SANTA CLARA UNIVERSITY

Department of Bioengineering

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ENTITLED

PREDICTIVE MODEL FOR DESIGN OF A 3D DEVELOPMENTAL NEUROTOXICITY PLATFORM

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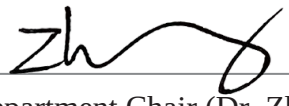

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PREDICTIVE MODEL FOR DESIGN OF A 3D DEVELOPMENTAL NEUROTOXICITY PLATFORM

By

Emma Barrett-Catton, Murial Ross, Cameron Read

SENIOR DESIGN PROJECT REPORT

Submitted to
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Abstract

Exposure to developmental toxins during gestation have been shown to be linked to neurological disorders such as epilepsy, schizophrenia, and dyslexia [1]. In this report we describe efforts that represent the ground work to develop a predictive neurotoxicity model to test developmental toxicity on early neuronal differentiation from drugs and toxins for human consumption or exposure. Developmental toxins are toxins that prevent stem cell differentiation into neurons by impacting neural development [2]. Currency technologies used to evaluate a compound's potential as a developmental toxin are centered around culturing stem cells in a two-dimensional environment or exposing animal models to the compound. The stem cells are then monitored for changes in proliferation, differentiation, and death. These classes of experiments proved not only to be expensive, but also extremely time consuming and ineffective in some cases. These technologies do not accurately mimic the in vivo environment, which uses ECM proteins and cell-cell interactions to regulate cellular functions such as migration, apoptosis, and gene expression. Our predictive model would provide a more biologically accurate alternative of the human system compared to two-dimensional cell culture and animal models. Our model would further improve the quality and relevance of developmental neurotoxicity research, reduce the number of animal experiments and overall cost to evaluate the potential for a compound to act as a developmental toxin.

Keywords: Developmental Toxicity, Predictive Model, Three-Dimensional, Stem Cells

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- Santa Clara University School of Engineering for access to MATLAB for our statistical analysis.
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List of Abbreviations

EPA: Environmental Protection Agency
NIH: National Institutes of Health
SCU: Santa Clara University
COVID-19: Coronavirus Disease-19
2D: Two-dimensional
3D: Three-dimensional
DNA: Deoxyribonucleic acid
cDNA: Complementary deoxyribonucleic acid
mRNA: Messenger ribonucleic acid
qPCR: Quantitative polymerase chain reaction
RT-PCR: Reverse transcription polymerase chain reaction
MTT: (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
BCA: Bicinchoninic Acid
PVDF: Polyvinylidene Fluoride
ECM: Extracellular matrix
P19 Cells: Murine cell line
MAP2: Microtubule-associated protein-2
GFAP: Glial fibrillary acidic protein
CAM: Cell adhesion molecule
NGF: Nerve growth factor
BLEBB: Blebbistatin
kPa: Kilopascal
MATLAB: Matrix Laboratory
PCA: Principal component analysis

CHAPTER 1: Introduction

1.1 Introduction

For our Senior Design project, we worked towards designing a predictive model to identify neurotoxic compounds, particularly focusing on developmental neurotoxins. Developmental toxins prevent stem cell differentiation into neurons by impacting neural development through many possible avenues, such as by damaging DNA, impacting gene expression, modifying signaling proteins, and many others [2]. This type of toxicity is distinct from stem cell toxins, which are those that are cytotoxic to stem cells, and neurotoxins, which are either cytotoxic or functional toxins to neurons. We are interested in studying developmental neurotoxins due to their profound impact on brain development, and therefore on people's lives. In the US, about one in six children are affected by developmental disabilities, many of which are related to neurological development [3]. The exposure of the brain to various agents can lead to developmental neurotoxicity. These alterations can have long-lasting impacts, such as causing a number of other neurological disorders, like epilepsy, schizophrenia, and dyslexia [4]. Many commonly-found agents, such as metals like lead and mercury, pesticides, nicotine and ethanol, are known developmental toxins [2]. The EPA estimates that less than 1% of chemicals in the environment have been tested for developmental neurotoxic effects due to slow and expensive testing [1]. The prevalence of developmental neurotoxins in our environment, along with the profound impact these toxins can have on individuals and communities highlights the need for an increased understanding and awareness of developmental neurotoxicity. With our model, we hope to make it easier to identify and study developmental neurotoxins, thus helping to reduce their impact.

Currently, neurotoxicity testing uses several 2D assays to test toxicity of compounds for human topical use, consumption and exposure [5]. Existing assays, such as cytotoxicity, cell viability, and functional assays as well as qPCR and cell morphology, utilize stem cells, stem cell derived neurons and cultured neurons to analyze the impact of toxins on cell fates [6–9]. While the previous technologies discussed are useful, cell culture experiments represent a very costly class of experiments that do not always produce reliable results. For this reason, we propose creating a model based upon data collected from 3D culture systems in order to predict the cellular fate of early neurons. By studying developmental toxicity in 3D, we can overcome the limitations of 2D culture, which does not mimic in vivo cell-cell and cell-matrix interactions [10]. This would allow the production of data that better represents the in vivo environment in order to make more sound predictions about the microenvironmental factors that influence early neuronal differentiation. While data included in this report is focused on neuronal differentiation, we postulate that the methods developed could be applied to various cell types.

1.2 Background and Literature Review

1.2.1 Why 3D Culture?

Cell culture is an indispensable tool in areas of developmental biology, tissue engineering, and protein pharmaceutical production. All early cell culture techniques are composed of two-dimensional environments, where cells attach to plastics or extracellular matrix (ECM) attachment molecules shown in Figure 1. In vivo, cells are in constant interaction with a variety of ECM molecules that regulate cellular functions (migration, apoptosis, gene expression, etc) which cannot be fully represented in the 2D environment [10]. The current drug development pipeline costs anywhere from $800 million to $2 billion and can take up to 15 years to bring to

market [10]. The process begins with a screening of compounds in a 2D cell culture environment, followed by animal models and finally human clinical trials. This pipeline only brings approximately 10% of initial leads through clinical development. Additionally, some therapeutics make it all the way to phase III clinical trials before proving inefficacious, at which point millions of dollars have already been allocated to research and development. A rapidly growing field of literature has suggested that 3D cell culture systems promise to address these challenges by providing cells a more realistic extracellular environment shown in Figure 1 [10].

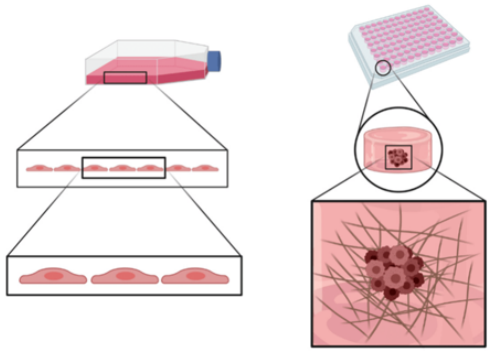


Figure 1: Typical 2D (Left) and 3D (right) polymer matrix culture systems (adapted from [11])

3D culture has been shown to produce superior and more relevant results compared to 2D in a variety of applications. One example is when cell culture systems are used as a model for drug development, various studies have illustrated that pharmacaiduals that show promise in 2D have reduced or no efficacy when tested in 3D culture systems. For example, a study performed by Edmonson *et. al***.** showed that an anti cancer drug, Melphalan, killed ~100% of an intestinal cancer cell line at concentrations of 100uM but when the same concentration of the drug was tested in a 3D spheroid culture system only ~20% of the cells were killed [12]**.** These results indicate the need for cell culture techniques that better represent the invivo environment. As 3D culture platforms offer a more robust way to culture cells and study their cellular functions, it represents a new modality to understand the effect of compounds on the differentiation of stem cells to early neurons.

1.2.2 Existing Technologies

Currently, neurotoxicity testing uses several 2D assays to test toxicity of compounds for human topical use, consumption and exposure [5]. Existing assays, such as cytotoxicity, cell viability, and functional assays as well as qPCR and cell morphology, utilize stem cells, stem cell derived neurons and cultured neurons to analyze the impact of toxins on cell fates [6–9]. The next several subsections will elaborate on specific examples of these 2D neurotoxicity assays and the type of data collected.

1.2.2.1 Cytotoxicity and Cell Viability Assays

Succinate dehydrogenase activity assay, also known as a MTT assay, is a common cytotoxicity colorimetric assay that measures cell viability and proliferation by enzymatically reacting with succinate dehydrogenase in the mitochondria [13]. Essentially, the occurrence of mitochondrial respiration catalyzes the reduction of the MTT dye into insoluble crystals [13]. Color produced from the cells is proportional to the number of viable cells after the cells are lysed and processed [13]. A great example of a cell viability assay is dye exclusion. This test allows researchers to

determine the number of viable cells and dead cells [14]. Dyes, such as trypan blue, eosin or propidium, are introduced into a cell suspension and will only dye cells without intact cellular membranes [14]. Since viable cells have intact membranes, they will remain clear while dead cells are dyed [14]. Using a hemocytometer, researchers can count a small fraction of the overall cell suspension and calculate an estimate for the overall number of viable and dead cells [14]. MTT assays and dye exclusion allows researchers to determine the dose- and time-dependent cytotoxic effect of their drug or compound of interest [13,14].

1.2.2.2 Functional Assays

Functional assays measure the neuron's ability to function, such as generating action potentials or creating calcium influxes to release neurotransmitters [15,16]. Two commonly used functional assays are calcium imaging and patch clamp recording. Calcium imaging is used to analyze neuronal signaling by allowing researchers to image or record the occurrence of action potentials in neurons [15]. Calcium is used by neurons in their axon terminals to trigger exocytosis of neurotransmitters, releasing them into the synaptic cleft and passing the signal to postsynaptic neurons [15]. In order to capture neuron signaling, a bioluminescent calcium indicator such as aequorin, derived from bioluminescent marine organisms, or chemical calcium dyes are used. Due to aequorin's large size it must be loaded into each cell by a micropipette or transfected into the cells via genetic engineering [15]. Similarly, chemical dyes need to be introduced by micropipettes. Once introduced, neuronal signalling can be imaged by high-speed confocal microscopes [15].

Patch clamp recording is another type of functional assay to measure neuron activity. It can be performed on single neurons, brain slices or live brains in sedated animals [16]. Researchers place a glass micropipette electrode directly on a small area of the cell membrane and use suction to firmly seal the tip of the pipette to the cell [16]. As the cellular membrane changes voltage during action potentials, the electrode will be able to record the change in voltage. The tight seal creates very high resistance, allows detection of small voltage changes and blocks external currents from surrounding cells [16]. This method allows researchers to measure the neuronal activity of individual cells [16].

1.2.2.3 Gene and Protein Expression

Reverse Transcription Polymerase Chain Reaction (RT-PCR), Western Blots and Immunocytochemistry allow researchers to analyze the gene and protein expression of cells as a result of exposure to drugs or environmental compounds [17,18]. Researchers can identify cell differentiation, maturity and up/down regulation of a gene or protein of interest [17,18].

