent then that neuronal growth, specifically axonal development, is influenced by the mechanical and chemical properties of the substrate on which the cell grows.

The general goals aforementioned are broken down into the following specific experimental goals. The results presented in this thesis will represent one portion – achievable within the timeframe of the final year of the Honors College curriculum - of a larger research endeavor that will be carried on by subsequent undergraduate researchers in the Cell Mechanics and Tissue Engineering Laboratory at the University of Maine. The data collected in the course of this Honors Thesis is thus presented in reference to the full set of experimental goals and plans in order to provide a more meaningful framing.

General Goal

Determine the nature of influence on neuron growth by substrate stiffness and adhesivity. Investigate a wide range of substrate rigidities and ligand densities to identify an optimal set of conditions for neuronal growth and explore the interplay between the two effects.

Experimental Goal 1: Investigate Substrate Rigidity

Specific Aim1: Develop a substrate that is non-toxic and non-inhibitory of neuronal growth, which allows for the neurons to be exposed to a tunable range of substrate rigidities.

Specific Aim 2*:* Develop and execute observational methods by which neuron growth (especially axonal growth) can be both qualified and quantified, yielding meaningful data from which the nature of the durotaxic response of neurons to substrate stiffness can be determined.

Experimental Goal 2: Investigate Substrate Adhesivity

Specific Aim1: Develop a substrate that is non-toxic and non-inhibitory of neuronal growth, which allows for the neurons to be exposed to a tunable range of adhesive ligand density.

Specific Aim 2*:* Develop and execute observational methods by which neuron growth (especially axonal growth) can be both qualified and quantified, yielding meaningful data from which the nature of the haptotaxic response of neurons to substrate-bound molecules, specifically fibronectin and laminin, can be determined.

Secondary Objectives:

- Well-document every procedure and method developed to ensure future efforts are performed with the highest possible fidelity to the original work, expanding on this foundation to refine the techniques and broaden the scope of the project.

- Establish effective culturing methods for neurons to facilitate future research on this type of cell.
- Develop novel growth substrates with tunable gradients of stiffness and ligand coatings for cell mechanics studies with other types of cells.

Background

Organ Level: The Nervous System

The *nervous system* is an organ system found in most multi-cellular animals. The tissues of this system consist of a network of specialized cells - *neurons* - that transmit signals between different parts of the body via cell-cell interactions [6]. This signal transmission not only allows for coordination and regulation of different parts within the body, but also the functioning of the body as a whole in interacting with the external environment.

The nervous system of vertebrate animals is made up of two parts – the central and peripheral nervous system. The central nervous system consists of the brain – the center of the nervous system that controls the organs of the body – and the spinal cord – the main pathway between the brain and the rest of the body. The peripheral nervous system, consisting of sensory neurons and clusters of neurons (ganglia) serve to further connect the central nervous system and ultimately the brain to the organs and limbs of the body. In addition, the enteric nervous system serves to control the gastrointestinal system, and can function independently in doing so even when physically severed from the central nervous system [6].

Tissue Level: Neural Tissue

The next level of organization by which an organ system can be understood is that of *tissues* (in this case *nervous tissue*): a collection of cells that share a common origin and perform the same specific functions. Nervous tissue is thus composed of the two basic cell types found in the nervous system: neurons and neuroglia cells (glial cells, or simply *glia*). Neurons transmit the electrochemical impulses that allow for communication across nervous tissue to different parts of the body, while neuroglia cells assist in this transmission and provide nutrients necessary for neurons to grow. Many specific varieties of neurons exist within these tissues, which are classified by their function and structure (intrinsically interrelated) [6].

Cell Level: Neurons

Neurons are the specialized cells that in addition to neuroglia cells make up the nervous tissue that in turn makes up the nervous system. These cells are composed of a centralized cell body (or soma), which contains the genetic information of the cell within a nucleus as well as the necessary organelles for the translation of said genetic material and synthesis of proteins. From this central body extend many fibers by which the neuron can receive and send out electrochemical signals to other neurons. The majority of signal input to a neuron occurs via one kind of fiber – dendrites – highly branched cellular extensions that protrude from the soma in number and all directions. Another type of cellular extension – the axon – is a finer fiber that can extend hundreds or thousands of times the diameter of the soma (on the scale of hundreds of micrometers) by which signals can be carried from the soma to other cells [6].

While most neurons only have one axon, this extension can also be highly branched, allowing one neuron to send signals to many other neurons. These signals are propagated as electrochemical waves that travel along the axon to cell-cell junctions called synapses, where they trigger the release of neurotransmitter chemicals to pass the signal to the next cell. A cell receiving such a signal can be inhibited, excited into some form of action, or have some cellular function otherwise manipulated. As an example relating back to the function of the organ system as a whole: sensory neurons that are activated by a physical stimuli acting on them send signals along these cell-cell connections throughout the nervous tissue, ultimately relaying information about the state of that region of the body back to the brain in the central nervous system [6].

Neurons are commonly classified by the structure and orientation of their dendrites and axons. *Multipolar* neurons have three or more cellular extensions including at least one axon, extending in any direction. This is by far the most common type of neuron. *Bipolar* neurons have an elongated cell body with an axon extending from one end and a the major dendrite from the other, while *unipolar* neurons have cellular extensions in only one place, which splits into an axon and dendrites at a point that is close to the main body of the cell [6].

The Axon

As the main route by which a neuron sends out signals to other cells, the axon is of particular interest in understanding how these cells grow and function. Apart from differing from dendrites in function - axons usually sending signals while the latter usually receive signals - axons can also be distinguished from the other cellular

extrusions of the neuron by shape and length. Axons usually maintain a constant thickness while dendrites taper. The axon's terminus is also marked by growth cone whilst growing or by a synapse once a cell-cell interaction is formed. While the length of dendrites is usually limited to a radius of similar scale to the size of the cell body, axons can extend for hundreds or thousands of times longer than the diameter of the central body of the cell [6].

Culture Methods

In the lab, low-density cultures of neurons are far less complex than neural tissue and thus provide a promising opportunity to understand single-cell mechanics through phase-contrast microscopy and fluorescence imaging techniques. Primary cell cultures are used almost exclusively for this type of research as clonal, immortalized cell lines are found to be unable to form well-defined axons and synapses [7]. This study will use primary neuron cultures taken from the hippocampus of a rat brain. The hippocampus has become widely used as a source for primary neuron cultures because of its largely homogenous population of neuron cells, making ideal for isolation of such cells without contamination by other cell types [7].

