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Response of stretch-injured schwann cells

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ABSTRACT

RESPONSE OF STRETCH-INJURED SCHWANN CELLS

By
Stephanie Iring

Axon fibers are covered by myelin sheath. After axonal damage, demyelination follows with the production of debris. In the Central Nervous System many studies have been performed to observe and analyze stretch injured axons, but very little has been done to study the white matter axonal tracts, oligodendrocytes. Schwann cells can help take a first look into stretch injured glia cells from the Peripheral nervous system. In order to observe changes in Schwann Cells a stretch injury device is used to produce the effects of severe and moderate injuries. Schwann Cells are stretch injured in both their undifferentiated and differentiated stages. In characterizing both states of Schwann Cells we observe different outcomes for the influx values in the presence of a calcium-containing buffer and non-calcium containing buffer. In undifferentiated Schwann cells we observe a clear transient influx while in differentiated we see minimal response. To observe morphological changes in myelinated cells after injury we induce Schwann cell differentiation. In differentiated Schwann cells images are taken and analyzed as follows: preinjury, 1 hour after injury, 4 hours after injury, and 24 hours.

RESPONSE OF STRETCH-INJURED SCHWANN CELLS

**by
Stephanie Iring**

**A Thesis
Submitted to the Faculty of
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STRETCH RESPONSE OF STRETCH-INJURED SCHWANN CELLS

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To my husband, Justin Sanchez and parents. Thank you for your support and encouragement.

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CHAPTER 1

INTRODUCTION

1.1 Traumatic Brain Injury

Traumatic brain injury (TBI) is an injury that does not heal like other injuries. Recovery after a brain injury is functionally based on mechanisms that remain indeterminate (Traumatic Brain Injury, 2004-2006). The consequence of each type of brain injury differs according to severity and specific damage inflicted on the brain. Research shows that about half of severely injured patients need surgery to repair, or completely remove hematomas. Injuries may also result in disabilities and are dependent on the following: severity, location, age, and the overall health of the individual. Disabilities that abrupt from a head injury include issues with cognition, sensory processing, communications, and behavioral or mental health (National Institute of Nuerological Disorders and Stroke, 2015). Statistics show that there are about 1.7 million people that suffer from traumatic brain injury annually. Of those 1.7 million: 52,000 die, 275,000 become hospitalized, and 1.365 million are treated and released from emergency rooms (Centers for Disease Control, 2015)

Traumatic brain injury has been divided into two major categories: focal trauma and diffuse trauma (Smith and Meany et al., 2000). Focal trauma occurs when injury is direct to an area in the head, which results in injuries such as hematomas. Conversely, diffuse trauma occurs when there is a rapid rotational change, which occurs over a more widespread area of the brain. Diffuse trauma can cause an acceleration/deceleration in the

brain creating inertial forces that can lead to shear deformation and injuries such as diffuse axonal injury (Thibault et al., 1992; Meaney et al., 1995; Smith et al., 1999).

1.2 Properties of the Brain

Research has proposed that the brain has viscoelastic properties (Smith & Meaney, et al., 2000). It is of great importance to look at the mechanical properties of viscoelastic materials when studying deformation. Viscoelastic materials exhibit both viscous and elastic properties when undergoing deformations (Palsson & Bhatia, et al., 2004; Callister, et al., 2007). Elastic materials deform immediately following the application of stress, but can also completely recover. However, viscoelastic materials have a time-dependent strain response, which can recover only over long periods of time. Thus, the study of strain rates are considered following deformations due to TBIs.

On a microscopic level shear deformation can be translated to uniaxial stretching, which is caused by forces between the brain (viscoelastic) and the skull (rigid). Diffuse axonal injury (DAI) is one of the most common TBIs, which falls under the category of diffuse trauma. One of the major causes of secondary brain injury is the inflammatory response after a TBI. There is very little information on DAI and inflammation, but it is currently a growing area of interest.

Uniaxial stretching and compression after rapid rotational head motions lead to DAI. In severe cases there is damage to the brain leading to disconnections between axons from the initial tearing of the injury. In less severe cases disconnections may occur over time, which is known as secondary axotomy (Maxwell, et al., 1993). Following an injury

axons undergo deformations, studies have purposed that the amount of elongation axons experience after injury depend on the amount of applied strain (Smith, et al., 1999).

It has been difficult making DAI diagnostics specifically in mild or moderate cases because they are better observed at cellular level and cannot always be identified through medical imaging. This makes microscopic observation advantageous because it allows the observation of pathological changes in the brain due to degeneration. The study of in-vitro culture models aid in making a better analysis of changes on a microscopic level.

1.3 Myelination and De-myelination

Wallerian degeneration is the degeneration of the axon distal to the site of transection. It occurs in both the PNS and CNS and begins 24-36 hours after a lesion. In the PNS, macrophages enter the transected area to eliminate myelin and axonal debris. Then within 96 hours Schwann cells synthesize growth factors (DeArmond, 2004) . In the CNS oligodendrocytes produce myelin sheaths. It has been proposed that myelin debris accumulates in the brain for a period of time after diffuse axonal injury (Liang Wen, et al., 2014).

Axon fibers are covered by myelin sheath produced by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) (network glia, 2011). In the CNS oligodendrocytes produce myelin sheath to produce insulating sheath of axons (BradI et al., 2009). In the PNS Schwann cells myelinate large-diameter axons and non-myelinating Schwann cells envelop and support sensory axons (Kim et al., 2013).

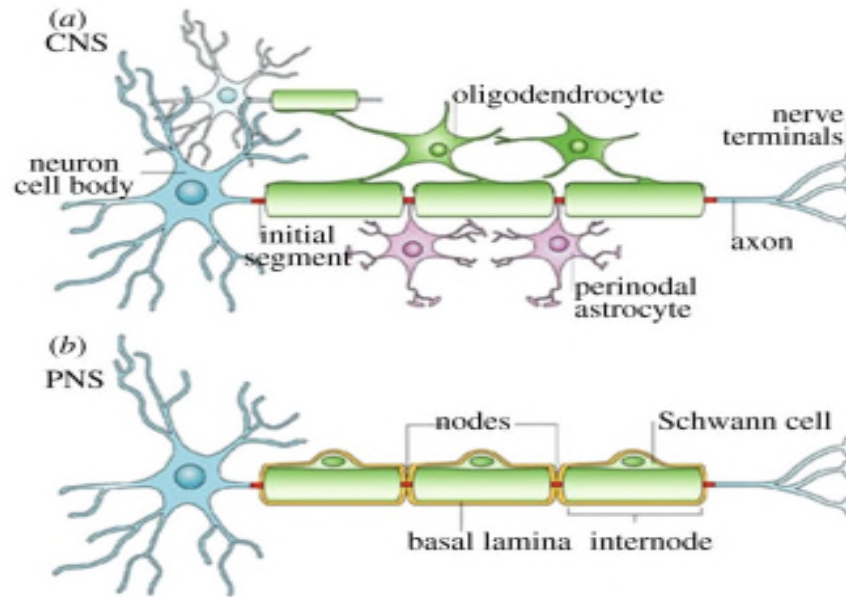


Figure 1.1 (a) CNS neuron myelinated by oligodendrocytes, which can myelinate several cells at once, (b) PNS neuron is myelinated by Schwann cells, which myelinate in a one-to-one ratio and are surrounded by basal lamina.

Source: Siddique, Rezina. "Investigation of Nerve Injury through Microfluidic Devices." *Interface*. The Royal Society Publishing, 2013.

Myelination is a complete cellular process in which glial cells wrap its membrane multiple times around the underlying axon resulting in myelin formation (Bhat, et al., 2009). Myelinated axons are commonly known as white matter in the brain and spinal cord. Myelin is made up of layers that shield the axon underneath, which allow signals to travel along segments called nodes of Ranvier.

When myelin sheath is damaged, nerves cannot conduct normal electrical signals to and from the brain. In unmyelinated neurons signals travel about one meter per second as opposed to myelinated neurons that travel at 100 meters per second. Following axonal damage, axons undergo a process of demyelination or myelin breakdown and have an increased production of myelin debris. When demyelination occurs it can cause deterioration of the axons.

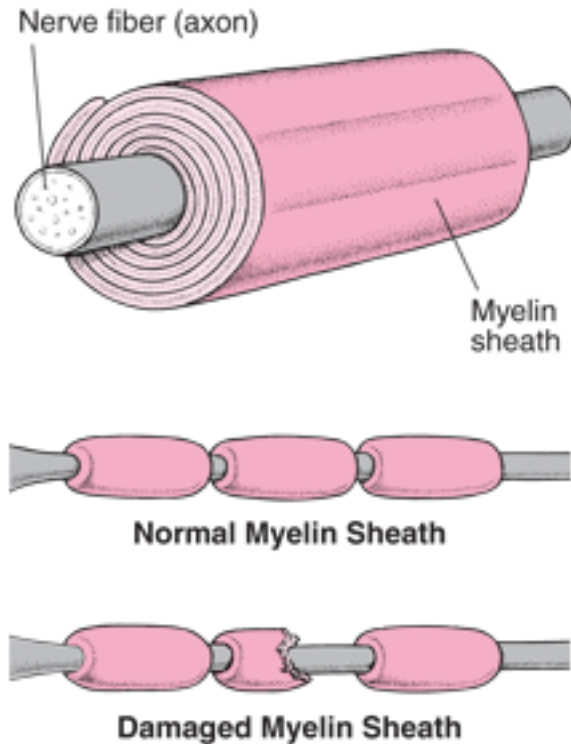


Figure 2.2 Insulated Nerve Fiber.

Source: "Overview of Demyelinating Disorders - Brain, Spinal Cord, and Nerve Disorders." *Merck Manuals Consumer Version*. N.p., n.d. 2015.

It is suggested that myelin sheaths are subject to the same proteolysis of myelin basic protein (MBP). So axonal injury leads to secondary damage to the adjacent membrane, causing MBP degradation. This sets off myelin instability and myelin breakdown, promoting axonal degradation (Liu et al, 2006). Thus, making myelin breakdown the outcome of different conditions following disease or injury to the nerve. In Guillan-Barre syndrome (GBS) symptoms appear within a few hours and it is due to inflammation of Peripheral Nerves. Multiple Sclerosis is another demyelinating disease, in which white matter in the brain and spinal cord undergo the formation of plaques or lesions. (Case-Lo et al., 2014).

1.4 Importance of Oligodendrocytes

Many studies have been performed to observe and analyze stretch injured axons, but very little has been done on the myelinating white matter axonal tracts from the CNS in-vitro. However, relevance of this white matter has become abruptly important because myelinating cells specifically oligodendrocytes ensheath axons to electrically stimulate structure and induce clustering sodium channels. Studies have even shown that axonal transport and neuronal viability depend on proper myelination. They also provide trophic support for neurons through the production of glia cells. It is important to study the effects traumatic brain injury has on myelin. When injury is directly inciting against oligodendrocytes, sharply demarcated plaques of demyelination are induced, causing the loss of myelin sheath. Oligodendrocyte cell bodies maybe preserved within active lesions (BradI et. al, 2010).