RT-PCR identifies and magnifies the presence of genes of interest allowing the comparison of gene expression pre- and post-exposure to the compound [17]. After cells have been exposed to the compound, mRNA is isolated and prepped for RT-PCR. Primers are selected to identify key genes for cell fate, such as beta 3 tubulin which is a marker for immature neurons [18] or MAP2 which is a marker for mature neurons [17]. During RT-PCR, the mRNA is reverse transcribed into cDNA, which is then amplified by taq polymerase [17]. The chosen primers will only bind to complementary sequences on the cDNA, amplifying the genes of interest to detectable levels [17]. Researchers can use either gel electrophoresis or primers with a fluorescent tag to detect the gene [17].

Western Blots are used to detect the presence of a protein of interest [18]. After compound exposure, the cells are lysed and processed to isolate protein. A BCA protein assay is used to determine overall protein concentration [18]. Then, gel electrophoresis is used to separate the protein mixture and transferred to a PVDF membrane to be stained with antibodies to detect the protein of interest [18]. To detect fluorescence from the antibody stain a fluorescence microscope, such as a confocal microscope, is used [18]. Images are taken of the membrane and image processing software is used to analyze protein expression [18].

Immunocytochemistry is another technique to detect the presence of a protein of interest. After cells are exposed to the toxin, they are fixed with paraformaldehyde and stained with fluorescent primary and secondary antibodies for specific proteins of interest [18]. Multiple proteins can be stained at the same time. Similar to western blots, a fluorescence microscope is used to image the cells and an image processing software is used to analyze protein expression [18]. Immunocytochemistry produces similar results as western blots in addition to allowing researchers to identify the protein location in the cell and morphology [18].

1.2.2.4 Morphology

Lastly, morphology can be used to identify the effect of the neurotoxin on stem cells or neurons. Researchers can measure the change in cell size, fragment length per cell, branches per cell, and total length per cell [19]. These morphological changes indicate the impact of the toxin on cellular differentiation and signs of cytotoxicity [19]. For instance, Crumpton *et. al.* used morphology in their study to identify the most sensitive period during differentiation for which the toxin had the greatest effects on the stem cells [19]. They concluded that lead had the greatest effect during the early initiation events of differentiation [19]. Although morphology is the simplest neurotoxicity detection platform explored in this section, it is a cheap and powerful tool that should not be ignored.

1.3 Proposed Goals

1.3.1 Mission

Our mission is to develop a three-dimensional neurotoxicity platform to test developmental toxicity on early neuronal differentiation from drugs and toxins for human consumption or exposure. Our model will provide a more biologically accurate alternative of the human system compared to animal models, currently used for clinical and pharmaceutical research.

1.3.1 Initial Project Goals

During the spring and summer of 2020, we designed our initial project to be performed completely in the lab. Our goal was to design a 3D cell culture system to test the developmental neurotoxicity effect of acrylamide on neural differentiation. In order to perform this experiment, we would culture and differentiate P19 cells in 3D alginate hydrogels. At various time points during differentiation, different concentrations of acrylamide would be added to the system. Then, we would analyze the morphological changes using microscopes, imaging and image analysis as well as cell proliferation.

1.3.2 Revised Project Goals Due to COVID-19

As a result of COVID-19, our project has gone through a number of revisions. Our initial project would have been largely conducted in the lab. However, we realized over the summer prior to our senior that this would not be feasible due to COVID-19, so we created the initial project goals outlined above. These goals still relied on some lab time, though, and by the end of Fall 2020, it became clear that we would not be able to complete any meaningful work in the lab due to the lab restrictions. At this time, we created revised project goals. The revised projects goals are as follows:

1. Collect data from literature on how various factors affect neural differentiation, to identify factors that have an impact on neural differentiation.
2. Analyze the combined data collected from literature using G-Tests, χ^2-tests and logistic regression to determine significance of these factors to show the effect of the variables on differentiation. Analyze large data sets using other techniques such as Principal Component Analysis (PCA).
3. Combine significance tests and other data analysis into a model to inform future research into developmental neurotoxicity tests in 3D culture.

CHAPTER 2: Project Overview

2.1 System Overview

In order to collect data to use in our model, we followed a general procedure that took place in three steps: literature review, data collection and unification, followed by statistical hypothesis testing. An initial literature review and data collection consisting of 50 articles regarding 7 different variables known to influence neural differentiation was narrowed down to 14 articles regarding three different variables: 2D vs 3D culture environment, toxin presence, and matrix stiffness outlined in Table 1. The final data set used for statistical analysis was chosen due to their most comparable experimental setups and data collection methods.

We performed χ^2-test(s), G-Test(s) of Independence, Logistic Regression, and PCA on some or all of the subsystem variables depending on the suitability of each data set outlined in Table 1. All of the statistical tests performed were used to determine if there is or is no association between the subsystem variable and neural differentiation with the exception of the PCA analysis. The PCA analysis was performed to reduce the dimensionality of one of the datasets from the stiffness subsystem variable (see section 5.2.2.2).

Table 1: Outline of Subsystem Data Collection and Analysis

Sub System	Data source / Number of Articles Used	Statistical Tests Used	Hypotheses
2D vs 3D	1. Huang et al., *Neuro Regen Res* (2013) 2. Brannvall et al, *Journal of Neuroscience Research* (2007) 3. Zare-Mehrjardi et al., *Int J Artif Organs* (2011) 4. Bozza et al., *Biomaterials* (2014) 5. Oritinau et al., *BioMedical Engineering OnLine* (2010)	1. Chi Square Test (χ^2-test)	*Null Hypothesis:* There is no association between the subsystem variable and neural differentiation. *Alternative Hypothesis:* There is an association between the subsystem variable and neural differentiation.
Toxin	1. Engstrom et al., *Toxicol In Vitro* (2016) 2. Lin et al., *Chemosphere* (2021) 3. Tasneem et al., *Toxicol Lett* (2016)	1. χ^2-test 2. G-Test	
Stiffness	1. Banerjee et al., *Biomaterials* (2009) 2. Leipzig et al., *Biomaterials* (2009) 3. Rammensee et al., *Stem Cells* (2017) 4. Ali et al., *Acta Biomaterialia* (2015) 5. Her et al., *Acta Biomaterialia* (2013) 6. Engler et al., *Cell* (2006)	1. χ^2-test 2. G-Test 3. Logistic Regression 4. PCA	

2.1.1 2D versus 3D

One factor we investigated was two-dimensional versus three-dimensional matrices. As outlined in section 1.2.1, various research groups have found that cells cultured in 3D matrices have a higher rate of stem cell differentiation as well as cell survival and proliferation [20–25]. We investigated the significance of the impact of matrix dimensions on stem cell differentiation using a χ^2-test.

2.1.2 Toxin

Research has shown that compounds that do not act as cytotoxins can still act as neural toxins. However, the EPA estimates that less than 1% of chemicals in the environment have been evaluated for their potential to cause developmental neurotoxicity [1]. For this reason, in addition to investigating the influence of 2D and 3D matrices on neuronal differentiation, we also investigated the effect of particular toxins on neuronal differentiation, proliferation, and death. Data was collected on the effect of Acrylamide and Lead exposure on cellular characteristics such as gene expression and cellular morphology and was analyzed for significance using χ^2 and G-tests.

2.1.3 Stiffness

Another factor that we investigated in regards to neuronal differentiation was matrix stiffness. A wide variety of researchers have investigated the impact of stiffness on stem cell differentiation. Overall, researchers have found that lower stiffnesses, like that of the brain, cause stem cells to differentiate into neurons [26–34]. Due to its importance, we wanted to incorporate the impact of stiffness into our predictive models. We investigated the impact of stiffness on differentiation by combining various datasets collected from literature, analyzed them with statistical techniques to determine significance, and completed Principle Component Analysis (PCA).

2.2 Systems Integration

The overall goal of this project was to create two models, as shown in Figure 2. The first would input microenvironment cues (2D vs. 3D, stiffness, and developmental toxin), and output cell fate. The second would input cell characteristics (morphology, cell viability and proliferation, and gene or protein expression) and output toxin type (developmental, neuronal, stem cell or no toxin). In order to create these models, we would need to do further data collection and lab work.

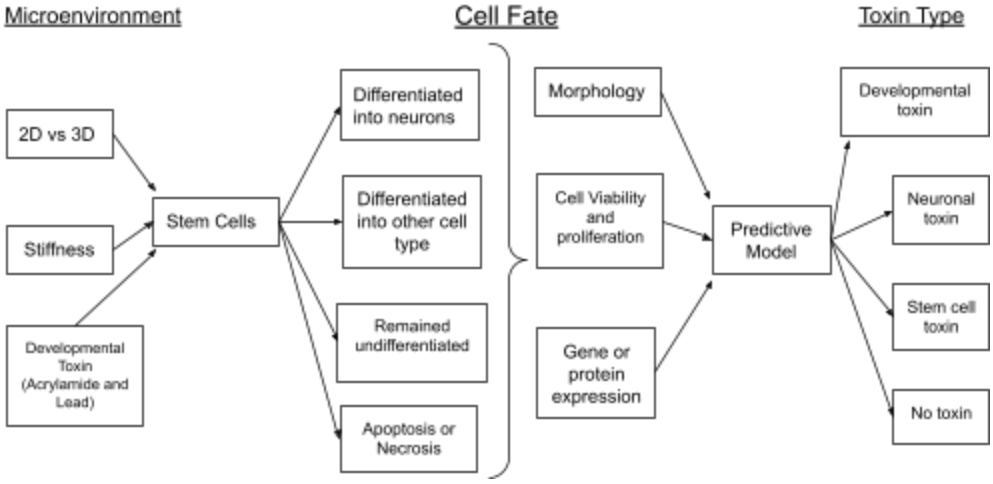


Figure 2: Diagram of the Predictive Models

2.3 Team and Project Management

2.3.1 Schedule

Due to COVID-19, we had to shift to an entirely virtual project at the end of fall quarter. As a result, we spent time during the fall working on preparing for lab work as well as developing our model before pivoting to solely working on modeling in the winter and spring. In fall 2020, we completed literature review, applied for funding, determined materials to purchase, and began data collection from literature. In winter 2021, we continued to collect data, began to analyze our data using contingency tables (χ^2-tests, G-tests, and linear regression), and PCA, reviewed mathematical models in biological systems, and began to write our thesis. In spring 2021, we completed our statistical analyses, senior design presentation and thesis. See the Gantt Chart in the Appendix D for an overview of our progress throughout the year (Figure 11).

2.3.2 Budget and Materials

In the beginning of the year, we planned to perform lab experiments as outlined in section 1.3.1 and submitted our proposed budget for funding from Santa Clara University School of Engineering located in Appendix C (Table 19 and Table 20). Due to COVID-19 restrictions, we transitioned our project Winter quarter 2021 to a fully virtual format. Table 21 in Appendix C outlines the finalized list of materials and cost.