Neuron Growth

The development of neurons in culture, with specific regard to differentiation (outgrowth) of the axon, can be characterized by four major stages of growth [8]. Once the cells are revived and introduced to a substrate they attach and a continuous lamella forms around the cell body (*Stage 1*). In *Stage 2* several cellular extensions 20-30μm in length (minor neurites) begin to emerge from the cell body. These will later differentiate into dendrites

and an axon. These minor extensions undergo a period of growth and retraction until the neuron enters *Stage 3*, where one neurite grows continuously for approximately 3 hours, becoming several times longer than all other extensions, and thus differentiating into the **axon**. *Stage 4* is marked by the continued extension of the axon to hundreds of micrometers long, and the growth and branching of dendrites showing their characteristic taper.

It is important to note that the transition between each stage does not occur synchronously with every neuron in the population. Under optimal growth conditions, over 50% of the neurons in a seeded population will reach Stage 3 24 hours after plating, with 80% following by 48 hours [8]. It is also important to note that frozen cultures that are revived are observed to develop slower than neurons taken directly from primary tissue sources [9]. Since the growth process - particularly the differentiation of the axon - is of primary interest to the research project, the ability to identify and observe neurons entering Stage 3 will be key.

Rigidity

Observing cells seeded on hydrogels with tunable stiffness has shown that this mechanical parameter influences cell behavior and growth [10]. Furthermore, Georges shows that neurons of the ganglia and spinal cord "exhibit a preference for growth on soft materials by increased neurite length and branching" [1] and in general neurons extend cellular process (dendrites and axons) better on soft gels (50 Pa) than on stiffer materials [11]. Summarily, it is understood that since neurons are found *in vivo* in the relatively

very soft tissue of the nervous system, they thrive on similarly soft materials in *in vitro* [12]. The relative 'softness' of nervous tissue has been quantified by a Young's elastic modulus range of 0.1-1kPa [13]. This project seeks to further validate this understanding by investigating the response of growing neurons to substrate stiffness values both close and far from the stiffness range associated with native nervous tissue.

Adhesivity

Selak and Liesi show that laminin is required for neural migration in developing nervous tissue [14,15]. The extracellular-protein fibronectin is also known to support survival and migration of neurons during their development [16]. While it has been shown that axon differentiation orients in the direction of increasing densities of such extracellular ligands [17], less is understood about how different ligand densities affect the rate of axon differentiation. This project will investigate the response of neurons during growth (with specific regard to axon differentiation) to different densities of adhesive ligands such as laminin and fibronectin.

Methods

Culturing Primary Hippocampal Neurons

Cryo-preserved primary rat hippocampal neurons are obtained from Invitrogen and are stored in liquid nitrogen. While primary cultures offer a better subject for axon growth studies, they also pose limitations on the experimental procedures to be applied. Chiefly, as non-immortalized cells this neuron culture cannot be passaged or frozen down into subcultures, negating the possibility of long-term cultures and the seeding of cells onto growth substrates at multiple time-points. Thus, the experimental procedures

implemented require reviving a new vial of primary hippocampal neurons for each experiment. These experiments must then be designed to yield the most complete set of data possible in regards to observing the axon growth rates of the neurons. Short-term culture does in turn have its own advantages, chiefly the reduced risk of contamination and ease of handling and maintenance.

Upon revival of a cell line, the neurons are seeded onto gels with the experimental conditions of a given trial. As directed for this cell line, they are kept in an incubator at 36-38°C and 5% CO_2, and supplied with appropriately supplemented Neurobasal Medium (Invitrogen), as directed by the instructions included by Invitrogen. Specifically, Neurobasal Media is supplemented with 2% B-27 supplement, 100µg/mL penicillin/streptomycin antibiotics, 200mM GlutaMAX (glutamine), and 25µM L-Glutamate. The neurons will be revived, seeded onto substrates, and maintained via the short-term culture protocols outlined by Meberg and Miller [9].

Development of Substrates

Before any neurons were revived, a specific set of substrates had to be developed in order to present the neurons with the extracellular conditions of interest in a way that would yield the most interesting data in the highest quantity, since they are a (very expensive) primary cell line. For both the stiffness and adhesivity experiments, 'bi-rigid' or 'bi-adhesive' substrates were created respectively, having two distinct regions of either stiffness or ligand density with the boundary between the two being distinct even on the scale of an individual neuron (50-100µm). This 'bi' format allows one substrate to yield

two sets of data in terms of stiffness or ligand density, and also allows for the observation of how developing neurons interact with a change in extracellular conditions that can be sensed by a single cell.

Bi-Rigid Polyacrylamide Gels

To investigate the influence of substrate stiffness on the growth of neurons, neurons will be grown and observed on a polyacrylamide hydrogel prepared on glass coverslips via chemical crosslinking [9]. The stiffness of this gel substrate can be tuned by altering the amount of cross-linking agent in the gel mixture [18]. Polyacrylamide gels are attractive substrates for this application because the mechanical properties are so tunable and post-synthesis washing yields an inert, non-toxic material that can be easily coated with extracellular proteins like lamanin and fibronectin. As aforementioned, the range of stiffness investigated, as quantified by the Young's Modulus will mirror the typical range of stiffness one would find in neural tissue samples - specifically 0.5 to 20 kPa. In order to observe the difference in neuron growth on substrates of different stiffness, as well as the ability of a cell to respond to such differences, gels will be prepared such that one half has a distinctly different stiffness than the other. This is accomplished by preparing two different gel mixtures with different amounts of cross-linker (thus different stiffness) and sandwiching adjacent aliquots of these solutions between a chloro-salinated glass slide and an amin-salinated glass coverslip during subsequent chemical synthesis of the gel [9]. The chemical treatments applied are such that the former piece of glass is extremely hydrophobic and will not adhere to the synthesized gel, which the later glass coverslip is adhesive to the gel, yielding a glass coverslip with a thin gel coating. A clear boundary

between the to regions is delineated by the inclusion of fluorescent beads in the stiffer of the gel mixtures, such that one half of the gel has a uniform distribution of embedded beads that can be imaged via fluorescent techniques, while the other half is pure hydrogel (Figure 1 below).

