ABSTRACT

AN IN VITRO STUDY TO CHARACTERIZE A NEW AUTOMATED HIGH THROUGHPUT NEURONAL STRETCH INJURY SYSTEM

by
Nicolae Valerian Hususan

Traumatic brain injury (TBI) is one of the most prevalent long-term neurological conditions and its overall economic consequences rival that of stroke. Considering the importance of the topic, new models for studying TBI are often designed and created. Based on a model developed by the University of Pennsylvania, NJIT constructed a fully automated version, which injures neuronal cell cultures in uniaxial regime and provides the high experimental yield required in pharmaceutical and neuroscience studies.

In this investigation, a new methodology for culturing primary cortical neurons of rat origin was established. Viable cell cultures developed for the first time in NJIT laboratory were successfully injured using the new NJIT device. The data recorded here may allow us to understand better the complex aspects of head trauma mechanisms and to develop potential therapeutic agents.

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by
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To my family

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CHAPTER 1

INTRODUCTION

1.1 Aims

The vital importance of studying and understanding the head trauma mechanisms is supported by the great challenges the condition presents for patients, families, physicians, therapists, and society. In the United States, the traumatic brain injury is more prevalent than cases of multiple sclerosis, HIV/AIDS, and breast cancer combined. The total direct (medical) and indirect (lost productivity) costs associated with the disease are high, being estimated at about 60 billion dollars annually [1, 2].

Traumatic brain injury (TBI) is a grave disease. Of the approximately 1.4 million people experiencing various types of head traumas and admitted in hospital emergency rooms every year, one quarter experience a form of moderate or severe TBI. The death rate is around 50,000 people and another 80,000 people survive with disabilities for the rest of their lives [2].

Considering the significance of the topic, new *in vivo* and *in vitro* TBI models are often designed and developed, the research being funded by various governmental and nongovernmental organizations. It is surprising though, that the only one *in vitro* model that mimics the real mechanical deformation of the brain during TBI has been designed and used by the University of Pennsylvania and its collaborating laboratories [3]. The Penn device considers the rotational motion of the head, caused by direct or indirect force, as being the main reason of TBI injury. Indeed, the rotational motion of the head produces shear deformation of the brain inside, that translates into uniaxial stretch of

neuronal fibers (axons).

While this model is a reliable research instrument offering valuable information to the scientist, it has inherent drawbacks. In order to correct some of its limitations, an improved version of the system (NJIT model) was constructed by our group. The original multi-well culture plate utilized with the NJIT device is high throughput, addressing the problem of low experimental yield that the Penn model presents. The large tissue samples often required in pharmaceutical and neuroscience studies are easily produced with this device. The cost efficiency of rather laborious studies (proteomic analyses, immunocytochemistry, or treatment screening) is achieved through reducing the investigation time and accelerating the process. The NJIT device is also fully automated, resolving the issue of difficult operation and handling, which the Penn model has.

The present work is part of a series of theses on NJIT injury device project. The objectives of this study were to establish viable cultures of primary cortical cells necessary in NJIT laboratory experimentation, test the biocompatibility of the injury device materials, and create the cellular architecture (uniaxially aligned axons) to mimic stretch injury. A procedure to isolate and culture rat cortical neurons, inspired by Dr. Kacy Cullen (University of Pennsylvania), was implemented and neurons could be maintained healthy for the entire length of the experiment. In addition, the healthy cultures confirmed the biocompatibility of the multi well culture plate made of PEEK and the other adjacent materials (silicone membrane, silicone glue). The method of attaching the silicone membrane (bottom) to the PEEK plate was also finalized. To demonstrate the operation of the NJIT device and the cultures' viability, six experiments were performed (three before and three after injury), and data was recorded and analyzed. Well dividers

were designed with ProE software and printed using a special technique. They were used to produce the cell free zone (axon gap) and axons were successfully grown across.

In summary, rat cortical neurons were successfully isolated and cultured in NJIT laboratories and subsequently injured using an original NJIT injury device. The data recorded here may allow us to understand better the complex aspects of head trauma mechanisms and to develop potential therapeutic agents.

1.2 Background

1.2.1 Traumatic Brain Injury

Statistics show that every 15 seconds in the United States somebody suffers a traumatic brain injury (TBI) [4]. Like other injuries, this is a sudden and acute event that may have a serious outcome depending on the severity of the damage at the brain level. Hippocrates, the father of medical science, considered all brain injuries significant, but not necessarily devastating [5].

TBI is a type of acquired brain injury, as opposed to the hereditary or degenerative brain injuries, caused by the violent contact of the head with an external object, which disrupts the brain function. If the object does not penetrate the skull, TBI is considered a closed head injury. In this case, the rotational movement of the head, generated by a rapid acceleration or deceleration, is the source of the brain damage [6]. The leading causes of TBI are: falls (28%), motor vehicle accidents (20%), struck by or against events (19%), and assaults (11%) [2].

There are a few different systems (Glasgow Coma Scale, Ranchos Los Amigos Scale, etc.) [7] that medical practitioners use to diagnose the symptoms of TBI. Loss of

consciousness (amnesia or coma) is one of the most common clinical symptoms. Based on its duration, TBIs are traditionally classified as mild, moderate, and severe. The initial loss of consciousness can be followed by other devastating events such as hematoma, diffuse axonal injury, neuronal death, cerebral hypoxia, ischemia, and edema. The consequences of TBI are very intricate, rarely limited to one set of symptoms. The specific nature of disabling conditions may be orthopedic, visual, aural, neurologic, perceptive/cognitive, mental/emotional, with serious impact on society [8].