2.3.31 Challenges

Throughout our project, we have run into issues related to obtaining raw data from articles, determining a similar metric across articles and the COVID-19 pandemic. The largest challenge was determining the feasibility of our original project due to COVID-19 restrictions. We originally wanted to perform laboratory experiments on the effects of acrylamide toxicity on neuronal differentiation in 2D versus 3D culture conditions using imaging and software to quantify differentiation. During Fall quarter, our team was not able to access the lab but focused on collecting protocols, researching background information for our project and collecting data for a predictive model. Unfortunately, a second stay-at-home order was put into effect in December, preventing Dr. Asuri from beginning to culture cells for our experiments and restricting our access during the Winter quarter. We transitioned our project scope to a fully computational project focusing on our predictive mathematical models.

To formulate our model, we gathered data from other's previous research on our variables of interest. Often the data was presented in graphs, requiring us to estimate the values using a grid overlay, mentioned in Chapter 3. During the Fall quarter, we emailed numerous laboratories for access to their raw data, but only one replied, proving it hard to obtain raw, high quality data for our model. Of the data we did collect, the metric for measuring each experiment varied by paper. Some researchers used normalized mRNA gene expression from RT-PCR, protein expression, percent cells differentiated, percent cells positive for a marker, fluorescence, change in neuron cell body area, number of neurite branches or neurite extension length to analyze the change in neuronal differentiation. We struggled to find a common metric across articles to be able to compare data between the papers for one variable. For instance, we collected papers analyzing the effect of matrix stiffness on differentiating stem cells. A common metric among several of the papers was the normalized expression of beta 3 tubulin. That selected data was then used in our contingency tables and expected value tables were created to perform χ^2-test and G-test. For

several of our variables, the expected values were less than 5 going against the general rule of thumb for a successful test.

CHAPTER 3: Subsystem 1: 2D vs 3D

3.1 Subsystem Overview

A number of researchers have found that cells cultured in 3D matrices have a higher rate of stem cell differentiation into neurons, as well as increased cell survival and proliferation, making matrix dimensions an ideal microenvironmental input to add to our model [20–25].

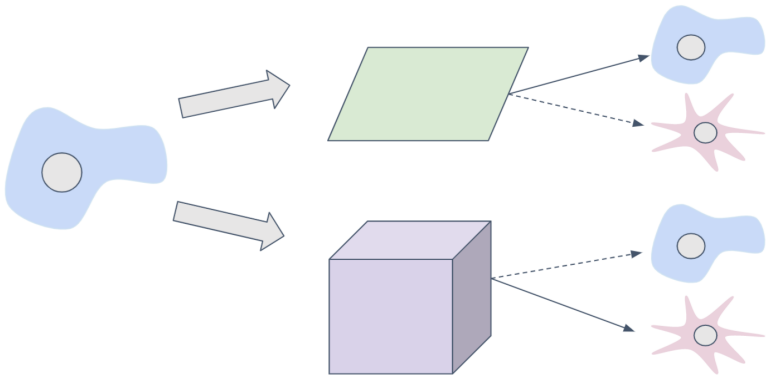


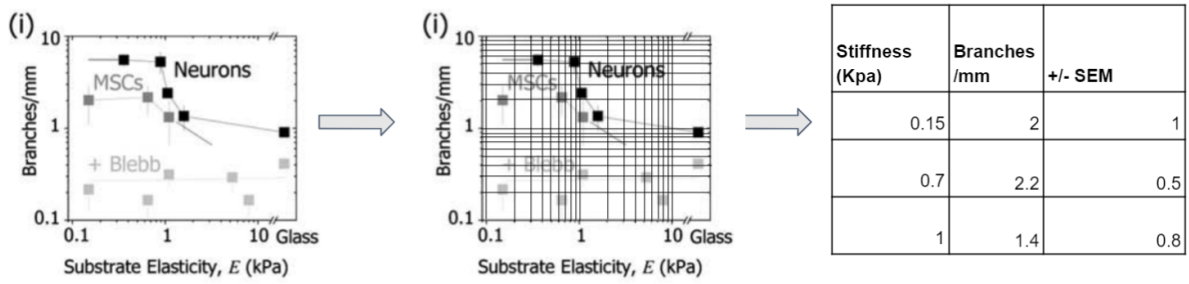
Figure 3: Diagram of the 2D versus 3D Subsystem. We collected data from articles that put stem cells into two dimensional and three dimensional matrices and measured neuronal differentiation rates.

3.2 Materials and Methods

3.2.1 Literature Review and Data Collection

For each subsystem, we started our process with literature review and data collection. We used various tools from the SCU library to collect data, particularly using the Interlibrary Loan system to get articles not owed by the library, and library database subscriptions, such as Engineering Village, PubMed and ScienceDirect and Google Scholar to access other articles. We also used search techniques such as boolean operators, narrowing down article types and years, and specifying keywords in the title and abstracts of the papers to find the articles.

After finding the articles, we then extracted data from the articles. If the data was present in tables, we then immediately transferred that data into our excel spreadsheet. However, in many cases the data was only presented in graphs and figures. In that case, we first reached out to the corresponding author of the paper to try and get the raw data from them. We did not hear back from many of the authors, though. As a result, when the data was not available we would extract images of the graphs, import the images into Google Drawings, and use grid lines to closely approximate the values and standard deviation (Figure 4). A similar extraction method has been used by other researchers [35–37]. Using this process, we collected data from six papers on stem cell differentiation into neurons in 2D and 3D matrices [20–25].



Stiffness (Kpa)	Branches /mm	+/- SEM
0.15	2	1
0.7	2.2	0.5
1	1.4	0.8

Figure 4: Diagram of the Data Extraction Method Using Gridlines (graph from [30]).

For our data analysis, we used five out of the six papers. These five papers all used beta III tubulin expression, a common neuronal differentiation marker, as the measure of neuronal differentiation [38]. We then calculated the fold of beta III tubulin expression for the matrices versus the control, using the following formula:

$$Fold\ change = \frac{experimental\ value - control\ value}{control\ value} \quad \text{(Eq. 1)}$$

We set a two fold change from the control as the cut off between differentiated and undifferentiated neurons. We made this cut-off based on a paper by Gurok *et al*. who studied the expression of various markers over the course of stem cell differentiation into neurons [39]. They found that there was roughly a two fold increase in beta III tubulin expression between stem cells and differentiated neurons [39]. As a result, we said that samples with a two fold or greater increase compared to the control were differentiated, and samples with less than a two fold increase were undifferentiated. Based on these categories and available data from literature review, we created a contingency table (Table 3) for statistical analysis. A contingency table showcases the distributions of multiple variables, in this case depicting the distribution of differentiated and undifferentiated cell samples for both 2D and 3D matrices.

Table 3: Contingency Table for 2D versus 3D

Dimensions	Differentiated (>2 fold increase)	Undifferentiated (<2 fold increase)	Total
2D	0	10	10
3D	21	7	28
Total	21	17	38

3.2.2 Statistical Analysis

We completed a Chi Square Test (χ^2-test) on the data combined in the contingency table. The resulting observed and expected tables for the χ^2 -Test are located in Appendix A. We were unable to complete other forms of analysis that we completed on the other subsystems, such as the G Test and Logistic Regression, because of the zero in the 2D-differentiated box. The zero results in undefined values and errors in the G Test and Logistic Regression computations.

3.2.3.1 Chi Square Test

A χ^2-test tests whether the distributions of variables differ from one another. In our case, it tests the distributions of differentiated and undifferentiated cells for 2D versus 3D matrix cultures. This test lets us know if there is a significant difference between the experimental group and the control in terms of differentiation [40]. The null hypothesis is that there is no relationship between matrix dimensions and neural differentiation, and the alternative hypothesis is that there is a relationship between dimensions and differentiation.

To complete the χ^2-test, we compared the observed and expected data. The observed data is the data in the contingency table, with an individual value designated as O_{ij} (observed count in row i and column j). We computed the expected values from the contingency table using the following formula:

$$E_{ij} = \frac{(row\ sum\ i)(column\ sum\ j)}{(table\ sum)} \quad \text{(Eq. 2)}$$

(expected value for row i, column j)

The expected values should all be at least 5 to complete a χ^2-test. However, one of our values (2D-undifferentiated) was less than 5, at 4.5. Even so, we still wanted to complete a χ^2-test to get a rough estimate of whether there was a significant difference between the relationships of the variables. However, it is important to note that our expected values did not entirely meet this benchmark. The resulting observed and expected values are found in Appendix A.

Next, we computed the χ^2-value using the following formula:

$$X^2 = \sum_{i \in rows} \sum_{j \in columns} \frac{(O_{ij} - E_{ij})^2}{E_{ij}} \quad \text{(Eq. 3)}$$

Finally, we used MATLAB to compute the p-value of the χ^2-value using the following code: *p=1-chicdf(x² value, degrees of freedom),* where *degrees of freedom=(i-1)(j-1)* and *i* and *j* are the number of rows and columns respectively.

3.3 Results and Discussion

The p-value from the χ^2-test is $4.2313*10^{-5}$. The p-value of $4.2313*10^{-5}$ is much lower than the cutoff value of 0.05, so we can reject the null hypothesis that there is no relationship between matrix dimension and neural differentiation. These results go along with all the individual papers, which all indicate that there is a higher rate of neural differentiation in 3D matrices versus 2D [20–25]. However, because one of our expected values does not meet the benchmark of 5, we would want to do further statistical analysis to confirm these results. Nonetheless, these results are promising, indicating that 3D matrices do have a significant effect on stem cell differentiation into neurons. We would want to keep this in mind in the development of our platform.

CHAPTER 4: Subsystem 2: Toxin

4.1 Subsystem Overview

A large body of literature has shown that the presence of particular compounds can influence cellular differentiation, proliferation and death (Figure 5), therefore toxins are a variable of interest for our model of developmental neuronal toxicity. Various factors such as the concentration of the compound and the time at which cells are exposed determine the compound's fate on cells [17,40,41].

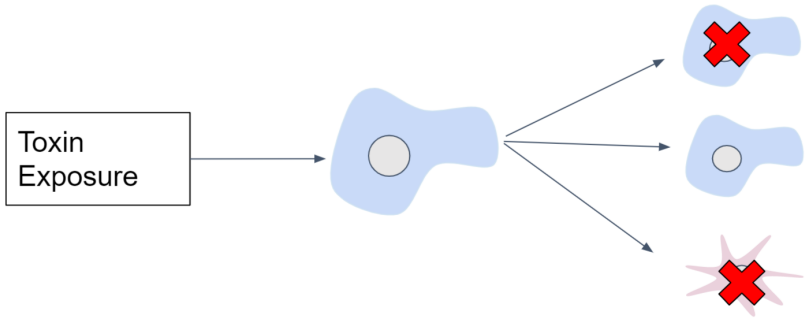


Figure 5: Diagram of the Toxin Subsystem. Toxins can can have one or more effect(s) on cells

4.2 Materials and Methods

4.2.1 Literature Review and Data Collection

As mentioned in section 3.2.1 the first step of each sub system was a literature review and data collection. For the toxin exposure subsystem, we began by collecting data on two different compounds previously shown to cause developmental toxicity: acrylamide and lead [6,13,39]. During the initial phases of literature review and data collection, data was collected from eleven papers for acrylamide and nine papers for lead.