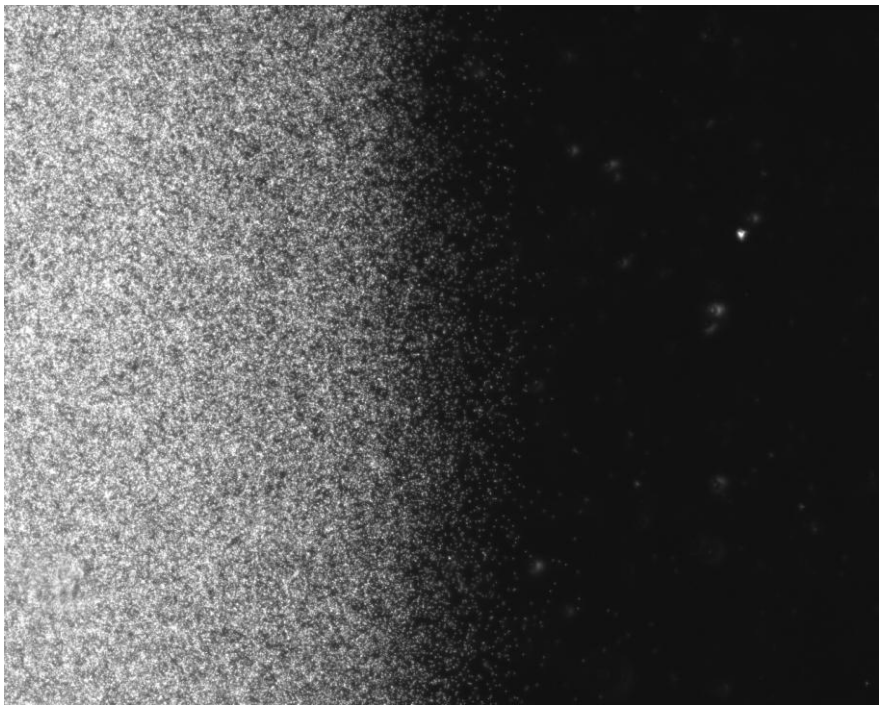


Fig. 1. Fluorescence image of bi-rigid gel boundary at 10X magnification, bead-impregnated 40kPa region on left and 0.5kPa region on right. Zone of bead diffusion at center is approximately 200μm wide.

Each gel in the stiffness experiments is coated with an identical concentration of neuron-adhesive molecule (laminin), this control value being chosen to most closely model concentrations found in the natural extracellular environment (50μg/mL). Directly after revival, neurons are seeded at a density of 3000-5000 viable cells/cm^2. This density has been suggested as optimal for individual cell visualization, as it prevents poor growth from insufficient density, and overcrowding leading to cell-cell interaction/interference [9]. Phase contrast video microscopy is then utilized to observe the growth and movement of single neurons over a time period appropriate to observe process

development and axon specification and growth. The observed growth will be characterized mainly by axonal growth rate, which will be measured and calculated with the aid of imaging software. Because each gel will contain two distinct regions of stiffness, observations can be made comparing axon growth under both conditions separately, and on the response of a developing neuron to an increase or decrease in stiffness (for those cells growing on the border between regions).

Bi-Adhesive Glass Coverslips

To investigate the influence of substrate adhesivity (bound-ligand density) on the growth of neurons, neurons will be grown and observed on glass coverslips coated with fibronectin or laminin. As with the 'bi-rigid' gels, 'bi-adhesive' coverslips are created with two distinct regions – either of different densities of bound ligand, or with one region presenting no specific ligand. The scope of the research completed in fulfillment of the Honors Thesis is limited to a coverslip that is has one half coated with fibronectin and the other half devoid of specific ligand interaction sites. This is accomplished by first entirely coating the coverslip with Poly-D-Lysine (PDL), a molecule that is shown to promote neuron adhesion in the absence of specific protein-protein interactions between the cell membrane and substrate [16]. One half of the coverslip is then physically excluded by firmly affixing a piece of PDMS to the glass. A solution of fibronectin with an *in vivo* –reflecting concentration can then be applied to the other half of the coverslip. Subsequent incubation at 37°C for 8 hours then promotes the binding of fibronectin to the glass. The boundary between the two regions can be visualized by using fluorescent rhodamine-tagged fibronectin, and shows the boundary is distinct and sharp, taking on the shape of the edge of the PDMS (Figure 2 below). This method can be similarly

employed to create a glass coverslip that has fibronectin or laminin coatings of different densities in two distinct regions of the same coverslip.

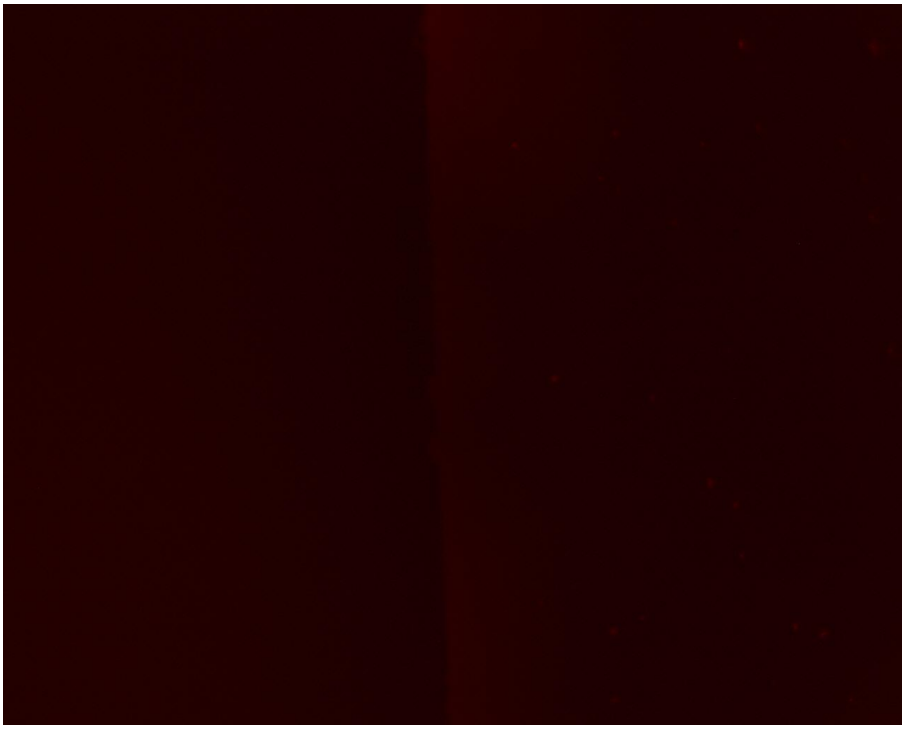


Fig. 2. Fluorescence image of bi-adhesive coverslip at 20X magnification. PDL-only region at left, PDL and rhodamine-tagged fibronectin region at right. Note the distinct boundary with the absence of any zone of diffusion.