Oligodendrocytes undergo an accurately timed cycle of proliferation, migration, differentiation, and myelination to finally produce insulating sheaths (BradI et. al, 2010). Oligodendrocytes only have a few hours to form myelin sheath around central nervous system axons and are difficult to control in-vitro. However, Schwann cells are successfully controlled in-vitro and may be used as substitute model to study injury in myelinating glia cells.

1.5 Schwann Cells

Professors Kristjan R Jessen and Rhona Mirsky from University College London's Research Department of Cell and Developmental Biology highlight the importance of Schwann Cells. It is reiterated that the nervous system is partially made up of nerve cells, and that the majority of the cells are glial cells, which are biologically and functionally different than nerve cells. Schwann cells are the main glial cells of the PNS. (Mirsky et al., 2013)

Schwann cells are either myelinating (differentiated), or un-myelinating (undifferentiating). Non-myelinating aid in maintaining axons, while myelinating form myelin around axons. As mentioned earlier Schwann cells support cells in the PNS by wrapping nerve axons, but unlike oligodendrocytes they wrap themselves around a single segment of an axon's myelin sheath. They have the function of creating myelin sheaths on axons of the PNS, clean PNS debris, and guide in the regrowth of PNS axons. They aid in the regeneration of axons by arranging themselves in cylinders that serve as guides (Sofka et al., 2015). Their role in nerve pathology is an interesting focus, they promote repair and function. They are one of the very few regenerative cell types within our bodies.

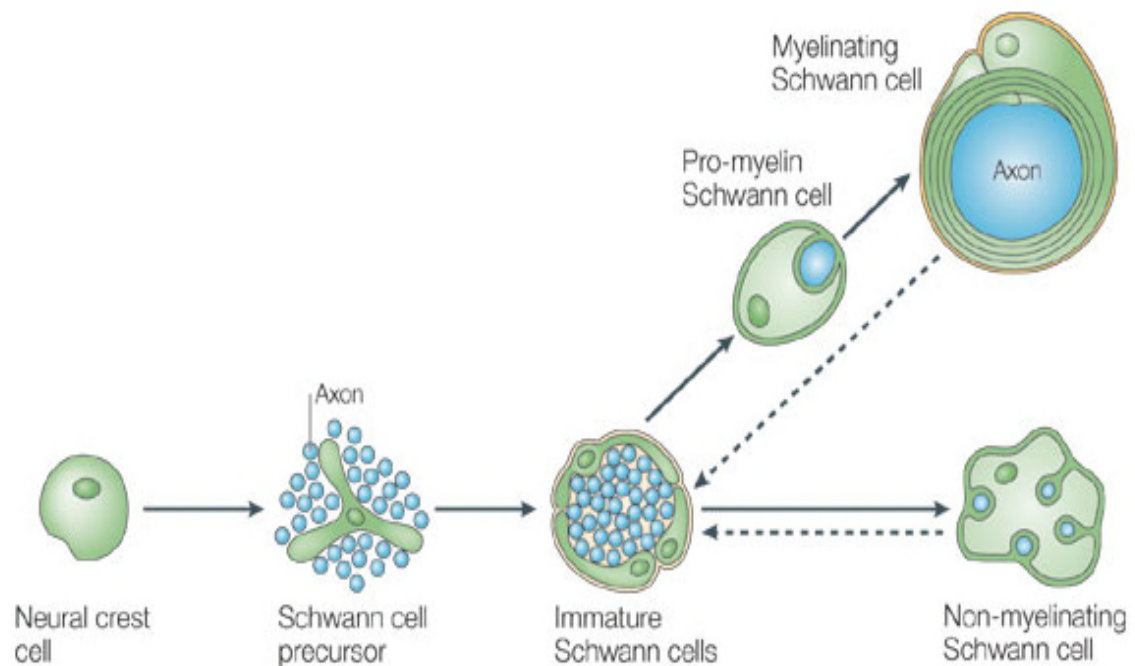


Figure 1.3 Shows an image of the pathway immature Schwann cells can take.

Source: Jessen, Kristjan. "The Origin and Development of Glial Cells in Peripheral Nerves." *Nature Reviews Neuroscience*, **6**, 671-682 September 2005.

Following an injury in the PNS, axons degenerate but a few weeks after, they begin to regenerate with myelin produced by Schwann cells. Professor Kristjan R Jessen and Rhona Mirsky are presently focusing on Schwann cell plasticity, which looks at the ability for Schwann cells to switch between differentiation states. They used the neural crest as seen in Figure 1.3 from a transient stem cell population which is found in the mid-term embryo. This then goes on to forming immature Schwann cells which then become myelinating or non-myelinating.

Repair within the PNS is highly dependent upon the ability for Schwann cells to dedifferentiate, exhibit direct axonal regrowth, and re-myelinate along with functional

recovery (Kim, et. al, 2013). Research has proposed that the ability for Schwann cells to switch between states is known as a transdifferentiation that generate Bugner cells which are dedifferentiated. These regenerative tracks, Bands of Bungner, guide nerve fibers so that function can be restored. The ability for Schwann cells to transdifferentiate make them important to study specifically in the way they respond to injury.

1.6 Calcium Response

Research has shown increased levels of intracellular calcium (Ca^{2+}) proceeding an injury in neurons. It has been suggested that the entry of calcium can cause secondary injuries, which include Wallerian degeneration and the activation of harmful proteases (Smith et al., 1999; Iwata et al., 2004; von Reyn et al., 2009). In a study done by Wolf, they used an in- vitro model of axonal stretch injury to analyze calcium entry after injury. A calcium sensitive dye was used to directly observe the increase in calcium levels after using the injury model described in Smith et al. (1999). Cultures where stained with fluorescent calcium indicator, Fluo-4AM, injured, and imaged before and after stretch injury. In calcium free extra cellular solutions there was no change in calcium concentration, which suggested that there was an extracellular source for increased levels of calcium post-trauma. After injury results also showed a increased levels of intra-axonal calcium.

Experiments performed by Smith, et al. similarly used neuronal cultures injured in both a calcium buffer and calcium free buffer. Fluorescence intensity was also measured using Fluo-4. Results showed that levels of calcium increased from the extracellular to the intracellular, rather than a release of intracellular stored calcium after injury.

1.7 Research Aims

The overall goal of this research is to achieve a set protocol to study Schwann cells using a stretch injury device along with serial phase contrast micrography to observe them in two states: myelinating and non-myelinating. Achieving set timelines for the protocol are extremely important in order to run injuries on healthy cultures. In addition, part of developing a set protocol requires testing the accuracy of the collected data, which is why a focusing test is performed. The focusing test will insure that re-focusing after injury does not affect the results obtained in calcium influx levels.

A strain of 60% is used to look at the effects of a severe injury in glial cells because in axon degeneration it occurs at this strain following the initial injury. Changes in intracellular calcium are measured in Schwann cells by measuring the calcium influx after cells have been stretch-injured. This is achieved using fluo-4, which is a calcium-binding indicator. In Undifferentiated Schwann cells injuries are performed with and without the presence of a calcium buffer. The change in calcium levels are measured in both differentiated and undifferentiated Schwann cells.

To observe changes in morphology, a timed analysis is performed after injuries. The goal for this study is to successfully induce differentiation on Schwann cell cultures growing on a silicone membrane, injure each of the cultures, and then observe any morphological changes that may occur after injury: re-myelination or degeneration. Based on this outcome future studies will be imposed to better determine the outcome of differentiated Schwann cells after injury.

Observing undifferentiated and differentiated Schwann cells in-vitro is an important component of this study. This study will allow a focus on the white matter of

CNS by studying myelinating cells. Schwann cells differentiate into non-myelinating or myelinating cells, but without axons they do not myelinate. Although the cells are incapable of forming myelin sheaths, we mimicked the molecular expression of myelinating cells by inducing differentiation on Schwann cells with cyclic amp, which made all studies possible.

CHAPTER 2

A HIGH-THROUGHPUT MODEL FOR *IN VITRO* STRETCH INJURY OF SCHWANN CELLS

2.1 Methods and Materials

2.1.1 In-Vitro Stretch Injury Model

This injury model is composed of Schwann cells cultured on an elastic silicone membrane within custom PEEK wells as seen in Figure 2.1. Cells are cultured on elastic silicone and deformed by a pressure-pulse of air. Schwann cells undergo a uniaxial stretch upon the application of the air pressure-pulse. The site of injury is determined by the deformation mask, which is made of acrylonitrile butadiene styrene plastic. The mask has a 2-millimeter gap in the middle and is placed under the well before the injury is applied. The masks are made with the use of a 3-dimensional rapid prototyping system (SST 1200es, Dimension, Inc., Eden Prairie, MN) using the Pro-Engineer software (PTC, Needham, MA).

To measure stretch injury in Schwann cells we measure strain; the percentage the membrane is deformed in relation to its original state. The strain rate is also calculated; the amount of strain over the time period of stretch. For Schwann cells we used a strain 60% over 20 ms, and a strain rate 30 s^{-1} .