Toxin data collected ended up containing many different markers used to measure the differentiation of cells, for example, one group would use the expression of a particular gene while another group would use morphological characteristics. Because different cell markers appear at different periods of cellular differentiation, we are unable to combine data of different gene markers. Furthermore, the timing of toxin addition proved to be highly variable between different groups which imposed further limitations when attempting to unify the data.

The changes in neurite length were represented as percentage changes with reference to negative control and positive control, along with calculating fold change using equation 1. The negative control was defined as 0 uM Lead t=0 without Nerve Growth Factor (NGF) and the positive control was defined as 0 uM Lead t=0 with NGF. Nerve growth factor has been shown to play a critical protective role in the development and survival of early neurons, so a culture without this factor is a suitable negative control [43]. In situations where negative control was not present in the data set, it was assumed to be zero fold change.

$$Percent\ Change = \frac{experimental\ value - negative\ control}{positive\ control - negative\ control} * 100 \quad \text{(Eq. 4)}$$

To arrange the neurite extension data into contingency tables, we set a one fold increase as the cut off between differentiated and undifferentiated neurons. This decision was made because a

one fold difference was equal to the difference between the positive and negative control (neurite extension with and without NGF at t=0). This means that any neurons that had over a one fold increase in neurite extension compared to the negative control were considered differentiated and any neurons that were less than one fold increase compared to the negative control were considered differentiated for the purpose of our contingency table (Table 4).

Table 4: Contingency Table for Toxin Type

Toxin Concentration	Differentiated (>1 fold increase)	Undifferentiated (<1 fold increase)	Total
0-0.09uM	4	12	16
0.1-2uM	1	5	6
Total	5	17	22

4.2.2 Statistical Analysis

For the toxin data that we collected and combined into a contingency table, we performed a χ^2-test and G-test. For the χ^2-test, we used the same methodology described in section 3.2.3.1. The resulting observed and expected tables for both the χ^2 and G-Test are located in Appendix A.

4.2.2.1 G Test

The G-test of Independence is a likelihood ratio test that is used to determine whether the number of observations in a specific category fits the theoretical expected value. The G-test is used when you have one minimal variable with two or more states and it allows you to see if the proportions of one variable different for different values of another variable [44,45].

The test generates a G statistic which can be used to calculate a p-value to determine if you can accept or reject the null hypothesis. The null hypothesis is that there is no relationship between the presence of a particular toxin and neural differentiation, and the alternative hypothesis is that there is a relationship between presence of a particular toxin and neural differentiation. To perform the test, a contingency table (Table 4) was created as described previously in section 3.2.2 and observed and expected values for contingency tables for analysis along with degrees of freedom were calculated in the same manner as for the χ^2-tests mentioned in section 3.2.3.1. Following the definitions of O_{ij} and E_{ij}, the G-statistic was calculated as shown below in equation 5.

$$G = 2\left(\sum_{i=1}^{r}\sum_{j=1}^{c} O_{ij} ln\left(\frac{O_{ij}}{E_{ij}}\right)\right) \quad \text{(Eq. 5)}$$

Finally, using the resulting G-statistic, a p-value is calculated in MATLAB by applying the chi-square cumulative distribution function as previously described in section 3.2.3.1.

4.3 Results and Discussion

As mentioned in section 4.2.1, the toxin data collected contained various different metrics of differentiation which removed the option of combining the data from dissimilar papers. As a result, we were unable to create a contingency table for any of the data collected by acrylamide papers and only able to create a contingency table for lead from three of the nine papers that we reviewed.

The p-value from the G test was 0.6714 indicating that we fail to reject the null hypothesis. The p-value from the χ^2-test was 0.6801 indicating that we fail to reject the null hypothesis. These results are not in agreement with published results, as the papers' data used to create the contingency table found that there was an effect of lead on neural differentiation. We hypothesize that the differences in our results from the published work could be caused by the process of over simplifying differentiation as a binary when creating our contingency table when in fact there are many stages of differentiation between a stem cell and a mature, differentiated neuron. Additionally, As mentioned in section 3.2.3.1, the expected values should all be at least five to complete a G or χ^2 test so the small sample size may also contribute to differences between the literature and our findings.

CHAPTER 5: Subsystem 3: Stiffness

5.1 Subsystem Overview

The impact of stiffness on cell differentiation has been heavily investigated and is well known to influence cell fate [26–33,46], making it a prime variable to incorporate into our predictive model. In general, cells differentiated on stiffnesses less than 1 kPa will express neurogenic biomarkers, while those cultured on 10 kPa surfaces will express myogenic biomarkers [30] as shown in Figure 6. In addition, those differentiated on 34 kPa surfaces will express osteogenic biomarkers [30].

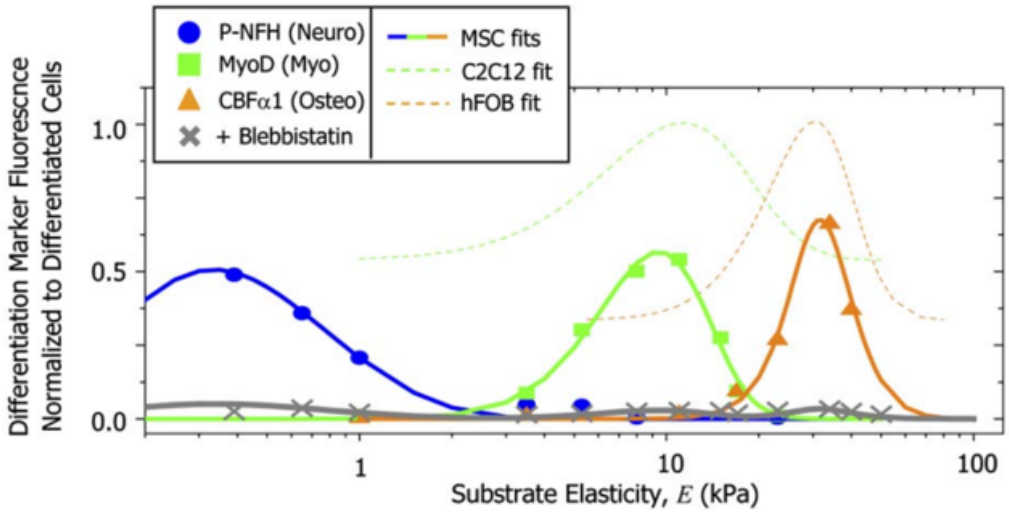


Figure 6: Impact of Stiffness on Fate of Stem Cell Differentiation Adapted from [30]

5.2 Materials and Methods

5.2.1 Literature Review and Data Collection

During our literature review, we read and collected data from 9 papers analyzing the impact of various 2D culture stiffness on neural differentiation. The same methodology for literature review and data extraction from graphs was followed, mentioned in section 3.2.1. Researchers measured gene expression such as beta III tubulin, MAP2 and GFAP, as well as neurite branching and extension to analyze the impact of stiffness on neural differentiation [26–33,46]. For our data analysis, we used data that measures beta III tubulin expression because this was the most common metric across 6 out of the 9 papers. The stiffness categories were decided based on Engler *et al.*'s research shown in Figure 6 [30]. The data was decided if it was differentiated or undifferentiated using a 2-fold threshold using the same methodology mentioned in section 3.2.2 [39]. Based on these categories and available data from literature review, we created a contingency table (Table 5) for statistical analysis.

Table 5: Contingency Table for Stiffness Data

Stiffness	Differentiated (>2 fold increase)	Undifferentiated (<2 fold increase)	Total
Low (<1kPa)	3	1	4
Medium (1-10kPa)	5	5	10
High (>10kPa)	1	5	6
Total	9	11	20

5.2.2 Statistical Analysis

For the stiffness data that we collected and combined into a contingency table, we performed a χ^2-test, G-test and Logistic regression. We performed Principal Component Analysis (PCA) on an extensive dataset provided by Engler *et al.* in their supplementary materials that measures the gene expression of 21 neural lineage markers over various stiffness with and without blebbistatin (BLEBB), a chemical that blocks mechanical signal transduction [30].

For the χ^2-test and G-Test, we used the same methodology described in section 3.2.3.1 and 4.2.2.1 respectively. The resulting observed and expected tables for both the χ^2 and G-Test are located in Appendix A.

5.2.2.1 Logistic Regression

Logistic regression analysis examines the association between categorical or continuous independent variables and with one binary dependent variable, producing an odds ratio and p value that indicates the strength and direction of association between the two variables [47]. This method is optimal for measuring the relationship between various stiffnesses, a categorical independent variable, with differentiation, a binary dependent variable. In order to perform logistic regression, we used the link function and standard equation shown below [48].

$$log\frac{p_i}{1-p_i} = \beta_0 + \beta_1 x_i \qquad \text{(Eq. 6)}$$
$$where\ i = 1,\ 2, \ldots . n$$

After performing logistic regression and solving for β_0 and β_1, equation 6 can be rewritten as equation 7 to solve for the proportion of cells differentiated per stiffness category.

$$p = \frac{exp(\beta_0 - \beta_1 x_i)}{1 + exp\beta_0 - \beta_1 x_i)} \qquad \text{(Eq. 7)}$$

We used MATLAB to perform logistic regression, provided by Santa Clara University Design Center. The data from the contingency table was input into MATLAB as a stiffness matrix with 1, 2, and 3 representing the three stiffness categories, the number of data points considered differentiated and the overall sample size per category. Using the generalized linear model

function, b values for equation 6, standard deviation and p-values were generated. The MATLAB code is private domain and can not be provided in this report.

5.2.2.2 Principal Component Analysis

Principal Component Analysis (PCA) is a helpful dimensionality reduction tool. PCA essentially identifies which variables are closely associated and which are the most unique, allowing the preservation of as much variability as possible while reducing dimensions [49,50]. We performed this test to identify which genes in Engler *et al.*'s dataset were the most unique and should be focused on for future laboratory research, reducing the number of future experiments that need to be run.