Results

The following is a summary of the results of the first set of experiments performed during the final semester of the Honors curriculum. While the goals of the broader research endeavor aforementioned involve a wide range of substrate stiffness and ligand densities, this first attempt looks at the two extreme ends of either spectrum. Replicates of both a bi-rigid gel and bi-adhesive coverslip were created and seeded with a vial of revived neurons, and then alternately observed via microscopy during the course of the cell population's development. The bi-rigid gels were created with one half 0.5kPa, representing the native neural tissue-mimicking stiffness that is hypothesized to be

optimal, and the other half 40kPa (with beads included), to represent a substrate far stiffer than the neurons would prefer. It was hoped that such a large difference in stiffness, presented at such a distinct boundary, would elicit an easily observable response by neurons growing across this boundary. To observe the effect of a large difference in adhesivity, multiple glass coverslips were prepared with one half coated only with PDL, and the other coated with PDL and 10µg/mL of rhodamine-tagged fibronectin.

Morphological Study of Neuron Growth

Once the neurons were revived and seeded on the substrates at the appropriate density, the first task was carefully image neurons in each stage of growth in order to develop an observational understanding of what these cells look like as they grow in culture. This is important since later data collection techniques for measuring axon growth rates will depend on identifying Stage 2 neurons that are likely to have an axon begin to differentiate. The following series of images were collected 48 hours after seeding, when the population of neurons included cells in each stage of growth. It is important to note at this point the unfortunate fact that successful culturing of neurons was only accomplished with the bi-rigid gels, and not the bi-adhesive coverslips. Thus, all subsequent data presented is in reference to the study of substrate stiffness.

Growth Stage 1: Attachment to Substrate

Fig. 3. Single neuron in Stage 1 of growth on 40kPa region of bi-rigid gel (imaged at 20X magnification). Diffuse light around cell body indicates extension of lamella prior to extension of minor processes.

As shown by Craig, once the neuron settles on the substrate upon which it is seeded, a thin lamella extends from the cell body as attachment to the substrate develops. All neurons took on this appearance directly after seeding, some remaining like this for the entire duration of the observation.

Growth Stage 2: Development of Minor Processes

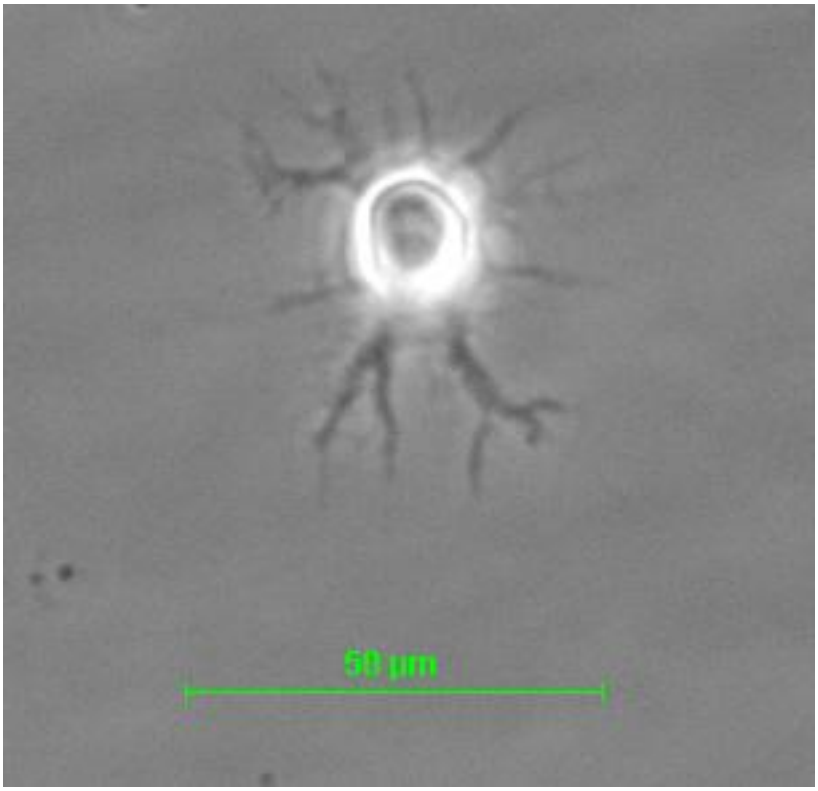


Fig. 4. Stage 2 neuron on 0.5kPa region at 20X magnification.

The image above image shows the development of minor cellular extensions in many directions characteristic of Stage 2 growth. In later attempts to capture time-lapse imagery of axon differentiation, it is important to image a group of neurons containing as many such neurons as possible, as they are the most likely to enter Stage 3 growth (especially those with one extension distinctly though not greatly longer than the rest).