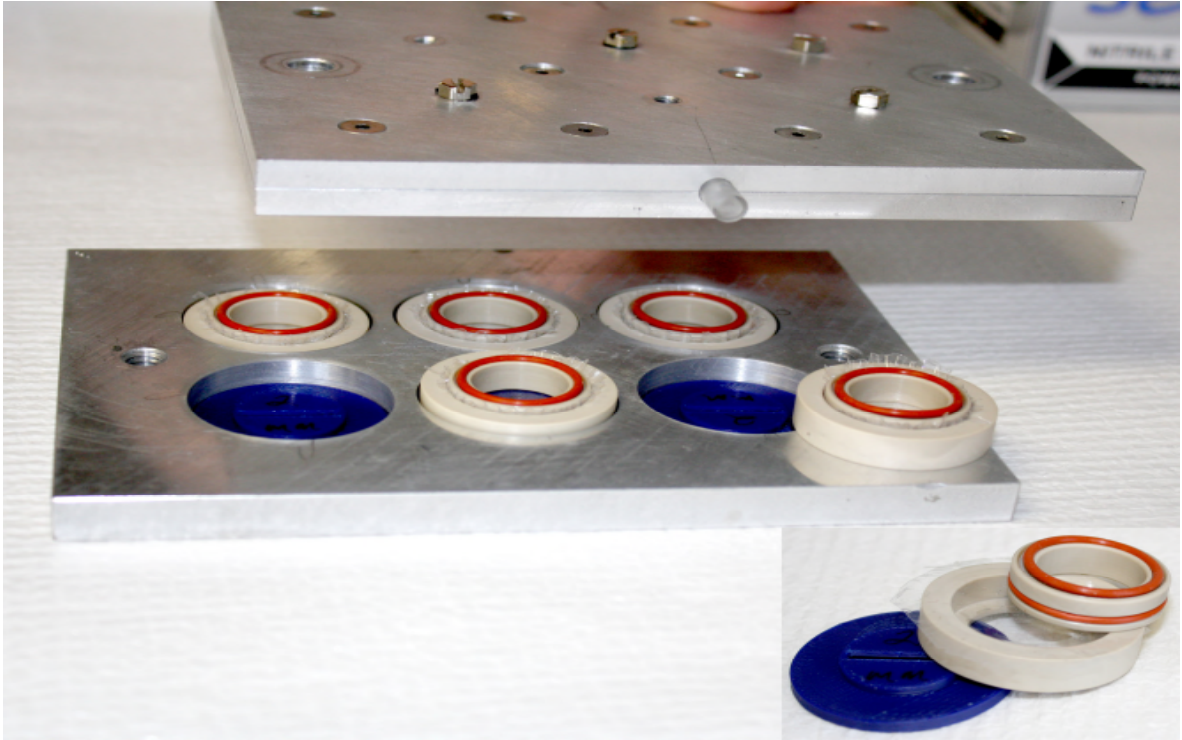


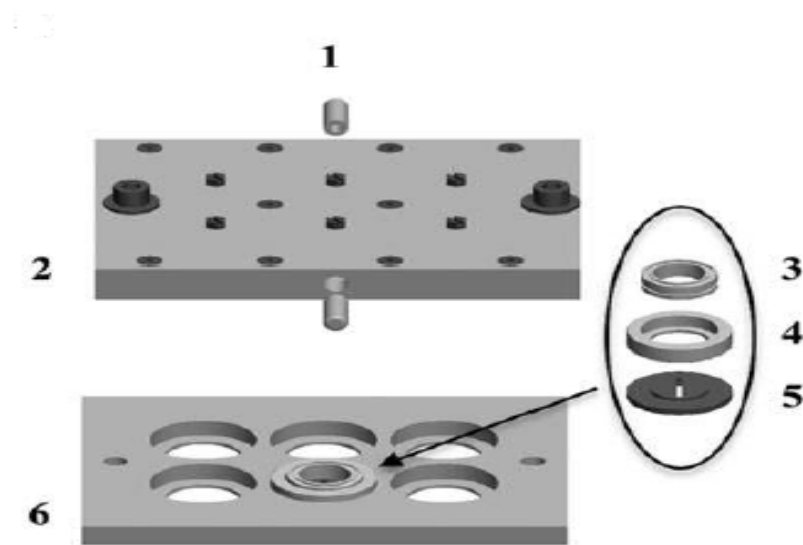
Figure 2.1 Pressure chamber with PEEK wells.

Source: Magou G.C., Guo, Y., Choudhury, M., Chen, L., Hususan, N., Masotti, S. & Pfister, B.J. (2011) Engineering a high throughput axon injury system. *The Journal of Neurotrauma*, 28, 2203-2218.

2.1.2 Stretch Injury Model Overview

The injury system has 4 main parts: 1) a control system that runs with LabVIEW (National Instruments, Austin, Texas), 2) an input pressure regulation system, 3) a valve control system, and 4) an injury chamber (Figure 2.2, Magou, et al., 2011). The schematic (Figure 2.3, Magou, et al., 2011) shows the control system which allows you to set an input for the reserve tank pressure and time for the valve depending on the injury. The electronic pressure controller (VSO-EP) controls the pressure input that you want and can range from 0-100psi. Once the chosen pressure is reached, the control system signals the valve driver circuit (OEM). LabVIEW allows the user to control the opening and closing of the valve controlled. Using the control system, the pressure over the

period of time can be determined. The valve has a three-way, solenoid valve, which connects the secondary tank to the chamber allowing the appropriate pressure pulse to travel to the Schwann cell cultures (Magou, et al., 2011) .



Module A (6-well)	
1	Pressure Inlet
2	Pressure Chamber (Top Plate)
3	6-well Vessel (Top Ring)
4	6-well Vessel (Bottom Ring)
5	Deformation Mask
6	Pressure Chamber (Bottom Plate)

Figure 2.2 6-Well Pressure Chamber with Removable Wells and Deformation Mask. The drawing includes (1) the air pressure inlet, (2) top plate of the chamber, (3) top ring, (4) bottom rings, (5) deformation mask with a 2 mm gap, (6) and the bottom plate of the chamber.

Image and image text taken from: Magou G.C., Guo, Y., Choudhury, M., Chen, L., Hususan, N., Masotti, S. & Pfister, B.J. (2011) Engineering a high throughput axon injury system. *The Journal of Neurotrauma*, 28, 2203-2218.

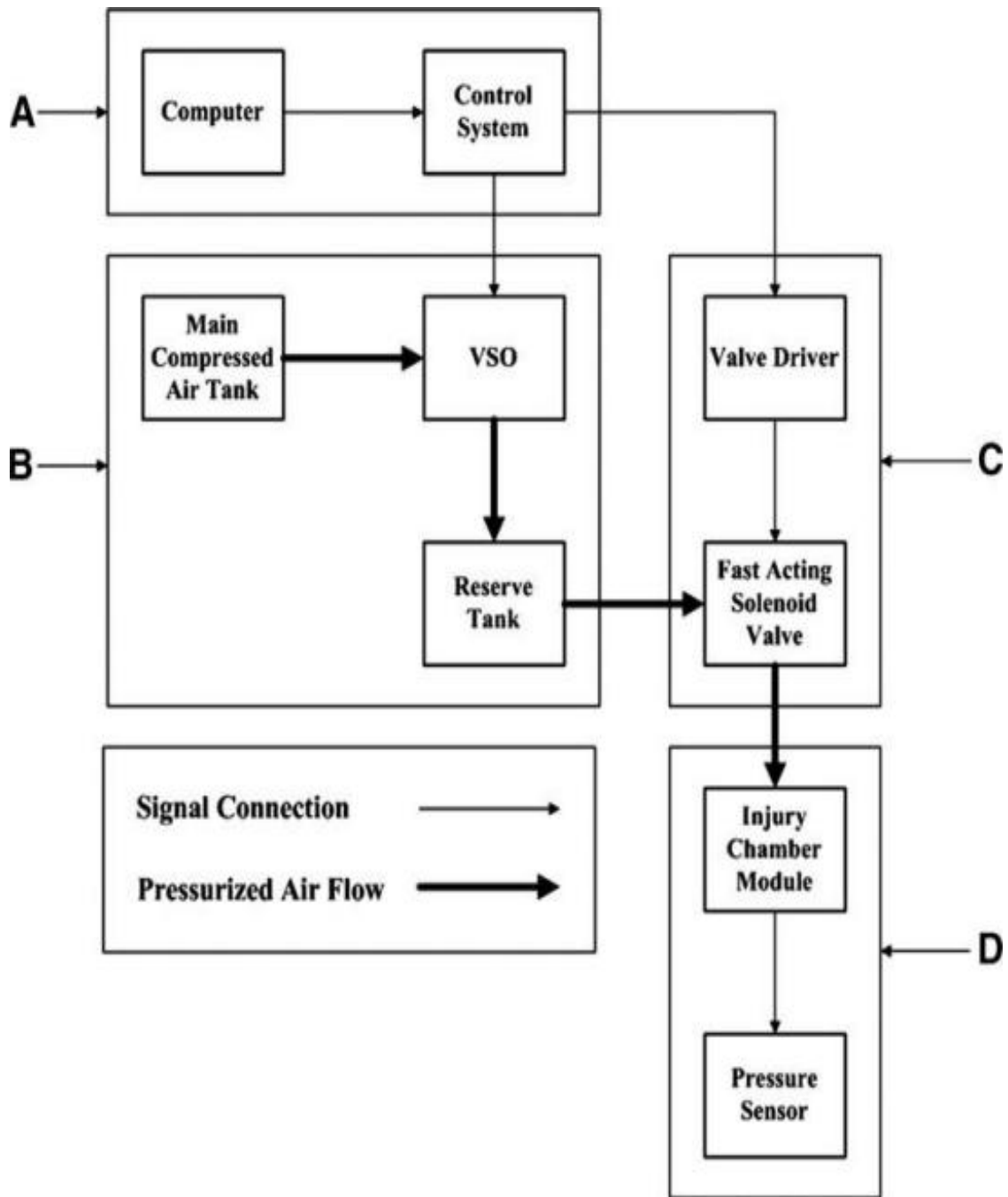


Figure 2.3 A schematic of the injury system (A) Control system using Labview. (B) Input pressure regulatory system. (C) Valve system, which delivers the injury pressure pulse. (D) Injury chamber module and pressure sensor.

Source: Magou G.C., Guo, Y., Choudhury, M., Chen, L., Hususan, N., Masotti, S. & Pfister, B.J. (2011) Engineering a high throughput axon injury system. *The Journal of Neurotrauma*, 28, 2203-2218.

2.1.3 Assembly and Preparation

To prepare wells for cell culturing, Sparkleen (Fisher Scientific Co.) a lab detergent is used to wash and scrub the wells using a toothbrush. They are then washed with dH₂O, sonicated 3 times with dH₂O for 5 minutes, changing water after every cycle. The silicone membrane is measured, cut out according to the desired amount, rinsed with dH₂O, and assembled to wells using Fit-016 silicone o-rings (McMaster-Carr). In order to create a cell free zone the silicone strips are cut to fit the wells at 2mm wide. They are sonicated once again and submerged in dH₂O and autoclave for 1hr.

2.1.4 Schwann Cell Culture Coating

Coating preparation is done the day before plating in injury wells. To prepare coat, aliquots of poly-D-lysine (PDL) (BD Biosciences, Bedford, MA) (in -20 C: 180 ug/100 uL aliquots) are thawed out for 2 minutes in a hot water bath. 100 uL of PDL is diluted in 6mL Hank's balanced salt solution (HBSS) containing Ca²⁺ and Mg²⁺. Wells are covered in PDL for 1 hour. After an hour, they are rinsed 3 times. Matrigel is diluted in autoclaved dH₂O, 1:35 (defrost on ice 20 minutes prior to being used). Wells are coated after being rinsed, with Matrigel solution (approximately ~600 uL for Injury Wells) and left overnight in incubator at 37° C to dry.

Coating preparation for plastic is done on the day of plating. To coat a 100 mm tissue culture dish thaw out PLL (60 μg/cm²) and dilute in autoclaved dH₂O 1:7. Then incubate at 37° C for 1 hour, rinse three times with sterile dH₂O and leave to dry under the sterile hood before coating.

2.1.5 Schwann Cell Culture: Plating, Passage, Freezing and Differentiation

The ability to study the development and myelination of Schwann cells has been enabled by the isolation of a pure population of primary Schwann cells. Present studies use cells between passages 4-6, a generous gift from Dr. Haesun Kim, Department of Biological Sciences at Rutgers University.