In order to conduct PCA, z-scores are computed for each variable (X_k), the covariance matrix is computed from the z-scores, eigenvectors are computed from the covariance matrix ($V(\lambda_k)$), then reduced dimensionality is computed using the following transformed equation [49,50]:

$$PCA = \sum_{K=0}^{n} V(\lambda_k) * X_k \quad \text{(Eq. 8)}$$

We analyzed a dataset from Engler *et al.* that measures the gene expression of 21 neural lineage markers over various culture stiffnesses ranging from 0.1kPa to 34kPa [30]. Engler *et al.'s* dataset is located in Appendix B. We used MATLAB to run PCA. The code is private domain and is not provided in this report. Although we ran several iterations, our final analysis focused on the dataset without the addition of BLEBB since we are interested in how mechanical signals influence cell fate.

After running our MATLAB code, two graphs were generated. The first graph is the Pareto of Effects, a bar graph which graphs percent of variance over variance. This graphic indicates how many distinct clusters of variables exist in the dataset after dimensionality reduction and what percent of variance the corresponding cluster represents in the dataset [51]. The second graph is a biplot, which graphs each gene where the cosine of the angle between the gene and axis indicates its importance [49]. The cosine of the angle between pairs of genes indicates their correlation [49]. Genes with high correlations will point in similar directions and can be stacked, while genes with low correlations will have large angles and can be perpendicular to each other [49].

5.3 Results and Discussion

The χ^2-test had a p-value of 0.1736, while the G-Test had a p-value of 0.15. Both of these values are well above the level of significance (p=0.05), therefore we did not reject the null hypothesis, which states that there is no association between stiffness and differentiation.

Logistic regression yielded the following model, where xi corresponds to 1, 2 or 3 depending on the stiffness category and p is the proportion differentiated:

$$p = \frac{exp(2.64–1.37x_i)}{1+exp(2.64–1.37x_i)} \quad \text{(Eq. 9)}$$

Equation 9 has a negative slope, indicating an inverse correlation between the variables. As stiffness increases, the odds of neural differentiation decreases, as seen in Figure 7. This trend was expected based on previous literature [26–31].

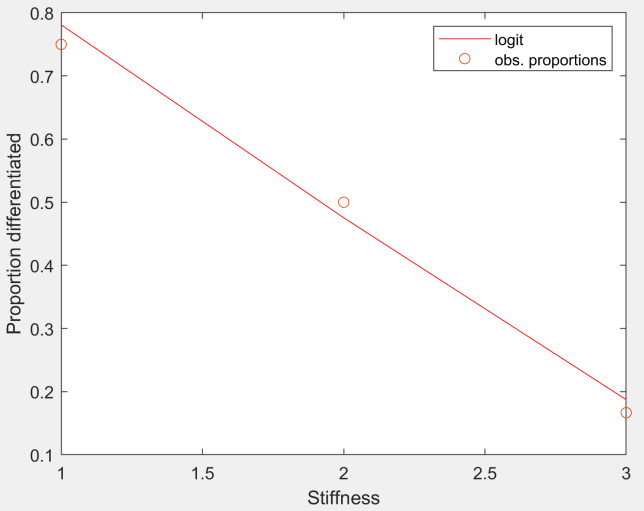


Figure 7: Graph of Logistic Regression Model (logit) Compared to Observed Values Extracted from Stiffness Contingency Table.

MATLAB generated p-values of 0.0847 and 0.1218 for the two coefficients incorporated into the logistic regression model. Neither of the p values are significant with an alpha of 0.05, therefore we fail to reject the null hypothesis.

These results from the χ^2-test, G-test and logistic regression were unexpected because all literature incorporated into our contingency table significantly showed that stiffness influences neural differentiation [26–31]. As mentioned in Chapters 1, 3 and 4, the expected value in each category must be greater than 5 in order for the statistical tests to accurately reflect the data.

After running our PCA script, the Pareto of Effects bar graph identified 3 unique clusters of genes shown in Figure 8. Cluster 1 makes up roughly 80% of the variance in the dataset, while cluster 2 and 3 consist of 20% of the variance.

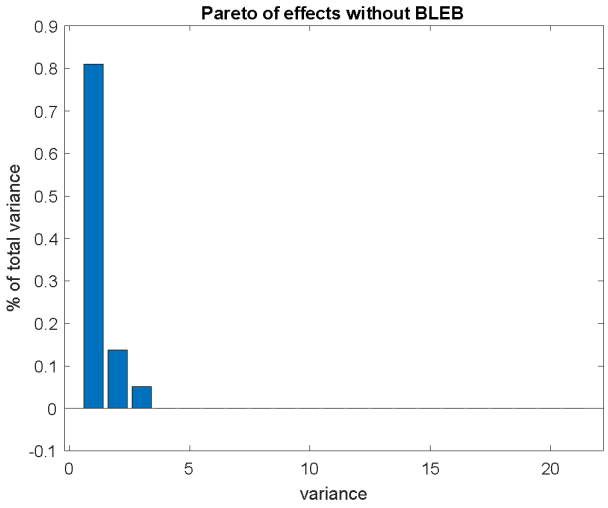


Figure 8: Pareto of Effect Representing Unique Clusters in PCA Data Set from Engler *et al.* [30]

The generated biplot identifies which genes belong to the three significant clusters as well as their importance shown in Figure 9. N-Cadherin is the most distinctly unique gene with the largest cosine of the angle between the gene and x-axis. N-Cadherin is commonly known as an adhesive protein, but it is also used by cells to promote neural differentiation and stabilize neural identity by dampening anti-neural signals [52]. The middle cluster contains 13 genes, indicating these are relatively similar and only one from this cluster needs to be investigated in future research. The bottom cluster contains 3 genes, Neural Cell Adhesion Molecule (CAM) 1 being the most significant due to the largest cosine angle value. Neural CAM 1 influences neuronal migration, axonal branching and synaptogenesis [53]. The red dots represent outliers in the data set identified from the PCA. Engler *et al.*'s dataset did not have any replicates, therefore no formal outlier analysis was conducted. In future experiments, N-Cadherin, Neural CAM 1 and one gene of choice from the middle cluster should be focused on.

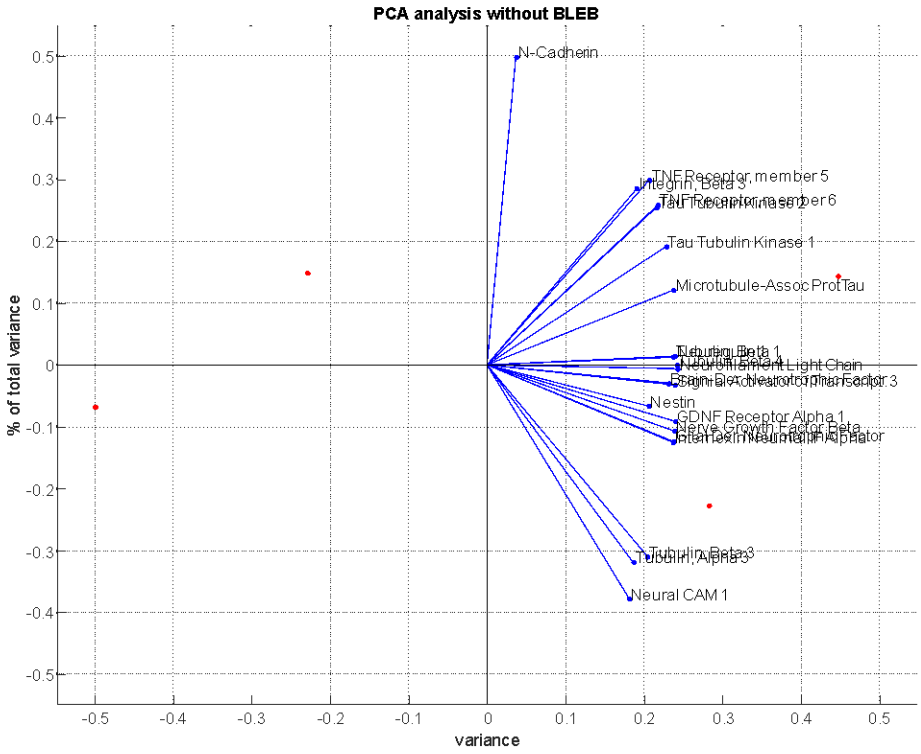


Figure 9: PCA Biplot Indicating Important Genes

CHAPTER 6: Engineering Standards

6.1 Ethical Justification

As engineers, we understand that it is vital to consider the ethical implications of our work. We have three main ethical justifications for our project. First, by consolidating and analyzing a wide variety of sources on the impact of substrate stiffness, matrix dimensions and neurotoxins on neural differentiation, we hope to help others reduce the number of experiments that they need to complete on neural cells. This will allow more researchers to complete research on neurons, as they will not be as prohibited by cost, which in turn will produce more data on developmental toxicity. As more research is done on this topic, we will be able to better understand the impact of these toxins on the brain, and reduce exposure to such toxins in commercial products. Further, reducing the number of experiments will reduce the number of stem cells that need to be used for research purposes.

Second, our project will allow researchers to begin to shift away from animal models. This shift allows researchers to better understand the workings of the human brain, as animal models do not mimic human brain development well [54]. This will further improve the quality of research being done on brain development and developmental neurotoxicity. Further, moving away from animal models removes the ethical quandaries regarding animal research. While there are ethical justifications for using animal models, such as the benefits to human health, moving away from these models will open up these benefits to more people who may feel repulsed by benefiting from animal research [55].

Third, we believe that the choice of project itself, studying developmental neurotoxicity, is ethically justified. Developmental neurotoxins can have large impacts on brain development, resulting in various neurological disorders later in life [56]. As a result, we believe that the time and energy invested in this project will have benefits for many people because it will help open the doors for more research and understanding on developmental neurotoxicity. Further, our findings on the impact substrate stiffness and dimensions on neural differentiation can aid in other neurological research, not only developmental toxicity, broadening the applicability of our research.

6.2 Environmental and Sustainability Implications

As mentioned in the Ethics section, our project will ideally be used by other researchers to reduce the number of experiments and animal models needed to be completed for research on developmental neurotoxicity. By reducing the number of experiments needed, we will reduce the environmental impact of researching developmental neurotoxicity. Further, animal research has a large environmental impact, so reducing the need for animal models will make developmental neurotoxicity research more sustainable [57].

6.3 Economic Considerations

By reducing the need for animal models and the number of experiments, it will make developmental toxicity research more economically feasible. Reducing the number of animal models will reduce the cost of developmental toxicity research, as animal research comes with many costs, such as creation of facilities to house and care for the animals [58]. Reducing the number of experiments will decrease the cost of completing research as well, as people will have

to purchase fewer materials and spend less time. As a result, our project will have positive economic impacts.

6.4 Health and Safety Implications

As discussed in section 1.1.1, using two-dimensional cells cultured during drug development has shown to be ineffective due to culturing conditions that are not similar to the in vivo environment, affecting proliferation and differentiation. Our project represents a more relevant way to study developmental toxicity than currently available methods by using three-dimensional cell culture. Which in turn will further improve safety of pharmaceutical drugs and other biotechnology products, as researchers will be able to better and more relevantly evaluate if materials or compounds are developmental toxins.