Growth Stage 3: Differentiation of the Axon

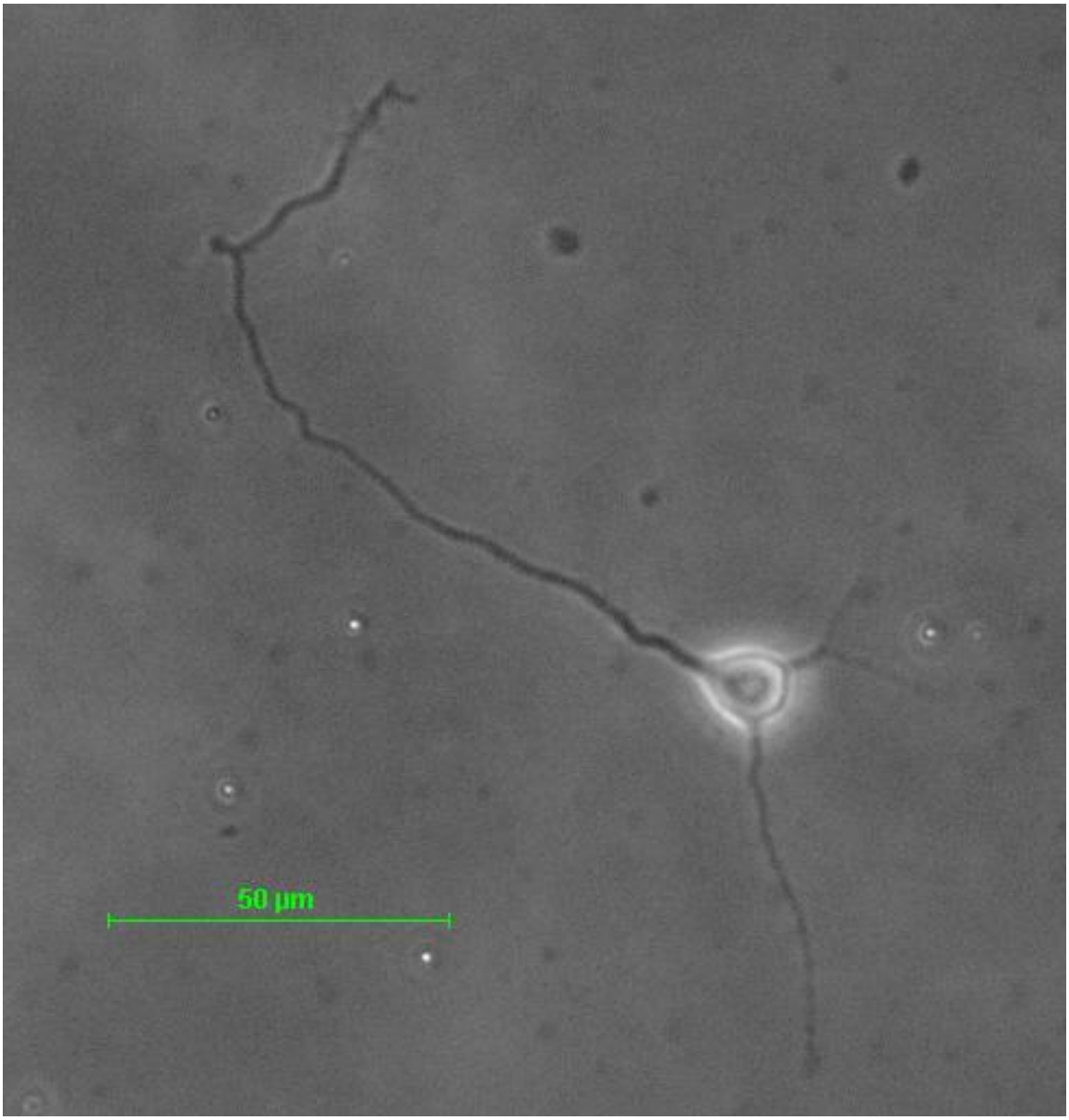


Fig. 5. Stage 3 neuron on 0.5kPa region at 20X magnification.

Figure 5 shows a neuron in Stage 3 of growth, where one of the minor processes has begun to develop into the axon. The axon can be distinguished both by its greater length and by its relatively constant width, in comparison to the more tapered extensions that are dendrites. The image below offers a comparison of Stages 2 and 3.

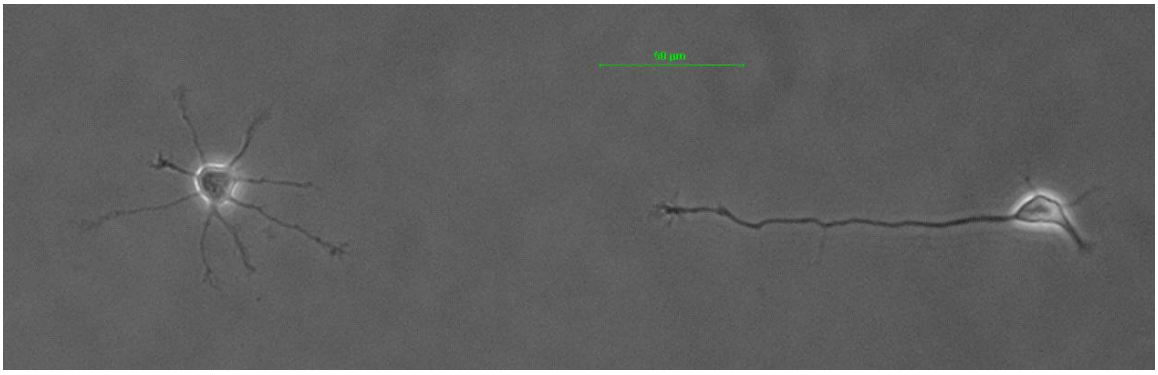


Fig. 6. Adjacent Stage 2 and Stage 3 neurons on 0.5kPa region at 20X magnification.

Growth Stage 3: Maturation of Axon and Dendrites

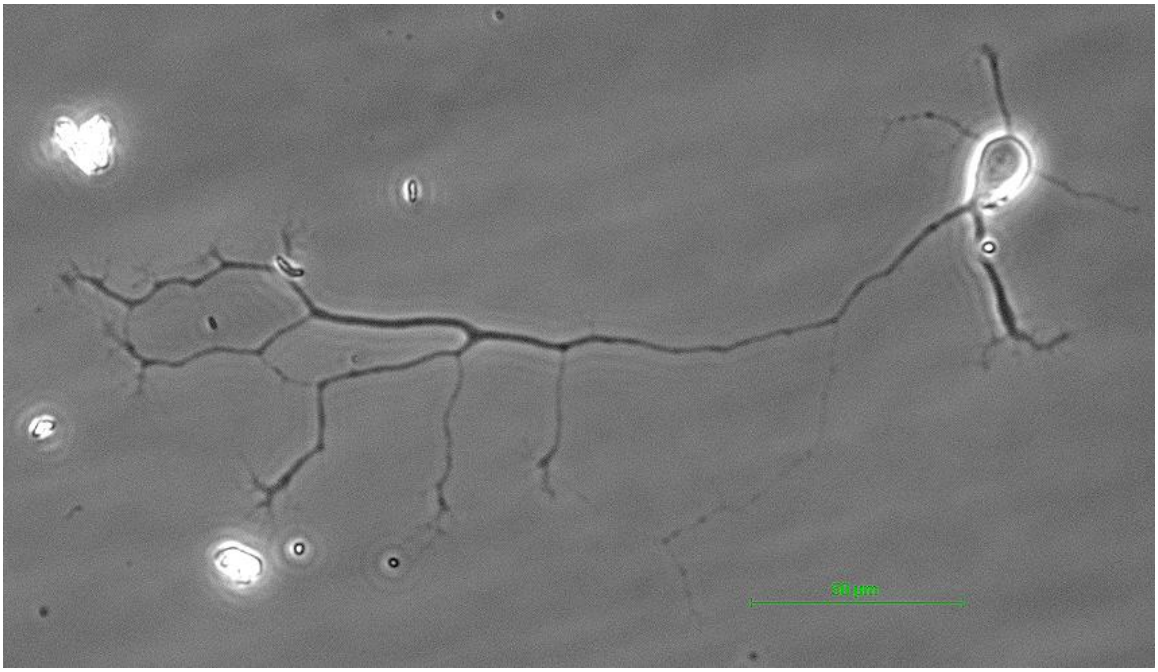


Fig. 7. Neuron considered to be entering Stage 4 of growth, on 0.5kPa region at 20X magnification.