Steps for expansion: Schwann cell media is made, and kept warm before plating. Schwann Cell media consists of Dulbecco's Modified Eagle Medium (DMEM), 1% penicillin-streptomycin, 1% Glutamax, and 10% FBS. For differentiated cells you use 2% instead of 10 % FBS. Schwann cells are isolated and purified using the methods described by Kim, et al. 1 mL vials of frozen SCs at Passage 3 (P3). The frozen vials (given to us by Dr. Haesun Kim, Department of Biological Sciences at Rutgers University) are defrosted briefly in a 37° C water bath for 2-3 minutes. Cells are transferred to a 15mL tube and 4 mL of 10% FBS Schwann cell media are added and centrifuged at 200 RPM for 5 minutes. After removal of supernatant cells are re-suspended in 1 mL Schwann cell media 10%FBS and 9 mL of Schwann cell media 10%FBS are added. After mixing well, 1 µL of 0.1 mg/ml Neuregulin (Nrg) (396-HB, R&D systems) and 5 µL 5 mM Forskolin (F6886, Sigma-Aldrich) are added. Cells are plated on a 100mm tissue culture dish and left in incubator for 3-5 days until confluent.

Once cells are 100% confluent, media is removed and cells are rinsed three times with HBSS without Ca^{2+} and Mg^{2+} . HBSS is replaced with 0.25% trypsin in HBSS with 75 mM EDTA without of Ca^{2+} and Mg^{2+} for 2 minutes in the incubator until cells detach from the surface of the dish. Remaining cells are detached by banging and shaking culture dish. Then media is added two times the volume of Trypsin into each dish to

neutralize. Cells are collected on 15mL tube and centrifuged for 5 minutes at 200 RPM. Supernatant is discarded and re-suspended in 9 mL growth medium and counted. Schwann cells are plated in injury wells coated with PDL and Matrigel at an average density of 250,000 cells/cm². Injury experiments are performed on cells between passages 4-6.

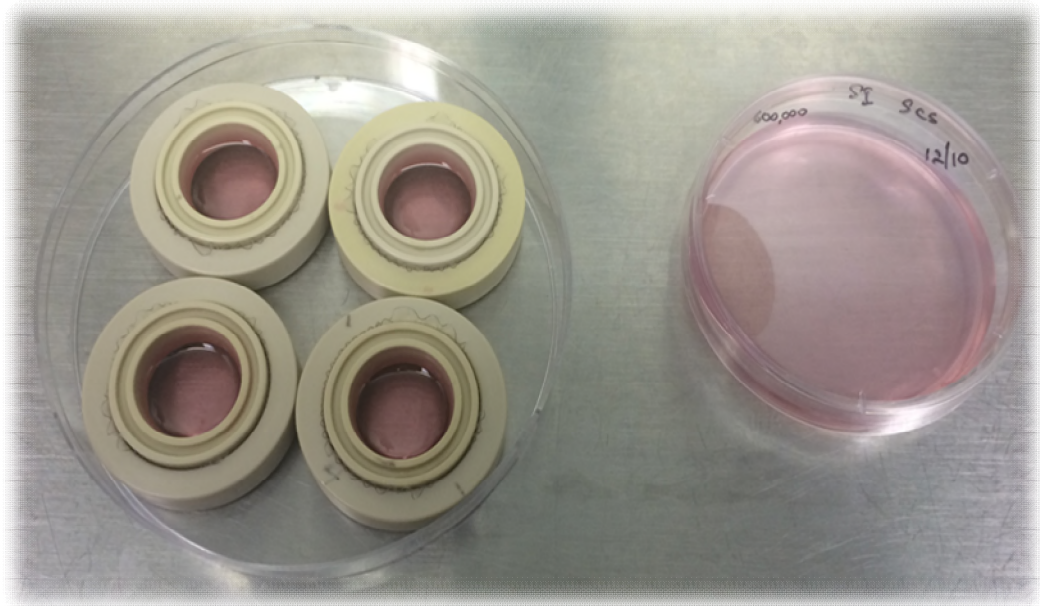


Figure 2.4 Plastic Dish with Schwann cell culture along with Schwann cell cultures in silicone injury wells.

Cells are counted using the hemocytometer. Before cells have a chance to settle, 0.5 mL of cell suspension is transferred on to a tube. 100 μ L of cells are transferred on to a tube along with 400 μ L 0.4% Trypan Blue, and are mixed gently. 100uL of solution are applied to hemocytometer and cells are counted with 10X objective. 4 sets of the 16 are counted and the equation below is used.

$$\text{Total cells/ml} = \frac{\text{Total cells counted} \times \text{dilution factor}}{\# \text{ Of Squares}} \times 10,000 \text{ cells/ml} \quad (2.1)$$

To freeze cells for storage, once cultures reach 80-90% confluency cells are washed twice with HBSS without w/o Ca^{2+} and Mg^{2+} , and replaced with 0.25% Trypsin + EDTA in incubator for 2 minutes. After detachment L-15/ 10% FBS solution two times the amount of Trypsin is added. Cells are collected on 15 mL tube and centrifuged for 5 minutes at 200 RPM. Supernatant is removed and re-suspended in 10 mL L-15/10% FBS. Cells are counted with hemacytometer then centrifuged again and suspended in ice cold freezing media (10% dimethyl sulfoxide (DMSO) + 90% FBS) with a final density of $\sim 2 \times 10^6/\text{mL}$. 1mL aliquots in cryovials are made and frozen overnight at -80°C . The following day they are moved to liquid nitrogen.

Differentiation is achieved after Schwann cells reach confluency. Cells are rinsed with DMEM one time and replaced with 2%FBS Schwann cell media. 24 hours later cells are rinsed once again and replaced with differentiation media (1% penicillin-streptomycin, 1% Glutamax, 2% FBS, 500 μM dbcAMP). Schwann cells are left incubation for three days; differentiation media is added every other day until differentiation is achieved. Injuries are performed immediately upon differentiation.

2.1.6 Differentiated Schwann cell Morphology: Timed study

Differentiated Schwann Cells were imaged using phase contrast microscopy. Before injury the preferred area of injury of the gap is defined and marked in a nearby location with a thin permanent marker on the opposite side of the silicone well (bottom location without cells). All Schwann cells are injured at 60% strain at a rate of 30s^{-1} . Each well is imaged before injury (pre-injury), one hour after injury, four hours after injury, and twenty-four hours after. Images were recorded using Nikon NSL elements software. Each well is analyzed using morphological markers within the images. Then single cells

are selected and observed individually for changes in morphology.

2.1.7 Stretch Injury Model Overview

Calcium measurements are recorded in both injured undifferentiated and injured differentiated Schwann cell cultures. Cells are rinsed 3 times for 3 minutes in HBSS with Ca^{2+} and Mg^{2+} before adding the stain. Schwann cells are stained with 4 μM Fluo-4 AM (488/515 nm), a cell-permeant calcium indicator (F-14201, Invitrogen). The solution is solubilized in 100% anhydrous dimethyl sulfoxide (DMSO) along with Pluronic F-127 a cellular detergent (0.01% w/v). The 2 μL of Fluo-4 and 2 μL of pluronic acid (4 μM stock solution + pluronic acid) are diluted in 4mL of HBSS with Ca^{2+} and Mg^{2+} and vortexed. Solution is added to cultures and put in incubator for 30 minutes. Following the 30 minutes cultures are rinsed once and stain solution is replaced with HBSS and returned to incubator for 30 minutes for full de-esterification. Finally, cells are rinsed once for 3 minutes immediately before imaging for optimal fluorescence. Cultures are also stained without calcium as a control (calcium free buffer solution), so the HBSS that was used was without Ca^{2+} and Mg^{2+} for both rinsing and diluting the Fluo-4 stock solution.

The calcium influx is recorded using the Nikon Eclipse TE2000 microscope with an objective of 20x with a 75 watt xenon arc lamp and Photometrics CoolSNAP EZ CCD Camera. Before and at the time of injury, images are taken every 2 seconds with an exposure time ranging from 400-600. To record the images Nikon NSL Elements software was used along with a control shutter, the Lambda SC Smart Shutter, Sutter Instruments. To lower photo bleaching of cells while increasing light collected by the camera, binning was adjusted to 2x2. The injury is then recorded over 1 to 3 minutes, this includes the time of injury and a substantial amount of time after injury.

2.1.8 Functional Calcium Image Analysis

The average intensity of Schwann cells within the field of view is measured via ImageJ software (Schneider, et al., 2012). Videos are opened, and the 'StackReg' plugin (Thevenaz, et al., 1998) is used to correct any displacement among the frames at the time of injury and after. Schwann cells are selected using the circle tool or free hand selection and then 't' is selected on keyboard to record all selection. The function 't' will number all the cells as you select them to be analyzed (Figure 2.5). The ROI manager function will have all the cells that are selected and will show three measurements of background intensity that are also selected in addition to the selected Schwann cells. The ROI manager function is used to analyze each of the selected cells and background. It analyzes each frame and makes a row for each selection (Figure 2.6). The measurements are copied on to an excel spreadsheet. The background data is averaged to a single value that is subtracted from each of the intensity values from the selected cells Figure (2.6). These values are normalized to the first measured average intensity by subtracting the background intensity and dividing each value from the cell of the first measured value, which is the baseline. The baseline is determined by the fluorescent intensity of the first frame of the video and should always be one (before injury). (F) represents the measure of fluorescence at each time point divided by the baseline (F0), and the baseline intensity is $F/F_0=1$. Excel is also used to plot the maximum intensity.

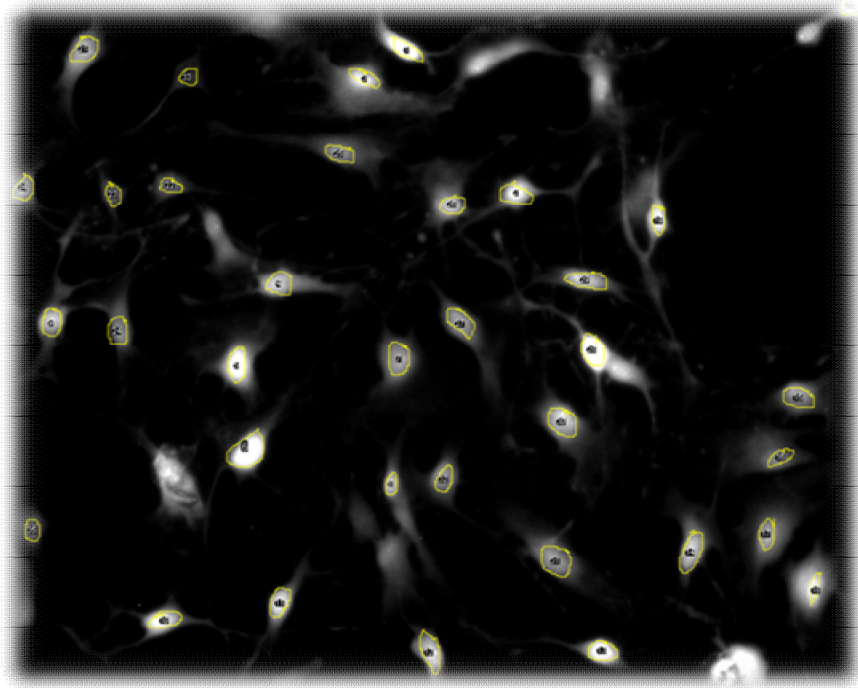


Figure: 2.5 Image J application showing the randomly selected Schwann cells using 't'. 30-50 cells are selected each time.