6.5 Social and Political Considerations

Federal government is the primary source of research and development funding; the NIH alone invests 41.7 billion dollars each year into medical research, much of which is related to drug development [59]. If proved effective, our model could serve as a pre-screening tool for drug development. This would greatly reduce the number of experiments required to bring a product or material to market and therefore reduce the cost of research and development to taxpayers. Additionally, as many developmental toxicology studies make use of stem cells, some cell lines have ethical sourcing complications. Our project has additional social considerations as it reduces the use of these types of cells during experimentation.

CHAPTER 7: Summary and Conclusions

7.1 Summary of the Project

Our senior design project had three main stages. The first stage was literature review. In this stage, we researched the current available methods to study developmental toxicity as well as the different microenvironmental factors that impact neuronal differentiation. From this initial search, we narrowed down our pool of microenvironmental inputs to three main inputs: matrix stiffness, matrix dimensions, and toxin addition. The next stage of our project was data collection. We extracted data from over fifty papers on the impact of various factors on stem cell differentiation. We then combined the data from papers with comparable experimental methods into contingency tables. After data collection, we moved on to the final stage of our project: data analysis. We completed χ^2- and G-tests to determine significance of the microenvironment inputs. We also completed logistic regression to determine the correlation between stiffness and differentiation. Further, we used PCA to reduce the number of variables, thus simplifying further experimentation on neuronal differentiation. However, the only microenvironment input that was significant according to our tests was matrix dimensions. This result was surprising because our literature review indicated that both stiffness and toxin addition have an impact on stem cell differentiation into neurons [26–31]. We believe that this is due to our small sample sizes. As a result, we would like to do further research into this field.

7.2 Systems Integration and Future Work

In order to integrate the aforementioned subsystems, future work will need to be done to increase the sample size per category in the matrix dimension, toxin, and stiffness contingency tables. For variables that have a statistically significant influence on neural differentiation, these will be incorporated into our final predictive models. The goal of our project is to create two models as diagrammed in Figure 10.

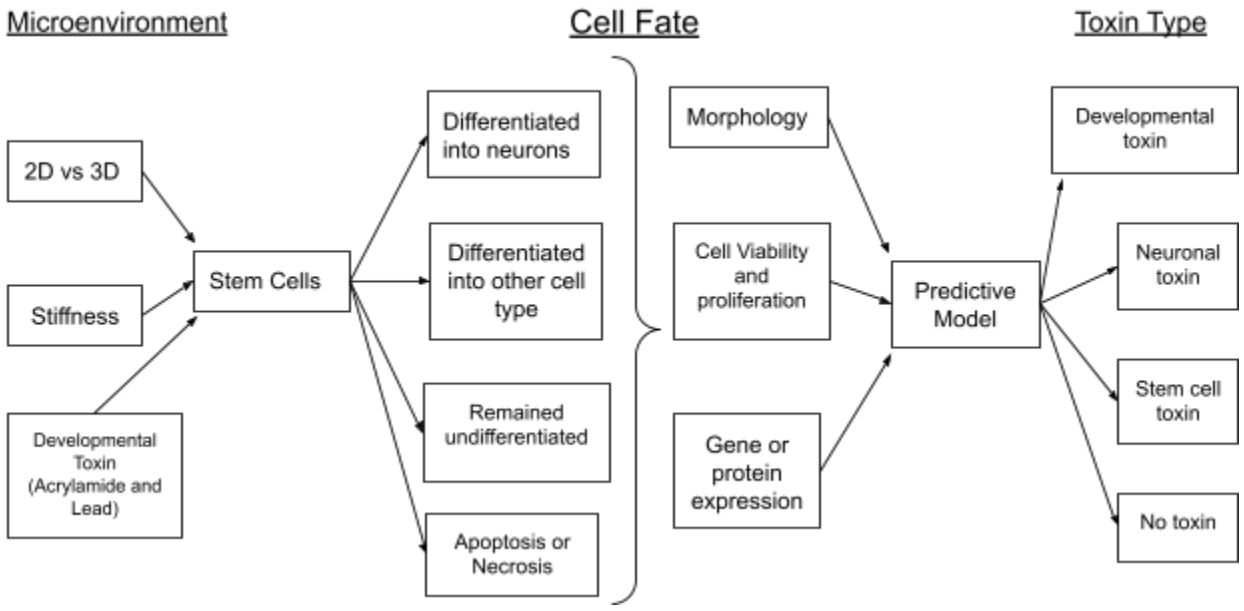


Figure 10: Diagram of Two Predictive Models to Predict Cell Fate and Toxin Type

In the first model, researchers will input data on selected microenvironmental cues of which stem cells will be subjected to. Then, the model will predict cell fate such as differentiation into neurons or other cell types, no occurrence of differentiation or occurrence of apoptosis or necrosis. In the second model, data regarding the cell fate such as morphology, cell viability, proliferation and gene or protein expression will be inputed. This data can take the form of neurite length, alive/dead cell counts or important biomarkers derived from literature or our PCA analysis such as beta III tubulin, N-Cadherin or Neural CAM 1. Once the data is input into the model, it should predict the toxin type added to the system which can include developmental toxins, neural toxins, stem cell toxins or no toxins. These two models can be used individually or as a system to predict the influence of a chemical on neural differentiation, each providing important information on cell fate and toxin type respectively.

In order to complete the predictive models, there is more work to be done in the future. First, we would generate more data in the lab regarding the impact of stiffness and toxin addition on stem cell differentiation to fill in the holes in our data collection from literature. Additionally, we would like to collect data on other variables such as diffusion and adhesion sites on stem cell differentiation from literature. Then, we would continue to fill in gaps in the contingency tables by generating data from the lab. Second, we would begin to develop the predictive models using statistical analysis of the lab generated data and the data from papers. The goal is to have two models: one where researchers input microenvironmental cues and the model predicts the stem cell differentiation, and another where researchers input cell characteristics and the model outputs toxin type. Finally, we would validate the models in the lab to ensure that they accurately predict stem cell differentiation and toxin type.

7.3 Lessons Learned

Our team learned a number of invaluable lessons from this project. First, we increased our proficiency in literature research. We learned how to use various search techniques and data bases to get a wide variety of sources that apply to our research. Second, we improved our data analysis techniques. We were able to practice χ^2-tests and logistic regression, which we had learned in prior courses. Further, we learned new techniques such as G-tests and PCA, which we will be able to use for future research projects. Finally, throughout this process, we improve our teamwork and communication skills, figuring out how to allocate work well. All of these skills will be indispensable as we all move forward in our careers.

CHAPTER 8: Citations

1. US EPA, O. Evaluating Developmental Neurotoxicity Hazard: Better than Before Available online: https://www.epa.gov/sciencematters/evaluating-developmental-neurotoxicity-hazard-better (accessed on 3 June 2021).
2. Song, C.; Kanthasamy, A.; Kanthasamy, A. Chapter 63 - Cell signaling mechanisms in developmental neurotoxicity. In *Reproductive and Developmental Toxicology*; Gupta, R.C., Ed.; Academic Press: San Diego, 2011; pp. 835–845 ISBN 978-0-12-382032-7.
3. Hayess, K.; Riebeling, C.; Pirow, R.; Steinfath, M.; Sittner, D.; Slawik, B.; Luch, A.; Seiler, A.E.M. The DNT-EST: A Predictive Embryonic Stem Cell-Based Assay for Developmental Neurotoxicity Testing in Vitro. *Toxicology* **2013**, *314*, 135–147, doi:10.1016/j.tox.2013.09.012.
4. Rice D; Barone S Critical Periods of Vulnerability for the Developing Nervous System: Evidence from Humans and Animal Models. *Environ. Health Perspect.* **2000**, *108*, 511–533, doi:10.1289/ehp.00108s3511.
5. IPSC Disease Modeling - Toxicology Testing Available online: https://www.appliedstemcell.com/research/services/neurotoxicity-testing/ipsc-derived-cells-compound-screening-assay-service-1 (accessed on 1 April 2021).
6. Mansel, C.; Fross, S.; Rose, J.; Dema, E.; Mann, A.; Hart, H.; Klawinski, P.; Vohra, B.P.S. Lead Exposure Reduces Survival, Neuronal Determination, and Differentiation of P19 Stem Cells. *Neurotoxicol. Teratol.* **2019**, *72*, 58–70, doi:10.1016/j.ntt.2019.01.005.
7. Sisnaiske, J.; Hausherr, V.; Krug, A.K.; Zimmer, B.; Hengstler, J.G.; Leist, M.; van Thriel, C. Acrylamide Alters Neurotransmitter Induced Calcium Responses in Murine ESC-Derived and Primary Neurons. *NeuroToxicology* **2014**, *43*, 117–126, doi:10.1016/j.neuro.2014.03.010.
8. Bu, Q.; Huang, Y.; Li, M.; Dai, Y.; Fang, X.; Chen, K.; Liu, Q.; Xue, A.; Zhong, K.; Huang, Y.; et al. Acrylamide Exposure Represses Neuronal Differentiation, Induces Cell Apoptosis and Promotes Tau Hyperphosphorylation in HESC-Derived 3D Cerebral Organoids. *Food Chem. Toxicol.* **2020**, *144*, 111643, doi:10.1016/j.fct.2020.111643.
9. Kang, S.; Chen, X.; Gong, S.; Yu, P.; Yau, S.; Su, Z.; Zhou, L.; Yu, J.; Pan, G.; Shi, L. Characteristic Analyses of a Neural Differentiation Model from IPSC-Derived Neuron According to Morphology, Physiology, and Global Gene Expression Pattern. *Sci. Rep.* **2017**, *7*, 12233, doi:10.1038/s41598-017-12452-x.
10. Jitcy Saji Joseph, Sibusiso Tebogo Malindisa and Monde Ntwasa *Two-Dimensional (2D) and Three-Dimensional (3D) Cell Culturing in Drug Discovery*; ntechOpen, 2018;
11. Saydé, T.; El Hamoui, O.; Alies, B.; Gaudin, K.; Lespes, G.; Battu, S. Biomaterials for Three-Dimensional Cell Culture: From Applications in Oncology to Nanotechnology. *Nanomaterials* **2021**, *11*, doi:10.3390/nano11020481.
12. Edmondson, R.; Broglie, J.J.; Adcock, A.F.; Yang, L. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. *Assay Drug Dev. Technol.* **2014**, *12*, 207–218, doi:10.1089/adt.2014.573.
13. MTT Assay - an Overview | ScienceDirect Topics Available online: https://www.sciencedirect.com/topics/medicine-and-dentistry/mtt-assay (accessed on 1 April 2021).
14. Strober, W. Trypan Blue Exclusion Test of Cell Viability. *Curr. Protoc. Immunol.* **2015**, *111*, A3.B.1-A3.B.3, doi:10.1002/0471142735.ima03bs111.