The classification of Stage 4 of growth sin somewhat ambiguous, as a neuron may continue to mature – its processes increasing in length and complexity of branching – for some time. However, the image above shows the more complex structure that the axon can take on as the neuron continues to grow. This highly branched fractal arrangement would allow a single neuron to transmit signals to multiple cells in the native nervous /

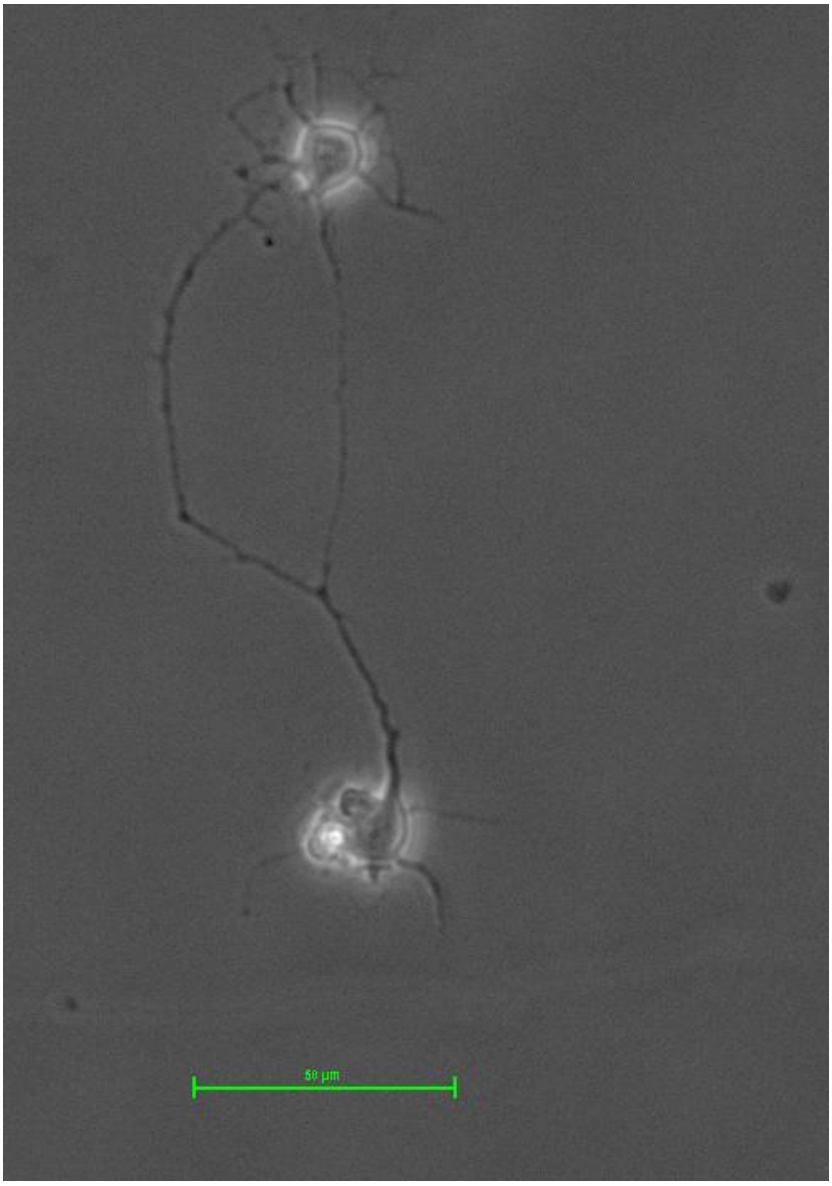


Fig. 8. Two maturing Stage 3-4 neurons that appear to be forming a synapse.

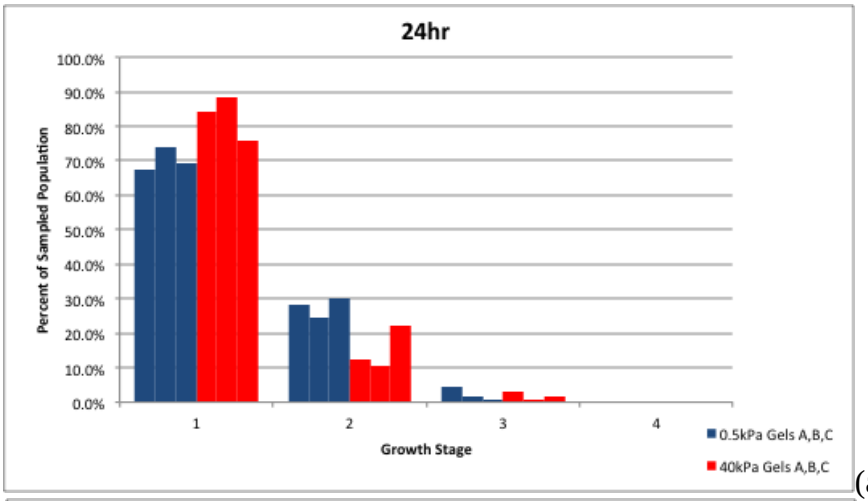
Quantifying Neuron Growth

Due to inadequacies in culturing techniques yet to be addressed, the neurons seeded in this experiment only grew for 72 hours before the entire population experience apoptosis, detaching from the substrates. During this time, data collection techniques were developed for the collection of quantitative information on neuron growth in the