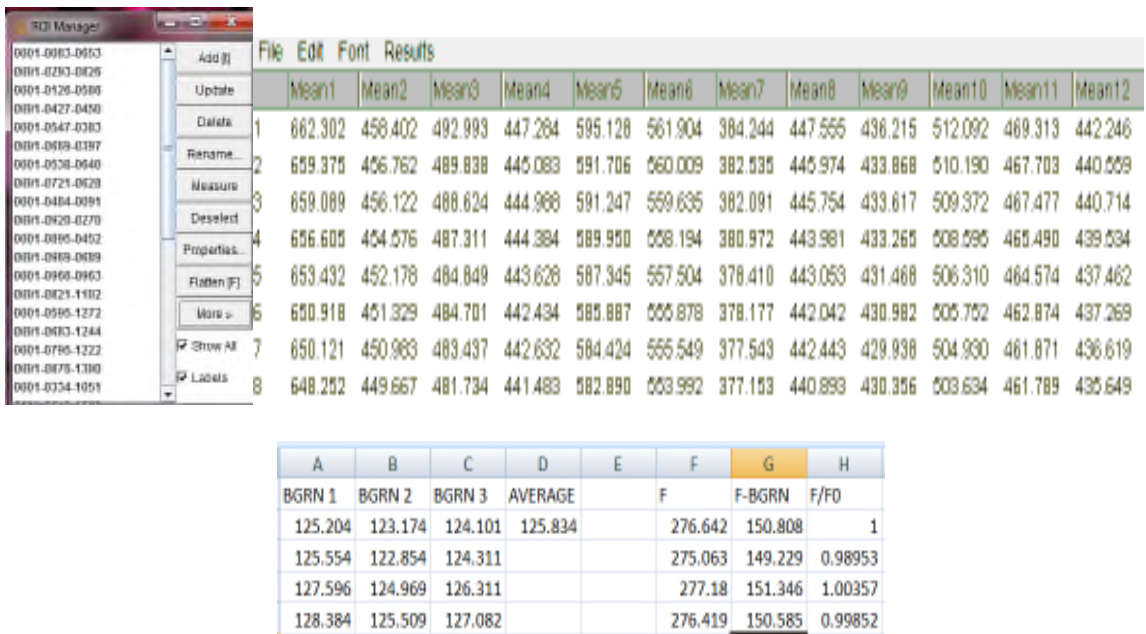


Figure 2.6 Upper Image: The ROI data from Image J showing Intensity values of the randomly selected Schwann cells (F). Lower Image: Analyzed data using Excel. BGRN (measurements of background intensity), AVERAGE(Average of 3 BGRNs), F (Intensity at each time point), FO (Baseline).

2.1.9 Analysis: Responding Cells and T-test

Fluorescent intensity is measured from each cell in the field of view. Since calcium influx was not observed in every cell after injury, there are cells that are considered “non-responding” cells ($F/F_0 < 1.1$). The percentage of non-responding cells is defined by the amount of cell within each subgroup that did not have calcium response divided by the total number of cells in the specific subgroup. Responding cells on the other hand are grouped into bins to analyze the percent of cells that fall into each level of calcium response. The bins look at four different types of responses: no response, transient response, immediate response and sustained (sustained1), and delayed response and sustained (sustained 2) This study was done for both undifferentiated and differentiated Schwann cells.

A statistical T-test was performed on the data for undifferentiated Schwann cells to compare whether the means of the two groups (Calcium Buffer and Calcium Free buffer response) statistically differ from one another. This test determined if the calcium is coming from internal stores as opposed to external.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}}} \quad (2.2)$$

Methods Flow Chart

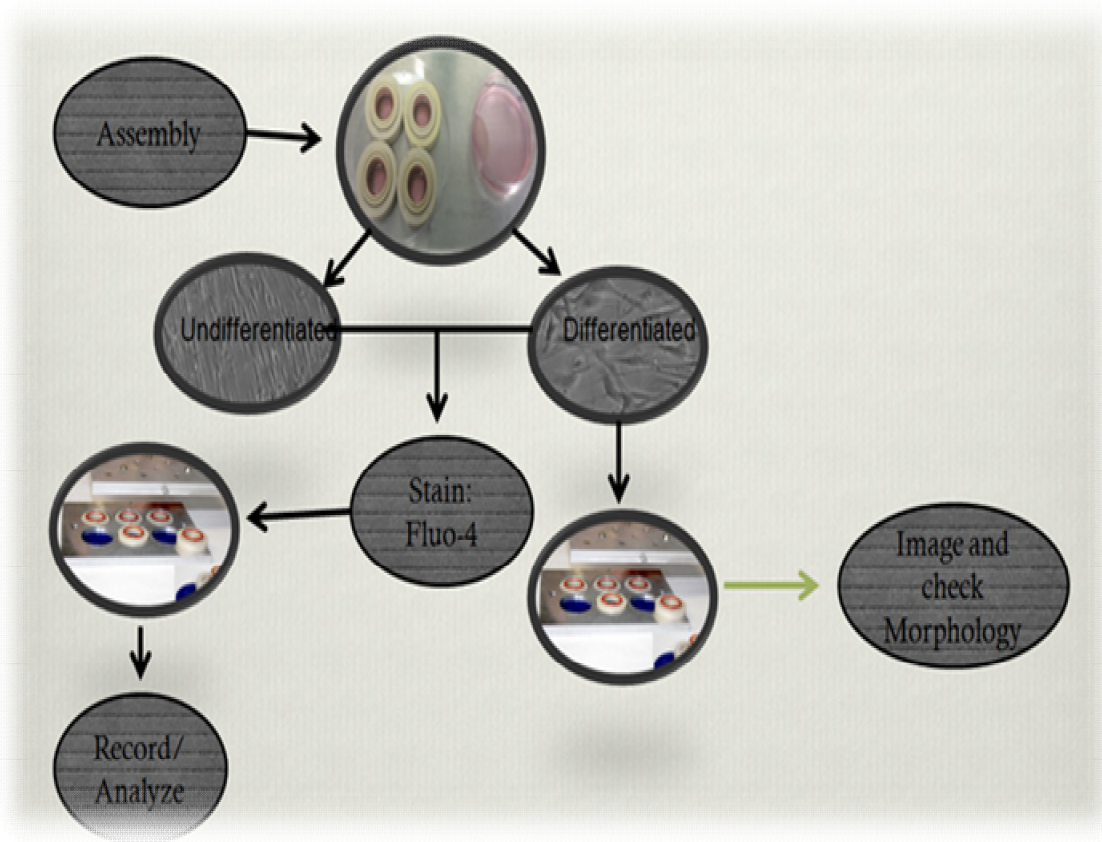


Figure 2.6 Methods overview: Wells are assembled and Schwann cells are cultured. The cells become undifferentiated, you add dbcAmp to induce differentiation. Both Schwann cell states are stained, injured and imaged. Differentiated cells undergo injuries and are imaged before injury, 1 hour, 4 hours, and 24 hours after injury.

CHAPTER 3

RESULTS

3.1 Undifferentiated and Differentiated Cultures

Cultures take about 3 days to become undifferentiated confluent cells and another 4 to 5 to induce differentiation. Images are taken when undifferentiated cells become confluent Figure 3.1A and after differentiation is induced. Figure 3.1B. Figure A shows undifferentiated Schwann cells which proliferate in the same direction while Figure B shows differentiated Schwann cells with a different morphology; cells are no longer in the same direction and are somewhat enlarged.

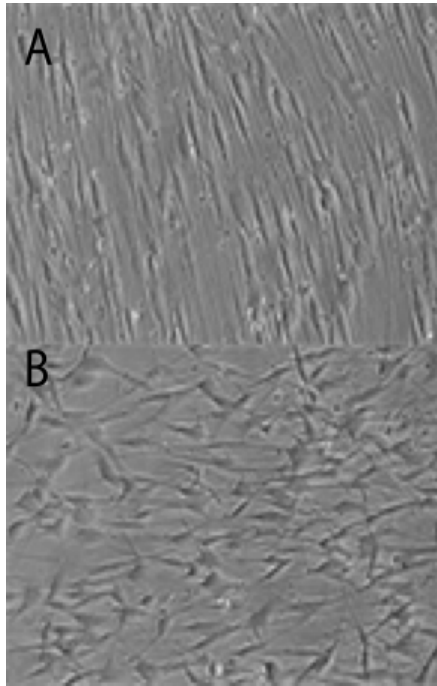


Figure 3.1 Cells at passage 4 (A) undifferentiated Schwann cells and (B) differentiated Schwann cells

3.2 CALCIUM RESPONSE

Calcium intensities following an injury were measured in 4-6 well cultures depending on the health of the cells. Recordings of the cultures were taken 10-30 seconds prior to injury and also include the injury to establish a baseline fluorescence (F_0) and calcium influx at injury (F/F_0). Culture populations are injured at a strain of 60% and at a rate of 30 s^{-1} .

3.2.1 Calcium Response in Undifferentiated Schwann Cells

To study calcium responses in undifferentiated Schwann cells, cells from passage 6 were used. In cells that were injured in the presence of a calcium buffer 59% of cells had a transient response (N=183 cells from 5 wells), where max calcium intensity values exceeded $F/F_0 = 1.1$ (Table 3.1). The majority of calcium changes took place between values of 1.2-1.6 of F/F_0 (Table 3.1. and Figure 3.2). In cells that responded, most showed a transient response with the exception of couple which exhibit sustained responses (Figure 3.2, Upper Figure). Cells in which calcium was sustained had two different responses; calcium increase after injury (sustained1) and calcium increase as a delayed injury responses. Sustained responses ranged between values of 2-3.5 in F/F_0 and did not return to baseline within the first minute. Figure 3.3 shows all 4 responses that are taken into consideration for analysis from randomly selected cells. From putting the sustained and non-responding cells into bins it is observed that 29.2% showed no response in a calcium buffer solution, 3.8% had increased intensities and where sustained, while 7.1% had delayed responses and where sustained (Table 3.2).

To observe differences in fluorescence among internal and external sources of calcium, cells were injured in a free calcium buffer solution. Cells that were injured in the

calcium free buffer had 52% cells with a transient response ($X=163$ cells from 4 wells), where maximum calcium intensity values exceeded $F/F_0= 1.1$. Similarly to the calcium buffer results (Table 3.1), the majority of the calcium responses took place between 1.2-1.6 F/F_0 . Cells responded transiently and sustained as well, with 34.9% showing no response in a non-calcium buffer solution, 9.2% sustained(1), and 3.1% sustained(2) (Table 3.2). Figure 3.3 shows the calcium response in one of the 4 wells, the cells that where randomly mainly have a transient response.