15. Grienberger, C.; Konnerth, A. Imaging Calcium in Neurons. *Neuron* **2012**, *73*, 862–885, doi:10.1016/j.neuron.2012.02.011.
16. Covey, E.; Carter, M.; Covey, P.E.; Carter, P.M. *Basic Electrophysiological Methods*; Oxford University Press, Incorporated: Cary, UNITED STATES, 2015; ISBN 978-0-19-993986-2.
17. Kermani, S.; Karbalaie, K.; Madani, S.H.; Jahangirnejad, A.A.; Eslaminejad, M.B.; Nasr-Esfahani, M.H.; Baharvand, H. Effect of Lead on Proliferation and Neural Differentiation of Mouse Bone Marrow-Mesenchymal Stem Cells. *Toxicol. In Vitro* **2008**, *22*, 995–1001, doi:10.1016/j.tiv.2008.02.009.
18. Engstrom, A.; Wang, H.; Xia, Z. Lead Decreases Cell Survival, Proliferation, and Neuronal Differentiation of Primary Cultured Adult Neural Precursor Cells through Activation of the JNK and P38 MAP Kinases. *Toxicol. Vitro Int. J. Publ. Assoc. BIBRA* **2015**, *29*, 1146–1155, doi:10.1016/j.tiv.2015.05.001.
19. Crumpton, T.; Atkins, D.S.; Zawia, N.H.; Barone, S. Lead Exposure in Pheochromocytoma (PC12) Cells Alters Neural Differentiation and Sp1 DNA-Binding. *Neurotoxicology* **2001**, *22*, 49–62, doi:10.1016/s0161-813x(00)00008-5.
20. Kothapalli, C.R.; Kamm, R.D. 3D Matrix Microenvironment for Targeted Differentiation of Embryonic Stem Cells into Neural and Glial Lineages. *Biomaterials* **2013**, *34*, 5995–6007, doi:10.1016/j.biomaterials.2013.04.042.
21. Zare-Mehrjardi, N.; Khorasani, M.T.; Hemmesi, K.; Mirzadeh, H.; Azizi, H.; Sadatnia, B.; Hatami, M.; Kiani, S.; Barzin, J.; Baharvand, H. Differentiation of Embryonic Stem Cells into Neural Cells on 3D Poly (D, L-Lactic Acid) Scaffolds versus 2D Cultures. *Int. J. Artif. Organs* **2011**, *34*, 1012–1023, doi:10.5301/ijao.5000002.
22. Ortinau, S.; Schmich, J.; Block, S.; Liedmann, A.; Jonas, L.; Weiss, D.G.; Helm, C.A.; Rolfs, A.; Frech, M.J. Effect of 3D-Scaffold Formation on Differentiation and Survival in Human Neural Progenitor Cells. *Biomed. Eng. OnLine* **2010**, *9*, 70, doi:10.1186/1475-925X-9-70.
23. Brännvall, K.; Bergman, K.; Wallenquist, U.; Svahn, S.; Bowden, T.; Hilborn, J.; Forsberg-Nilsson, K. Enhanced Neuronal Differentiation in a Three-Dimensional Collagen-Hyaluronan Matrix. *J. Neurosci. Res.* **2007**, *85*, 2138–2146, doi:10.1002/jnr.21358.
24. Bozza, A.; Coates, E.E.; Incitti, T.; Ferlin, K.M.; Messina, A.; Menna, E.; Bozzi, Y.; Fisher, J.P.; Casarosa, S. Neural Differentiation of Pluripotent Cells in 3D Alginate-Based Cultures. *Biomaterials* **2014**, *35*, 4636–4645, doi:10.1016/j.biomaterials.2014.02.039.
25. Huang, F.; Shen, Q.; Zhao, J. Growth and Differentiation of Neural Stem Cells in a Three-Dimensional Collagen Gel Scaffold. *Neural Regen. Res.* **2013**, *8*, 313–319, doi:10.3969/j.issn.1673-5374.2013.04.003.
26. Leipzig, N.D.; Shoichet, M.S. The Effect of Substrate Stiffness on Adult Neural Stem Cell Behavior. *Biomaterials* **2009**, *30*, 6867–6878, doi:10.1016/j.biomaterials.2009.09.002.
27. Banerjee, A.; Arha, M.; Choudhary, S.; Ashton, R.S.; Bhatia, S.R.; Schaffer, D.V.; Kane, R.S. The Influence of Hydrogel Modulus on the Proliferation and Differentiation of Encapsulated Neural Stem Cells. *Biomaterials* **2009**, *30*, 4695–4699, doi:10.1016/j.biomaterials.2009.05.050.
28. Rammensee, S.; Kang, M.S.; Georgiou, K.; Kumar, S.; Schaffer, D.V. Dynamics of Mechanosensitive Neural Stem Cell Differentiation. *Stem Cells Dayt. Ohio* **2017**, *35*, 497–506, doi:10.1002/stem.2489.
29. Ali, S.; Wall, I.B.; Mason, C.; Pelling, A.E.; Veraitch, F.S. The Effect of Young's Modulus on the Neuronal Differentiation of Mouse Embryonic Stem Cells. *Acta Biomater.* **2015**, *25*, 253–267, doi:10.1016/j.actbio.2015.07.008.

30. Engler, A.J.; Sen, S.; Sweeney, H.L.; Discher, D.E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **2006**, *126*, 677–689, doi:10.1016/j.cell.2006.06.044.
31. Her, G.J.; Wu, H.-C.; Chen, M.-H.; Chen, M.-Y.; Chang, S.-C.; Wang, T.-W. Control of Three-Dimensional Substrate Stiffness to Manipulate Mesenchymal Stem Cell Fate toward Neuronal or Glial Lineages. *Acta Biomater.* **2013**, *9*, 5170–5180, doi:10.1016/j.actbio.2012.10.012.
32. Jiang, X.-F.; Yang, K.; Yang, X.-Q.; Liu, Y.-F.; Cheng, Y.-C.; Chen, X.-Y.; Tu, Y. Elastic Modulus Affects the Growth and Differentiation of Neural Stem Cells. *Neural Regen. Res.* **2015**, *10*, 1523–1527, doi:10.4103/1673-5374.165527.
33. Stukel, J.M.; Willits, R.K. The Interplay of Peptide Affinity and Scaffold Stiffness on Neuronal Differentiation of Neural Stem Cells. *Biomed. Mater. Bristol Engl.* **2018**, *13*, 024102, doi:10.1088/1748-605X/aa9a4b.
34. Flanagan, L.A.; Ju, Y.-E.; Marg, B.; Osterfield, M.; Janmey, P.A. Neurite Branching on Deformable Substrates. *Neuroreport* **2002**, *13*, 2411–2415, doi:10.1097/01.wnr.0000048003.96487.97.
35. Parmar, M.K.; Torri, V.; Stewart, L. Extracting Summary Statistics to Perform Meta-Analyses of the Published Literature for Survival Endpoints. *Stat. Med.* **1998**, *17*, 2815–2834, doi:10.1002/(sici)1097-0258(19981230)17:24<2815::aid-sim110>3.0.co;2-8.
36. Dauga, D. Biocuration: A New Challenge for the Tunicate Community. *genesis* **2015**, *53*, 132–142, doi:https://doi.org/10.1002/dvg.22842.
37. Dretzke, J.; Edlin, R.; Round, J.; Connock, M.; Hulme, C.; Czeczot, J.; Fry-Smith, A.; McCabe, C.; Meads, C. *Extraction of Data from Published Graphs*; NIHR Journals Library, 2011;
38. Katsetos, C.D.; Del Valle, L.; Geddes, J.F.; Aldape, K.; Boyd, J.C.; Legido, A.; Khalili, K.; Perentes, E.; Mörk, S.J. Localization of the Neuronal Class III β-Tubulin in Oligodendrogliomas: Comparison with Ki-67 Proliferative Index and 1p/19q Status. *J. Neuropathol. Exp. Neurol.* **2002**, *61*, 307–320, doi:10.1093/jnen/61.4.307.
39. Gurok, U.; Steinhoff, C.; Lipkowitz, B.; Ropers, H.-H.; Scharff, C.; Nuber, U.A. Gene Expression Changes in the Course of Neural Progenitor Cell Differentiation. *J. Neurosci. Off. J. Soc. Neurosci.* **2004**, *24*, 5982–6002, doi:10.1523/JNEUROSCI.0809-04.2004.
40. Using Chi-Square Statistic in Research - Statistics Solutions Available online: https://www.statisticssolutions.com/using-chi-square-statistic-in-research/ (accessed on 19 May 2021).
41. Tasneem, S.; Farrell, K.; Lee, M.-Y.; Kothapalli, C.R. Sensitivity of Neural Stem Cell Survival, Differentiation and Neurite Outgrowth within 3D Hydrogels to Environmental Heavy Metals. *Toxicol. Lett.* **2016**, *242*, 9–22, doi:10.1016/j.toxlet.2015.11.021.
42. Lin, L.F.; Xie, J.; Sánchez, O.F.; Bryan, C.; Freeman, J.L.; Yuan, C. Low Dose Lead Exposure Induces Alterations on Heterochromatin Hallmarks Persisting through SH-SY5Y Cell Differentiation. *Chemosphere* **2021**, *264*, 128486, doi:10.1016/j.chemosphere.2020.128486.
43. Aloe, L.; Rocco, M.; Balzamino, B.; Micera, A. Nerve Growth Factor: A Focus on Neuroscience and Therapy. *Curr. Neuropharmacol.* **2015**, *13*, 294–303, doi:10.2174/1570159X13666150403231920.
44. Woolf, B. THE LOG LIKELIHOOD RATIO TEST (THE G-TEST). *Ann. Hum. Genet.* **1957**, *21*, 397–409, doi:10.1111/j.1469-1809.1972.tb00293.x.
45. Bruen, T.C.; Philippe, H.; Bryant, D. A Simple and Robust Statistical Test for Detecting the