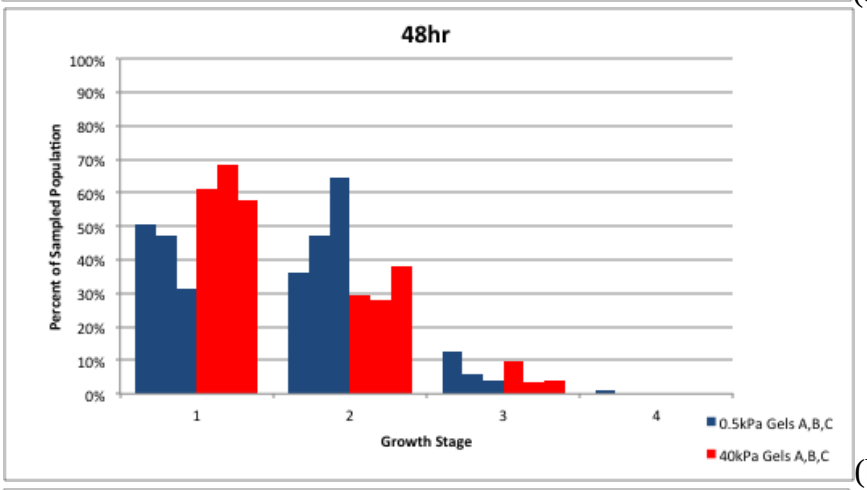
following forms. The general hypothesis of this research is that extracellular conditions which correspond to those found *in vivo* will result in optimal neuronal growth. In the case of the stiffness experiment, it is expected that the neurons will grow "better" on softer substrates that are similar to nervous tissue. The focus of the following trials was to develop several ways to characterize "better growth."

Population Statistics

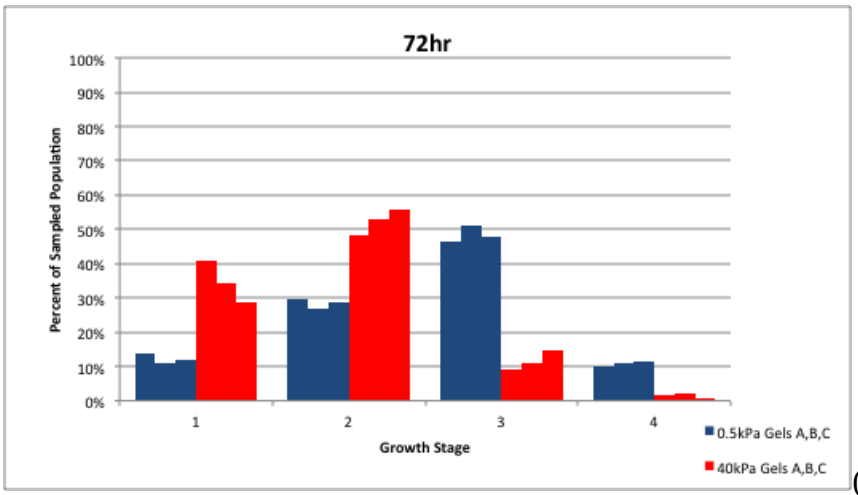
The means of characterizing "better" or "faster" growth is to determine how at what rate the population of neurons begins to develop axons – what percentage of the population is in each stage of growth after a given time. This was achieved by observing large numbers of neurons on each stiffness and visually determining the number of neurons in each stage of growth based on the characteristics of each stage. Counting was performed by scanning each region of the substrate in a uniform fashion to prevent re-counting, until sample sizes between 100 and 200 neurons for each region were collected. Further statistical significance was gained by repeating the process for three replicate gels. The figures below outline the population statistics for the series of 0.5/40kPa gels observed over three days of growth, with counting performed at 24, 48, and 72 hour time points (each corresponding to a separate panel of the figure respectively). Note that the three columns of like color in each series are data collected for that stiffness region from three replicate gels, and are placed adjacently to show the degree of variance.



(a)



(b)



(c)

Fig.9. Percentage of total number of neurons sampled in each stage of growth after 24, 48, and 72 hours (panel a,b, and c respectively)

The data collected in this population analysis supports the hypothesis that neurons will grow "better" as a collective on soft substrates as opposed to hard – in that more neurons

will begin to develop axons (transition from Stage 2 to Stage 3 and 4 neurons) as a function. After 24 hours it can be seen in Fig.9.a that although relatively few neurons have begun to mature beyond Stage 1, there are a higher percentage of Stage 2 and 3 neurons on the 0.5kPa substrate than on the 40kPa region, and correspondingly fewer Stage 1 neurons. The y-axis values in each graph represent the percentage of the total number of neurons counted on a gel, averaged for the three replicates. As the population matures 48hr after seeding, Fig.9.b shows that comparatively more neurons enter Stages 2 and 3 on the softer 0.5kPa gel, while the stiffer 40kPa region has more Stage 1 neurons that have yet to differentiate an axon or even extend minor processes. Fig.9.c shows the continuing trend, as the distribution of neurons in each Stage shifts to the right towards fully matured neurons at a comparatively increased rate on the 0.5kPa substrate than on the 40kPa. Unfortunately the neurons did not survive long for either population to consist entirely of Stage 4 neurons, as one might expect would happen, though it is hypothesized that at some time point the distribution of percentages for the 0.5kPa would shift completely to the right while the 40kPa would lag behind. The raw data collected, with associated percentage calculations can be found in the Appendix.

Axon Growth Rates

The second means of characterizing "better" growth developed in this project is compare the actual rate of growth of an axon as it develops. It is hypothesized that not only will a population of neurons begin to develop axons more rapidly on softer substrates, but that these developing axons will actually grow at an increased rate in comparison to stiffer substrates. Since the growth of the axon involves the extension of the growth cone at its

terminus across the substrate, it follows that the mechanical properties of the substrate with which the growth cone interacts have an effect on the rate of axon extension. In order to confirm this hypothesis, many neurons would need to be observed while the axon is differentiating. This must be accomplished via time-lapse imagery with the inverted microscope, since axon growth during the transition to Stage 3 happens on the order of several hours. Unfortunately, adequate numbers of neurons could not be grown to collect any meaningful amount of data comparing the soft and stiff substrates. This portion of the experiment served instead as a proof of methods, in that for a few collections of adequately dense neuron populations time-lapse imagery was successfully recorded with enough resolution to track the extension of minor neurites into axons in the case of a few cells. Imaging software was then employed to measure the starting and ending length of a specific axon over a given amount of time, and a growth rate could be calculated simply as the amount of growth divided by the amount of time the axon grew. To identify typical values and give some perspective of the scale of this growth, two neurons were successfully analyzed in this manner, yielding axon growth rates of approximately 8-10 μm/hr. A summation of the raw data involved in these calculations can be found in the Appendix.