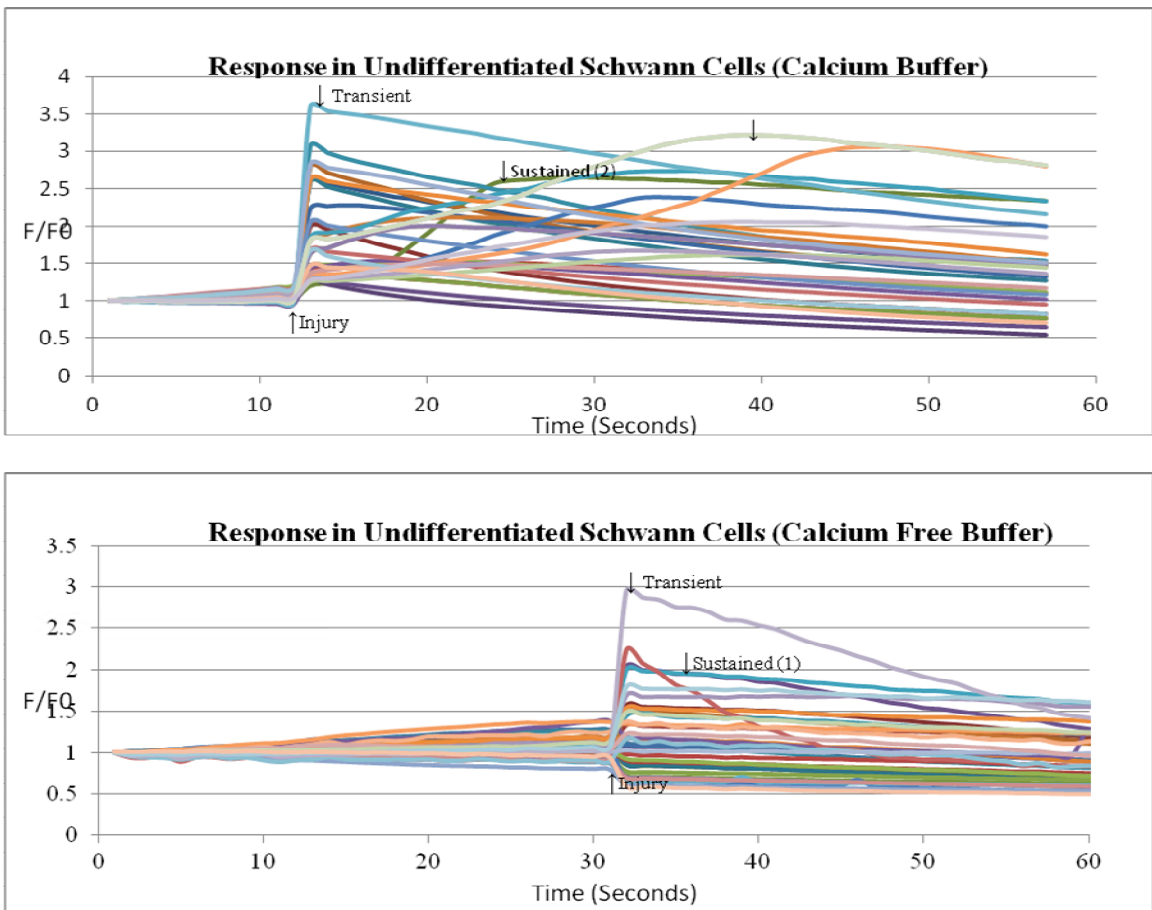


Figure 3.2 Displays 4 types of calcium traces from randomly selected Schwann cells at passage 6 within the first 60 seconds: Upper Image Calcium Influx in Undifferentiated Schwann cells with a calcium buffer (CSS) following injuries at a strain of 60% at a rate of 30 s^{-1} (injury at 11 seconds). Calcium traces from randomly selected Schwann cells at passage 6 within the first 60 seconds. Lower Image Calcium Influx in Undifferentiated Schwann cells with a calcium free buffer (CSS) following injuries at a strain of 60% at a rate of 30 s^{-1} (injury at 31 seconds). Calcium traces from randomly selected Schwann cells at passage 6 within the first 60 seconds.

Table 3.1 Percent (%) responders in calcium intensity for undifferentiated Schwann cells

Influx Value	1.2	1.4	1.6	1.8	2	2.2	2.4	2.6	2.8	<3.0
Calcium Buffer	8.2	25.4	7.8	5.9	3.2	2.8	0	3.4	2.1	1.1
Non- Calcium Buffer	9.1	15.9	4.9	3	6.1	2.4	2.4	3	2.4	3.6

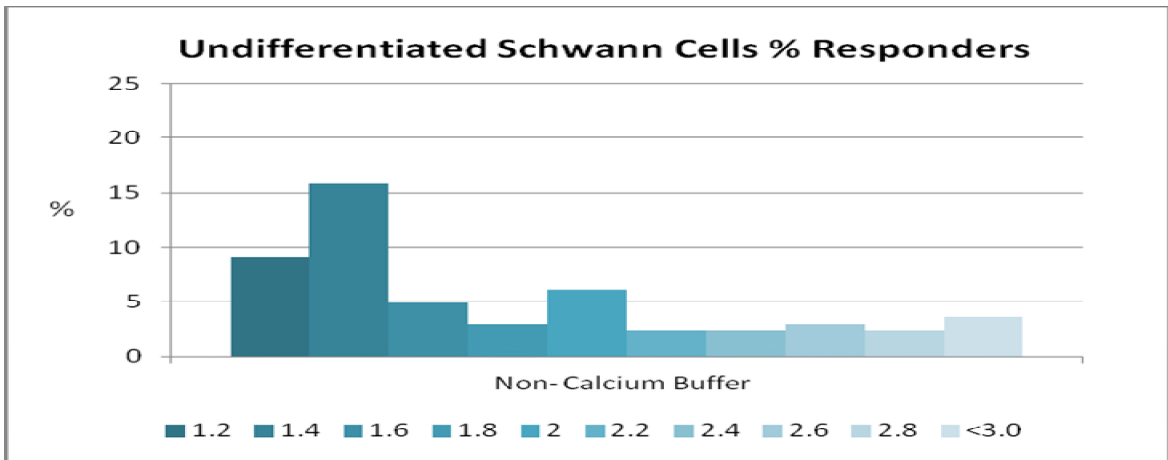
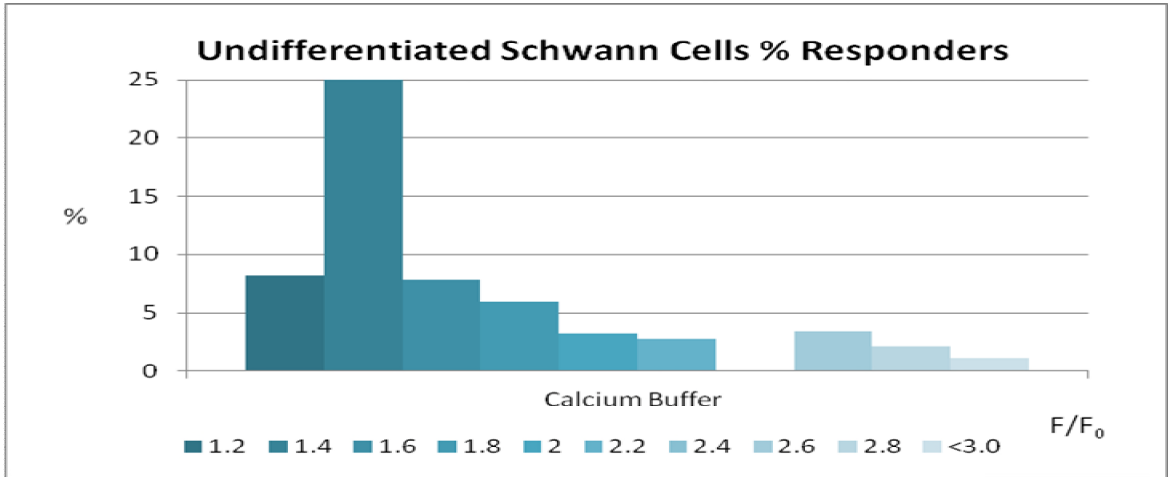


Figure 3.3 Upper Image: transient responding cells in a calcium buffer, Lower Image: transient responding cells in a calcium-free buffer.

Table 3.2 Percent (%) sustained and non-responding cells in calcium intensity

Response	Sustained (1)	Sustained(2)	No Response
Calcium Buffer	3.8	7.1	29.2
Non Calcium Buffer	9.2	3.1	34.9

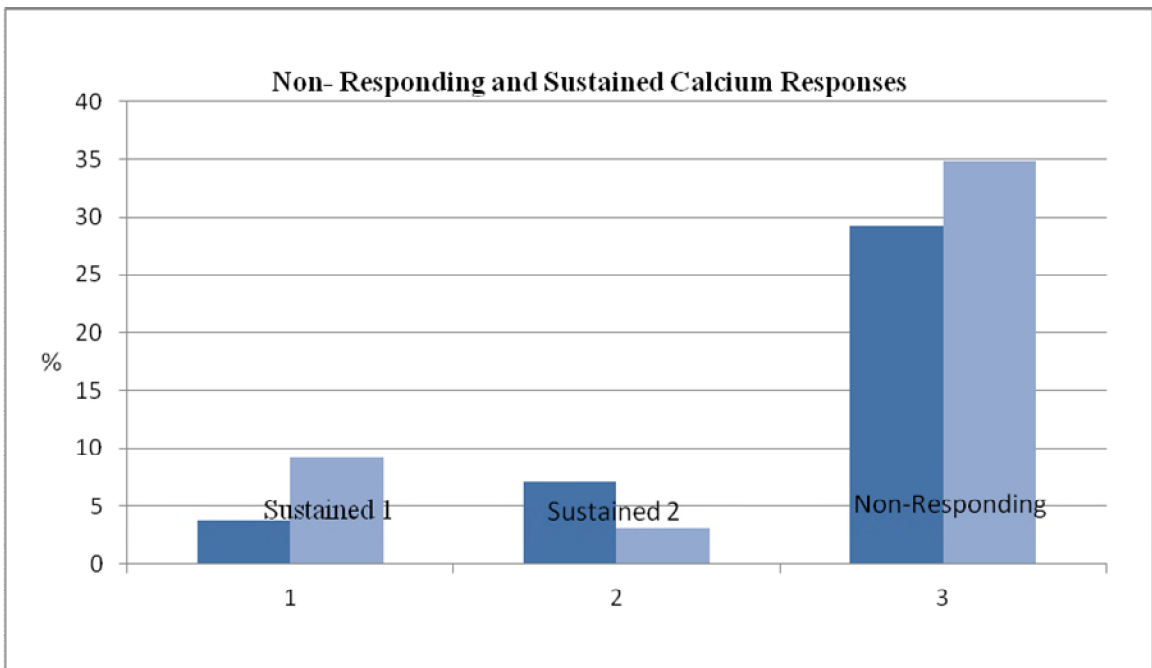


Figure 3.4 Shows sustained and non-responding cells in both a calcium buffer solution (dark blue) and buffer free (light blue).