Presence of Recombination. *Genetics* **2006**, *172*, 2665–2681, doi:10.1534/genetics.105.048975.
46. Pek, Y.S.; Wan, A.C.A.; Ying, J.Y. The Effect of Matrix Stiffness on Mesenchymal Stem Cell Differentiation in a 3D Thixotropic Gel. *Biomaterials* **2010**, *31*, 385–391, doi:10.1016/j.biomaterials.2009.09.057.
47. Leon, A.C. Descriptive and Inferential Statistics. In *Comprehensive Clinical Psychology*; Bellack, A.S., Hersen, M., Eds.; Pergamon: Oxford, 1998; pp. 243–285 ISBN 978-0-08-042707-2.
48. Mobed-Miremadi, M. Transition From Continuous Probability to Categorical Tests and Logistic Regression 2021.
49. Jolliffe, I.T.; Cadima, J. Principal Component Analysis: A Review and Recent Developments. *Philos. Transact. A Math. Phys. Eng. Sci.* **2016**, *374*, doi:10.1098/rsta.2015.0202.
50. Sadeghi, F. Dimensionality Reduction.
51. Pareto Chart of Standardized Effects Available online: https://support.minitab.com/en-us/minitab/20/help-and-how-to/statistical-modeling/regression/how-to/fit-regression-model/interpret-the-results/all-statistics-and-graphs/pareto-chart/ (accessed on 19 May 2021).
52. Punovuori, K.; Migueles, R.P.; Malaguti, M.; Blin, G.; Macleod, K.G.; Carragher, N.O.; Pieters, T.; van Roy, F.; Stemmler, M.P.; Lowell, S. N-Cadherin Stabilises Neural Identity by Dampening Anti-Neural Signals. *Development* **2019**, *146*, doi:10.1242/dev.183269.
53. Vukojevic, V.; Mastrandreas, P.; Arnold, A.; Peter, F.; Kolassa, I.-T.; Wilker, S.; Elbert, T.; de Quervain, D.J.-F.; Papassotiropoulos, A.; Stetak, A. Evolutionary Conserved Role of Neural Cell Adhesion Molecule-1 in Memory. *Transl. Psychiatry* **2020**, *10*, 1–13, doi:10.1038/s41398-020-00899-y.
54. Zhao, X.; Bhattacharyya, A. Human Models Are Needed for Studying Human Neurodevelopmental Disorders. *Am. J. Hum. Genet.* **2018**, *103*, 829–857, doi:10.1016/j.ajhg.2018.10.009.
55. Bennett, A.J.; Ringach, D.L. Animal Research in Neuroscience: A Duty to Engage. *Neuron* **2016**, *92*, 653–657, doi:10.1016/j.neuron.2016.10.034.
56. Giordano, G.; Costa, L.G. Developmental Neurotoxicity: Some Old and New Issues. *ISRN Toxicol.* **2012**, *2012*, e814795, doi:10.5402/2012/814795.
57. Groff, K.; Bachli, E.; Lansdowne, M.; Capaldo, T. Review of Evidence of Environmental Impacts of Animal Research and Testing. *Environments* **2014**, *1*, 14–30, doi:10.3390/environments1010014.
58. Powell, K. Replacing the Replacements: Animal Model Alternatives Available online: https://www.sciencemag.org/features/2018/10/replacing-replacements-animal-model-alternatives (accessed on 4 April 2021).
59. National Institute of Health NIH Budget. **2020**.

APPENDIX

Appendix A: Observed and Expected Tables for χ^2-test and G-Test

A.1 χ^2-Test Observed and Expected Tables for 2D versus 3D

Table 7: Observed Values for 2D versus 3D Contingency Table

Observed	2D	3D	Total
Differentiated (>2 fold increase)	0	21	21
Undifferentiated (<2 fold increase)	10	7	17
Total	10	28	38

Table 8: Expected Values for 2D versus 3D Contingency Table

Expected	2D	3D	Total
Differentiated (>2 fold increase)	5.5	15.5	21.0
Undifferentiated (<2 fold increase)	4.5	12.5	17.0
Total	10.0	28.0	38.0

Table 9: χ^2-Test Values for 2D versus 3D

χ^2-Test	2D	3D
Differentiated (>2 fold increase)	5.53	1.97
Undifferentiated (<2 fold increase)	6.83	2.44
	χ^2	16.76
	p	4.23E-05

A.2 χ^2 and G-Test Observed and Expected Tables for Toxin

Table 10: Observed Values for Toxin Contingency Table

Observed	0-0.09uM	0.1-2uM	Total
Undifferentiated (<1 Fold)	12	5	17
Differentiated (>1 Fold)	4	1	5
Total	16	6	22

Table 11: Expected Values for Toxin Contingency Table

Expected	0-0.09uM	0.1-2uM	Total
Undifferentiated (<1 Fold)	12.36	4.64	17
Differentiated (>1 Fold)	3.64	1.36	5
Total	16	6	22

Table 12: χ^2-Test Values for Toxin

χ^2-Test	0-0.09uM	0.1-2uM
Undifferentiated (<1 Fold)	0.01	0.03
Differentiated (>1 Fold)	0.04	0.10
	χ^2	0.17
	p	0.6801

Table 13: G-Test Values for Toxin

G-Test	0-0.09uM	0.1-2uM
Undifferentiated (<1 Fold)	-0.36	0.38
Differentiated (>1 Fold)	0.38	-0.31
	G	0.18
	p	0.6714

A.3 χ^2 and G-Test Observed and Expected Tables for Stiffness

Table 14: Observed Values for Stiffness Contingency Table

Observed	Low (<1kPa)	Medium (1-10kPa)	High (>10kPa)	Total
Differentiated (>2 fold increase)	3	5	1	9
Undifferentiated (<2 fold increase)	1	5	5	11
Total	4	10	6	20

Table 15: Expected Values for Stiffness Contingency Table

Expected	Low (<1kPa)	Medium (1-10kPa)	High (>10kPa)	Total
Differentiated (>2 fold increase)	1.8	4.5	2.7	9
Undifferentiated (<2 fold increase)	2.2	5.5	3.3	11
Total	4	10	6	20

Table 16: χ^2-Test Values for Stiffness

χ^2-Test	Low (<1kPa)	Medium (1-10kPa)	High (>10kPa)
Differentiated (>2 fold increase)	0.80	0.06	1.07
Undifferentiated (<2 fold increase)	0.65	0.05	0.88
		χ^2	3.50
		p	0.1736

Table 17: G-Test Values for Stiffness

G-Test	Low (<1kPa)	Medium (1-10kPa)	High (>10kPa)
Differentiated (>2 fold increase)	1.53	0.53	-0.99
Undifferentiated (<2 fold increase)	-0.79	-0.48	2.08
		G	3.76
		p	0.15

Appendix B: Principal Component Analysis Dataset

Table 18: Expression of Neural Lineage Markers due to Various Culture Stiffnesses [30]

Gene Description	Symbol	Lineage Marker	0.1 kPa MSC	1 kPa MSC	11 kPa MSC	34 kPa MSC	1 kPa MSC	11 kPa MSC	34 kPa MSC
Inhibitors Added							**Bleb**	**Bleb**	**Bleb**
(Normalization)			**Actin, MSC**	**Actin, MSC**	**Actin, MSC**	**Actin, MSC**	**Actin, MSC**	**Actin, MSC**	**Actin, MSC**
Microtubule-Assoc Prot. Tau	MAPT	N	5.56	7.63	3.70	1.51	1.45	0.88	1.02
Tau Tubulin Kinase 1	TTBK1	N	5.23	7.28	4.40	1.89	1.62	1.38	1.09
Tau Tubulin Kinase 2	TTBK2	N	3.56	4.89	3.50	1.74	1.29	0.97	0.93
Tubulin, Alpha 3	TUBA3	N	1.97	1.25	0.94	0.64	1.11	0.55	0.46
Tubulin, Beta 1	TUBB1	N	8.41	8.88	5.13	1.50	3.63	1.85	1.26
Tubulin, Beta 3	TUBB3	N	5.70	3.73	1.49	1.17	1.62	1.02	0.86
Tubulin, Beta 4	TUBB4	N	8.55	9.67	3.98	0.79	3.42	1.46	0.94
Glial Der. Neurotrophic Fctr	GDNF	N	9.88	9.67	2.65	1.15	2.18	1.38	1.20
GDNF Receptor Alpha 1	GFRA1	N	5.14	5.29	2.01	1.16	1.45	1.15	1.02
N-Cadherin	CDH2	N	2.19	4.70	6.38	1.62	1.52	1.58	1.14
TNF Receptor Member 5	CD40	N	3.73	6.51	3.72	2.10	1.82	1.39	1.24
TNF Receptor, Member 6	FAS	N	4.41	6.62	4.21	2.07	2.38	1.71	1.01
Brain-Der. Neurotrophic Fctr	BDNF	N	4.27	5.72	1.14	1.35	2.52	1.09	1.18
Neurofilament Light Chain	NEFL	N	3.62	4.13	2.00	1.23	1.97	0.93	0.86
Internexin Neuronal IFα	INA	N	6.53	6.17	2.52	1.33	2.13	0.64	0.79
Nerve Growth Factor Beta	NGF	N	4.48	4.46	2.33	1.75	1.20	0.66	0.94
Neuregulin 1	NRG1	N	4.38	4.67	2.97	1.58	2.12	1.15	0.95
Signal Activator of Transcrpt. 3	STAT3	N	4.57	5.41	2.07	1.64	1.56	0.73	0.98
Nestin	NES	N	3.30	4.25	1.38	2.22	1.27	0.44	0.96
Neural CAM 1	NCAM1	N	7.57	4.55	2.58	2.45	2.33	1.58	1.73
Integrin, Beta 3	ITGB3	N	0.59	0.72	0.58	0.56	0.78	0.59	0.55

Appendix C: Proposed Budget and Finalized Budget

C.1 Proposed Budget

Table 19: Hydrogel Materials

Material	Source	Quantity	Cost
Alginate	Abcam	1 kit (good for 100 tests)	$505
Crosslinker - Collagen	Sigma Aldrich	30 mg	$241
Acrylamide	Sigma Aldrich	100 mL	$43

Table 20: Cell Culture and Differentiation Materials

Material	Source	Quantity	Cost
P19 Cells	Dr. Zhang's Lab		
Dissociation Reagent trypsin- EDTA	Thermofisher	100 mL	$15
α-MEM	Thermofisher	1 L	$110
T-75 Flasks	Thermofisher	100 flasks	$337
Fetal calf serum	Thermofisher	100 mL	$171
Newborn calf serum	Thermofisher	100 mL	$32
Dulbecco's PBS without calcium and magnesium	Thermofisher	1 L	$49
All trans-retinoic acid	Sigma Aldrich	100 mg	$47
		Total	$1550

C.2 Finalized Budget

Table 21: Finalized Budget and Materials Used for Senior Design 2021

Material	Source	Quantity	Cost
MATLAB	SCU Engineering Design Center	N/A	$0
Journal Databases	SCU Library	N/A	$0
Google Suite	SCU	N/A	$0
		Total	$0

Appendix D: Project Schedule

Task	Prior	Oct. 7	Oct. 21	Nov. 4	Nov. 18	Dec. 2	Dec. 16	Dec. 30	Jan. 13	Jan. 27	Feb. 10	Feb. 27	Mar. 3	Mar. 17	Mar. 31	Apr. 14	Apr. 28	May 12	May 26	Jun 9
Preliminary Literature Review																				
Materials Research																				
Protocol Development																				
Data Collection from Literature																				
Contingency Tables																				
Significance Tests																				
Thesis Writing																				
PCA Analysis																				
Senior Design Presentation																				

Figure 11: Gantt Chart of Senior Design Project Progress