Neuron Interaction with Rigidity and Adhesivity Boundaries

The final and most novel type of data this experiment set out to collect was to quantify and characterize the response of developing neurons to the distinct border between regions of stiffness or adhesivity. Since the substrates were carefully prepared to present these changes in either stiffness or ligand density on a scale appreciable by the individual

neural cell, it was hoped that some response to this change could be observed. Unfortunately, the sparse distributions of cells that survived the revival and seeding did not provide adequate opportunity to make such observations and this goal will have to be passed on to future researchers in this ongoing endeavor.

Discussion

While problems with the culturing of neurons at consistent densities for longer than 72 hours prevented the collection of some forms of data, the accomplishments thus far are promising for future efforts. Firstly, the development synthesis techniques for novel 'bi-rigid' and 'bi-adhesive' substrates will allow future researchers on the project to expose cultured to a wide range of conditions, maximize the amount of data that can be collected with one cell line, and hopefully to observe the response of neurons to these novel boundaries between different conditions. The data that was gleaned from the neurons cultured does seem to validate initial hypotheses – with specific regard to the response of the population of neurons as a collective to different rigidities - and serve to further motivate future effort. While other data collection efforts were hindered by insufficient population densities, significant work was accomplished in developing methods for quantitative analysis of neuronal growth that can be utilized in future efforts to create a more complete understanding of how neuron axonal growth is influenced by substrate stiffness and adhesivity.

Looking towards the future, it is clear that culturing techniques must first be refined in order to provide consistently dense populations of neurons that will grow to full

maturation. While Meberg [9] reports that only 2/3 of revived-from-frozen neurons will be viable upon seeding, this percentage may be even lower if some aspect of short-term culture has been overlooked or if these frozen neuron stocks require specific considerations.

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Appendix

Table 1: Population Statistics Raw Data

Population Statistics by Count						
	Gel	Stage 1	Stage 2	Stage 3	Stage 4	Total
0.5kPa	A	76	32	5	0	113
	B	87	29	2	0	118
	C	85	37	1	0	123
40kPa	A	107	16	4	0	127
	B	101	12	1	0	114
	C	92	27	2	0	121

Population Statistics by Percentage					
	Gel	Stage 1	Stage 2	Stage 3	Stage 4
0.5kPa	A	67.3%	28.3%	4.4%	0.0%
	B	73.7%	24.6%	1.7%	0.0%
	C	69.1%	30.1%	0.8%	0.0%
	AVG	70.0%	27.7%	2.3%	0.0%
40kPa	A	84.3%	12.6%	3.1%	0.0%
	B	88.6%	10.5%	0.9%	0.0%
	C	76.0%	22.3%	1.7%	0.0%
	AVG	83.0%	15.1%	1.9%	0.0%

Population Statistics by Count						
	Gel	Stage 1	Stage 2	Stage 3	Stage 4	Total
0.5kPa	A	57	41	14	1	113
	B	55	55	7	0	117
	C	39	80	5	0	124
40kPa	A	77	37	12	0	126
	B	80	33	4	0	117
	C	76	50	5	0	131

Population Statistics by Percentage					
	Gel	Stage 1	Stage 2	Stage 3	Stage 4
0.5kPa	A	50%	36%	12%	1%
	B	47%	47%	6%	0%
	C	31%	65%	4%	0%
	AVG	43%	49%	7%	0%
40kPa	A	61%	29%	10%	0%
	B	68%	28%	3%	0%
	C	58%	38%	4%	0%
	AVG	63%	32%	6%	0%

Population Statistics by Count						
	Gel	Stage 1	Stage 2	Stage 3	Stage 4	Total
0.5kPa	A	14	30	47	10	101
	B	12	29	55	12	108
	C	16	38	63	15	132
40kPa	A	55	65	12	2	134
	B	50	77	16	3	146
	C	41	80	21	1	143

Population Statistics by Percentage					
	Gel	Stage 1	Stage 2	Stage 3	Stage 4
0.5kPa	A	14%	30%	47%	10%
	B	11%	27%	51%	11%
	C	12%	29%	48%	11%
	AVG	12%	28%	48%	11%
40kPa	A	41%	49%	9%	1%
	B	34%	53%	11%	2%
	C	29%	56%	15%	1%
	AVG	35%	52%	12%	1%

Table 2: Growth Rate Raw Data

Total Time
3.63 hr

Neuron 1	
Time (s)	Length (μm)
0	9.35
13085	48.05
Growth Rate	
0.0030	μm/s
10.6	μm/hr

Neuron 2	
Time (s)	Length (μm)
0	126.8
13085	155.35
Growth Rate	
0.0022	μm/s
7.9	μm/hr

Author Bio:

Eben grew up in the small rural town of Monroe, ME in a house built by his parents Grant S. Estell and Janet L. Estell. A profound interest in science and mathematics has defined Eben's academic endeavors since elementary school. Eben graduated second in his class with a GPA of 99.7, and was offered a full tuition scholarship at the University of Maine where he enrolled as both a Biological Engineering major and a student of the Honors College in the fall of 2008. Eben has continually sought experience in the research laboratory setting in addition to his rigorous academic schedule in order to prepare himself for his ultimate goal of obtaining a PhD in the field of biomedical engineering at an institution of high esteem, paving the way for a fruitful career in the scientific community at the academic, government, or private level.

Beginning with his employment in Dr. Mason's lab in the Department of Chemical and Biological Engineering, where Eben served as an undergraduate research aid in the synthesis of nanoparticles, Eben's research experiences have ranged from data analysis for Neutron Powder Diffraction at the Lujan Linear Particle Accelerator at Los Alamos National Labs, to investigating nanoparticle-impregnated hydrogels for applications as passive chemical sensors. Starting in the summer of 2010 and continuing throught the rest of his undergraduate career, Eben began employment in the Laboratory for Cell Mechanics and Tissue Engineering at the Univerisity of Maine under the guidance of Dr. Alireza Sarvestani, the terminal accomplishment of this employment being the research on neuron axonal growth submitted in satisfaction of this Honors Thesis.

Eben was recommended for High Honors in this endeavor, and also graduated Suma Cum Lade with the degree of B.S. in Biological Engineering. Eben plans on spending this coming summer enjoying the outdoors of Maine before he moves to the city of New York at the end of August, where he will be attending Columbia University as a PhD candidate in the Department of Biomedical Engineering on the MS-to-PhD track in cellular and tissue engineering. Someday, he hopes to perform tissue engineering research at the organ level, and entertains the idea of pursuing a dual MD/PhD degree so that he may grow organs in the laboratory and then implant them into patients himself.