Table 3.3 Statistical analysis for transient responding cells

T-Test	Mean	Variance	T-value
Calcium Buffer	5.6	47.64	0.278159
Non-calcium Buffer	4.9	15.69	

3.2.2 Calcium Response in Differentiated Schwann Cells

To study calcium responses in differentiated Schwann cells, cells from passage 4 were used. Differentiated cells were observed to see if there is a difference between the calcium intensity of undifferentiated cells and differentiated cells. In the case of differentiated cells, the response was different therefore, cells were only tested in a calcium buffer solution. Cells responded less frequently ($X=205$ cells, 5 wells) after injury with a total of 5.8% responders Table 3.1 and Figure 3.4. Figure 3.5 shows a control and a culture in which cells responded. From the few cells that did respond after injury at 30 seconds they have a increased intensities when F/F_0 is between 1.2 and 1.4.

Table 3.4 Percent (%) responders of calcium in differentiated Schwann cells

Influx Value	1.2	1.4	1.6	1.8	2	2.2	2.4	2.6	2.8	<3.0
Calcium Buffer	3.4	2.4	0	0	0	0	0	0	0	0

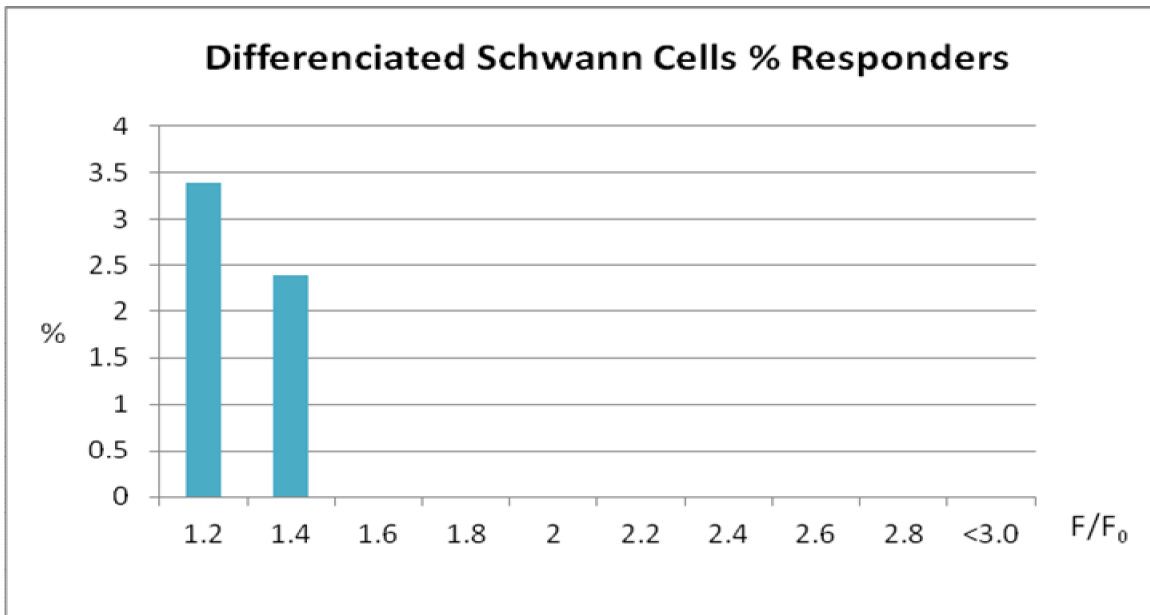


Figure 3.4 Calcium Increase percent (%) responders in Differentiated Schwann cells.

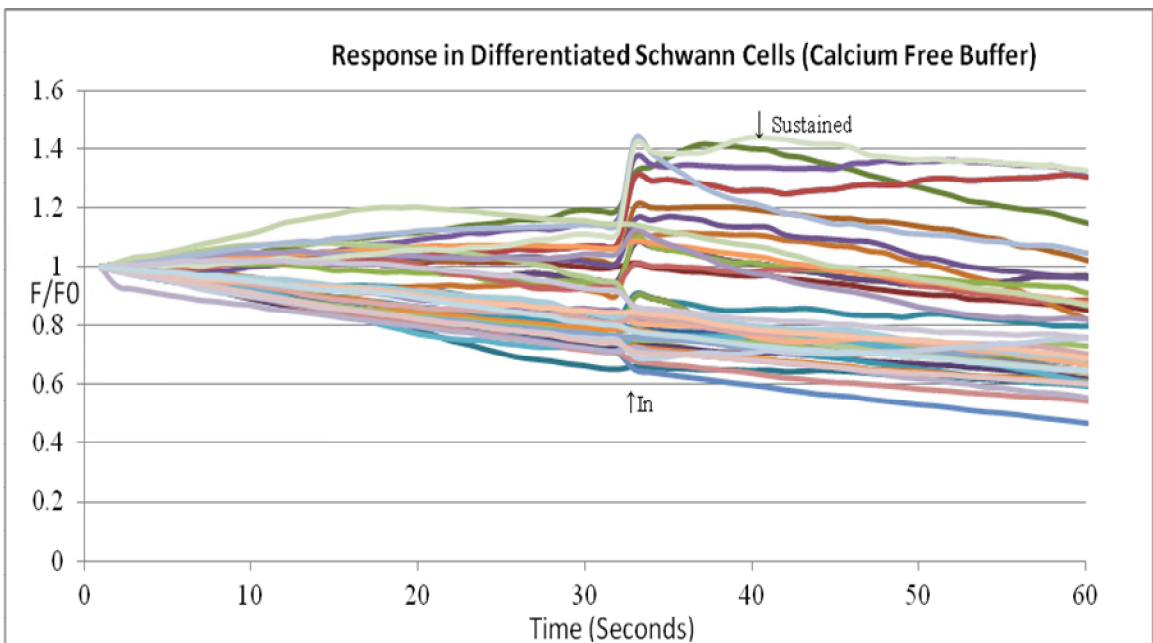
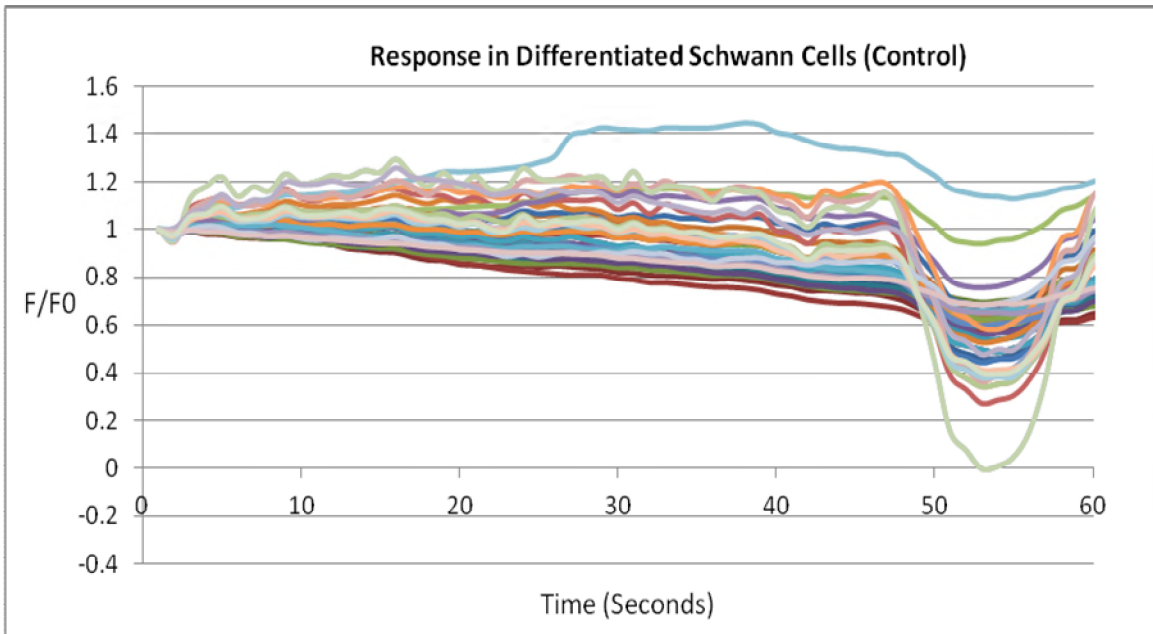


Figure 3.5 Calcium Influx in Differentiated Schwann cells with a calcium buffer following injuries at a strain of 60% at a rate of 30 s^{-1} (injury at 31 seconds). (Upper Image) Control. (Lower Image) Calcium traces from randomly selected Schwann cells at passage 5 within the first 60 seconds.

3.2.3 Focusing Test

Cultures between passages 4-6 underwent a focusing test to assure that the decrease in Florescence (F_0) wasn't a change due to re-focusing after the injury, but a normal decrease in intensity. It was also done to show that focusing in and out couldn't be done during the recording because it would cause artifacts in the data. Results proved the re-focusing did not significantly change the intensity within the first 60-90 seconds of recording after re-focusing(Figure 3.5) which is what needs to be done following an injury.

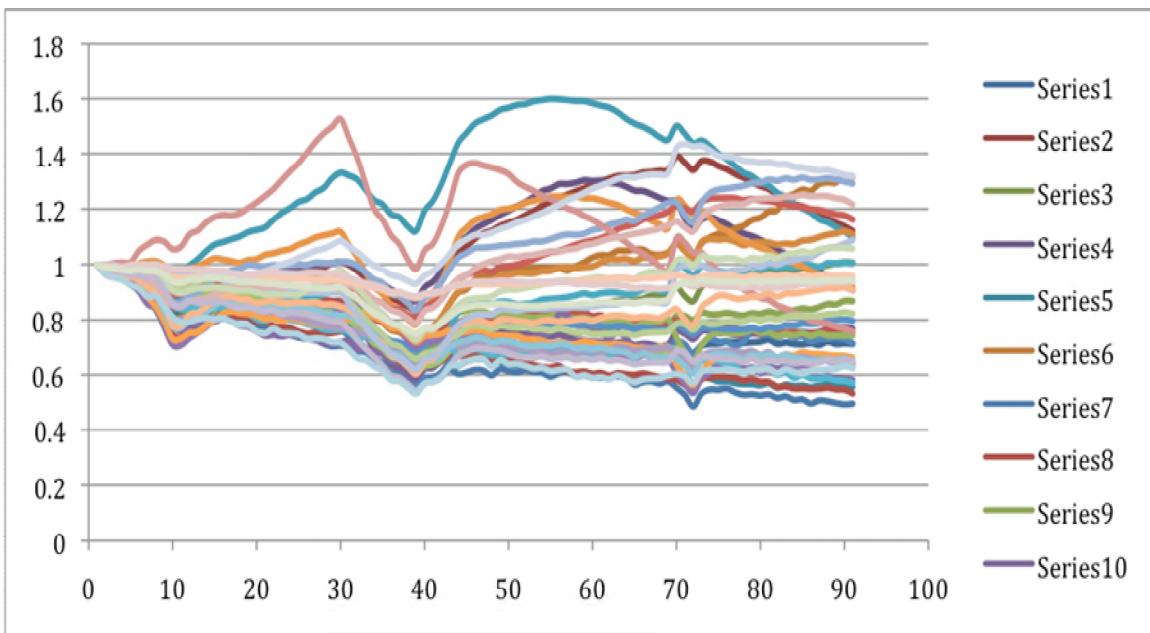


Figure 3.6 Focusing test, showing refocusing without a significant change in (F_0) intensity. Note that maximum intensity is 1.6.

3.3 Differentiated Schwann cell Morphology: Timed study

Cultures in passage 4 were expanded on a plastic dish and then plated onto 8 silicone injury wells. Once undifferentiated cells reached confluence, differentiation was induced following the protocol. Cells from experiment 1 (Figure 3.7) were only imaged up to an hour after injury because they began to degrade following the injury. It is possible that cells from experiment 1 underwent cell death an hour after injury because they were left in cyclic amp for too long.

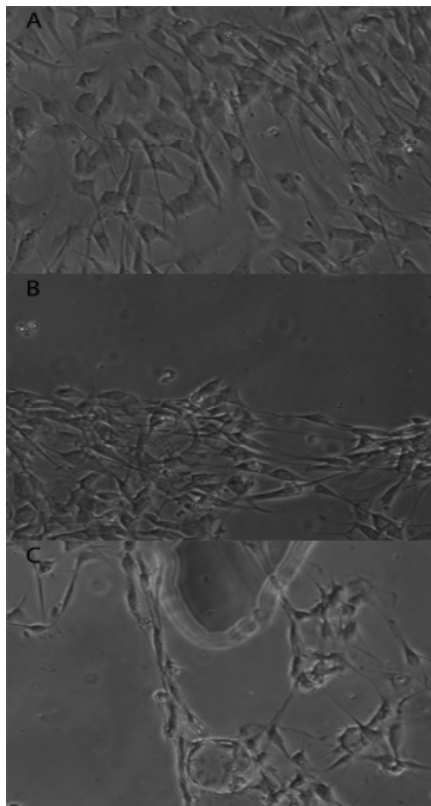


Figure 3.7 Experiment 1 (A) Differentiated Schwann cells before injury (B) Differentiated Schwann cells after injury (C) Differentiated Schwann cells one hour after injury

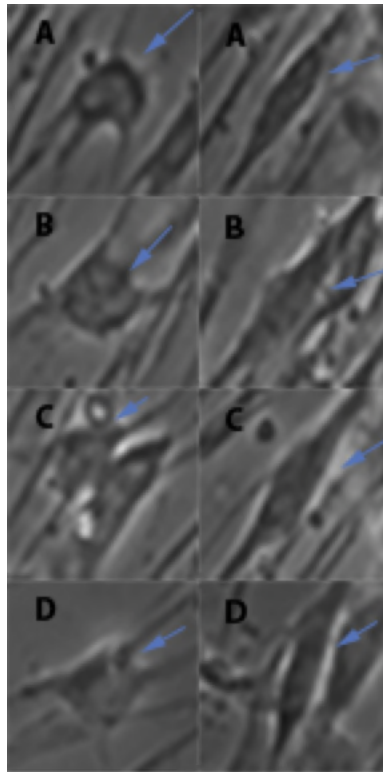


Figure 3.8 Experiment 2, Two Individual differentiated cells at passage 4: (A) preinjury, (B) 1 hour after injury, (C) 4 hours after injury, and (D) 24 hours after injury.

Individual wells are imaged before injury (pre-injury), and then injured at a 60% strain, with a rate of 30s^{-1} , cells were observed for changes after 1 hour, 4 hours, and 24 hours following the injury according to morphological markers and individual cells where compared at each time. From Figure 3.8 it can be observed that there are morphological changes in at each time. Looking at cell one, image C you can also see that cells are moving as well. Cells from this experiment where under cyclic amp for a total of 4 days.

CHAPTER 4

DISCUSSION

4.1 Calcium Influx Response

4.1.2 Calcium Intensities in Undifferentiated Schwann Cells

According to research similarly to neural cells and axons, large influx levels of calcium have been found to be harmful in the health and survival of Schwann cells (Trump and Berezsky, 1995). Additionally, it has been established that cells also have intracellular stores of calcium (Trump and Berezsky, 1995). Thus, making it possible to believe that these stores can influence the results in the sustain rise in cytosolic calcium.

In this research cells are injured in both a non-calcium buffer solution and a calcium buffer solution to explore the chance that internal stores might be affecting the calcium influx response. From the results it is concluded that there is an increase of intracellular calcium in undifferentiated cells under both conditions: calcium buffer and calcium buffer free solution. From the T-test results: The t -value is 0.27. The p -value is .39. The result is *not* significant at $p < .05$. This shows that the calcium traces after injury are coming from internal stores. Internal sources of calcium may have been the influence in some of the delayed responses, which are observed in both studies, calcium-free and calcium buffer solution.

4.1.3 Calcium Intensities in Differentiated Schwann Cells

Studies for differentiated Schwann cells had a different onset of outcomes when injured in a calcium buffer solution, which is why they were not injured under a calcium buffer free solution like undifferentiated cells. From the results in the few responding cells it is

observed that the influx levels are more evenly distributed in comparison to those in undifferentiated cells.

The induction of differentiation in Schwann cells could possibly change sodium channel properties within the cells, which is why the calcium influx results vary when compared to undifferentiated Schwann cells. It can be suggested that differentiated Schwann cells may be more resistant to the damaging effects of calcium influx, and perhaps resistant to extracellular calcium after the influx of an injury. It is also possible that cyclic amp may play a role in these results.

4.1.4 Percent Responders Following a Severe Stretch Injury

Research in Schwann cells in the PNS and glial cells in the CNS have shown that cells have intracellular stores of calcium (Miller, et al., 2009; Trump and Berezesky, et al., 1995). This study mainly focuses on intracellular calcium influx after a severe injury insult. Undifferentiated and Differentiated Schwann cells both underwent injuries of 60% strain at a rate of 30 s^{-1} .

In undifferentiated Schwann cells injured in a calcium buffer solution 59% of the cells showed a transient calcium influx response or sustained calcium influx response. In a calcium free buffer solution 52% responded with the majority having a transient influxes and similar calcium florescence as the cells injured in a calcium buffer solution. At the onset of severe injuries Schwann cells release intracellular calcium, which is why cells injured in the calcium buffer solution either had a sharp influx at injury or a slow response proceeding the injury (due to intracellular stores). From the bins it is observed that 10% of the calcium buffer Schwann cells had some sort of sustained response, and 12% of the non-calcium buffer Schwann cells had a sustained response.

The results for differentiated Schwann cells had a completely different outcome. Out of a study of 208 cells there are only 5.8% cells that have a calcium influx response after injury. Figure 3.3 shows a calcium trace in which you can clearly see that influx is evenly distributed and does not reach values of F/F_0 higher than 1.4. The control can be used to assure that cells responded, and to observe that there is a sustained response in the cells that did respond.

4.1.5 Focusing Test

Results from the focusing test can be used to assure the transient response in tells was due to a normal increase in F_0 intensity. Figure 3.2 shows a focusing test, in which you go in out of focus two times within 90 seconds. From the calcium traces it can be observed that there isn't a significant change in intensity after focusing in, which is typically what must be done preceding the injury insult to get a clear recording of the culture for the remainder of time

4.1.6 Morphology

Changes in morphology were studied in undifferentiated Schwann cells to observe if there are any of the following changes; re-myelination or degeneration following a severe injury on Schwann cells. Figure 3.7 shows degeneration in differentiated cultures an hour after injury. While figure 3.8 shows them at different times within 24 hours. The difference in both cultures is the **time** in which they were injured. Cultures from Figure 3.6 were injured 5 days later than cultures from Figure 3.8. This made them less resistant when they underwent stretch injury. Cultures from Figure 3.6 were under cyclic amp for too long.

Cultures from Figure 3.7 were studied over 24 hours. Following individual cells through morphological markers you can clearly see changes in cell morphology. Cells seem to change in size by the time they reach 24 hours. However, to clearly see changes in the injured cells, a stain should be used.

CHAPTER 5

CONCLUSION

Present Studies used Schwann cells to take a first look at glia cells undergoing a severe injury. Schwann cells are glia cells of the PNS, and can be used to determine the effects of calcium increases following an injury in glia cells. As previously mentioned, studies have shown that glia cells have intracellular stores of calcium. Cells were injured in a calcium free buffer solution and calcium buffer solution to study the effects of intracellular calcium in undifferentiated Schwann cells. From the results calcium response is coming from intracellular stores.

It is also very important to look at differentiated Schwann cells because after an injury to axons Schwann cells become myelinating cells. Results show that differentiated cells have a completely different response in calcium levels after injury, which may suggest that inducing differentiation may cause changes in sodium properties or calcium channels in undifferentiated Schwann cells. In this study there is no need to injure cells in a calcium free buffer since there are only 5.8% of cells that respond with a calcium increase. As mentioned earlier this can suggest that differentiated cells are more resistant to changes in calcium following an injury. It possible that these cells behave like this because they are regenerative cells.

The focusing test was done as a type of control to assure that changes in the levels of intensity whether sustained or transient, were due to the injury itself and not the refocusing that occurs directly after an injury insult. This test proved to be accurate judging from the results. It was also done to further study previous artifacts that where

seen in calcium traces due to focusing throughout the recording. Focusing should not be done while recording to avoid artifact in the data.

To further study changes in morphology in differentiated cells underwent a timed injury study. This study was primarily done to explore the following; 1) is there proliferation following an injury? 2) Are there any notable morphological changes in the cells following an injury? De-myelination? 3) From the results it is clearly observed that the cells do change in morphology following the injury. However, to really study morphological changes further studies will be needed. A suggested study would be Actin Staining with fluorescence in which cells will be able to be seen in phase contrast microscopy (Cytoskeleton Inc, 2015).

CHAPTER 6

FINAL STATEMENTS

Present studies in undifferentiated Schwann cells showed that calcium traces in both a calcium free buffer and calcium buffer solution, have an increase in calcium intensities upon injury and that intracellular stores of calcium are released upon injury. They both showed a similar response following an injury. However, for differentiated Schwann cells there was barely a response in calcium influx suggesting that inducing differentiation changes properties within the cells. Additionally, it should be suggested that differentiated Schwann cells are area that will require further studies especially because preventing demyelination and promoting myelination are an objective when studying Schwann cells. Focus should be on myelinating cells and how the morphology and pathology change after these cells are injured to understand the behavior after an injury.

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