At the microscopic level of TBI, a whiplash movement of the head, caused by an external force, starts a cascade of cellular events inside the brain. The magnitude of the acceleration and deceleration of the skull, especially during centrifugal motion, is transmitted as shear deformation between the layers of brain matter, which have different density (weight). The shear deformation causes uniaxial stretch to the axons aligned diagonally in the analyzed slab of brain tissue. In the rare case of very severe insult to the head, the mechanical stretch of the axons can be followed by total axotomy (Figure 1.1) [9].

Focal cortical contusion (FCC) and diffuse axonal injury (DAI) are the most common TBIs with the greatest implication on patient's long-term outcome [11]. In FCC the brain damage is restricted to a single area, as opposed to DAI, which is dispersed, affecting more than one region. DAI has higher frequency and graver effects. It was originally defined by Strich [12], as being produced by the mechanical forces that shear the fibers (axons) of the cerebral white matter at the time of the impact and immediately afterwards. Initially, the ionic homeostasis and permeability of the axolemma are modified. This avalanche of biochemical events is often followed by axonal

disconnection and failure. The degree of axonal damage can vary from superficial functional abnormalities (confusion, short loss of consciousness) to acute and widespread axonal disruption (prolonged coma) [13].

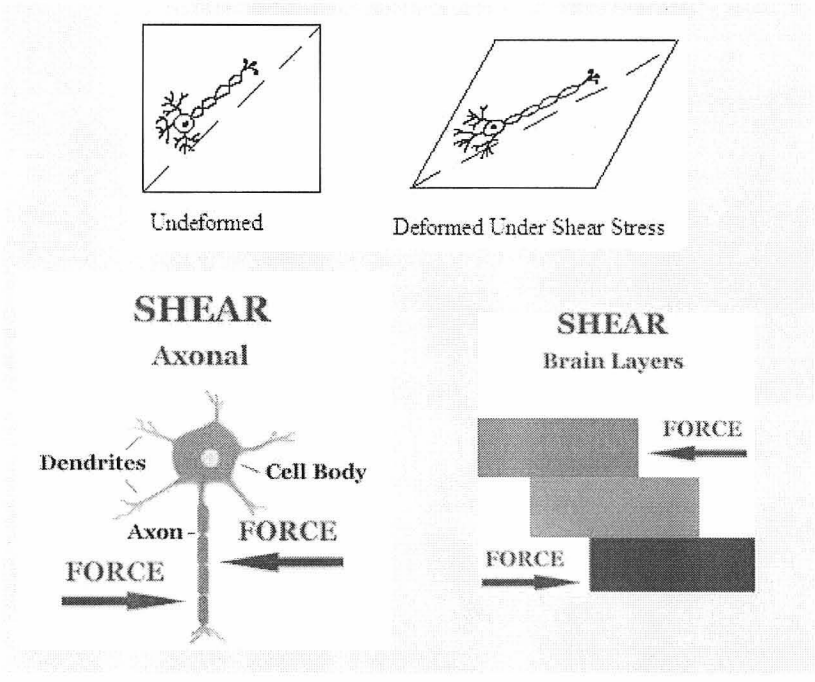


Figure 1.1 Shear stress of brain converted in uniaxial stretch of axon fibers [10].

TBI presents a serious health problem in the World today, and developing both *in vivo* and *in vitro* models is a necessary scientific approach to understand, prevent, and eventually treat the disease.

1.2.2 Traumatic Brain Injury Models

Numerous investigators have developed *in vivo* and *in vitro* models for studying traumatic injury of the central nervous system (CNS). Both models have advantages and disadvantages and are usually required before human trials occur. Typically, an *in vivo*

model is very complex, expensive (cost of animals), and difficult to interpret (many variables, variation between animals). It is successful in studying the overall effects of an experiment on its subjects but does not guarantee the same outcome in humans. The legal regulations and human concerns are other two reasons why scientific research has to begin with *in vitro* models. They simulate *in vivo* models providing data not obtainable otherwise. The *in vitro* counterparts are more focused (particular tissue or cell), less expensive, and highly productive (multiple samples). They provide real time information but require a great amount of work and have the contamination issue (lack of immune system). The *in vitro* models cannot completely replace the *in vivo* models but they allow the isolation and measurement of purely mechanical injury in the absence of secondary mechanisms [14].

Some of the models were simple, consisting of a culture dish in which the cells were scratched off with a stylet [15]. Other models were more complex, the neuronal cultures being injured by the means of: hydrostatic pressure to achieve a compression effect [16], applied load or acceleration [17, 18], and deformation of a cultured elastic membrane [9, 14, 19]. The NJIT model belongs to this last category, of which a brief summary of the most significant examples will be presented in the following paragraphs.

1.2.2.1 The Ellis Model [19]. Ellis et al. created a simple and reproducible (all parts are commercially available, Flexcell International, McKeesport, PA) *in vitro* model that can be utilized to injure mechanically and study brain tissue-cultured cells from a morphologic, physiologic, and biochemical point of view. Cortical astrocytes from 1- to 2-day-old rats were cultured in commercially available six-well Flex Plate. Each 25 mm-diameter well had a 2 mm-thick flexible silastic bottom coated with collagen.

The cultured plates were injured dynamically using a 94A Cell Injury Controller (Commonwealth Biotechnology, Richmond, VA) model. The Controller, used in a closed system, regulates the pressure (from 0 to 50 psi) and duration (from 1 to 100 msec) of the pulse (rapid positive pressure) through a valve (Figure 1.2).

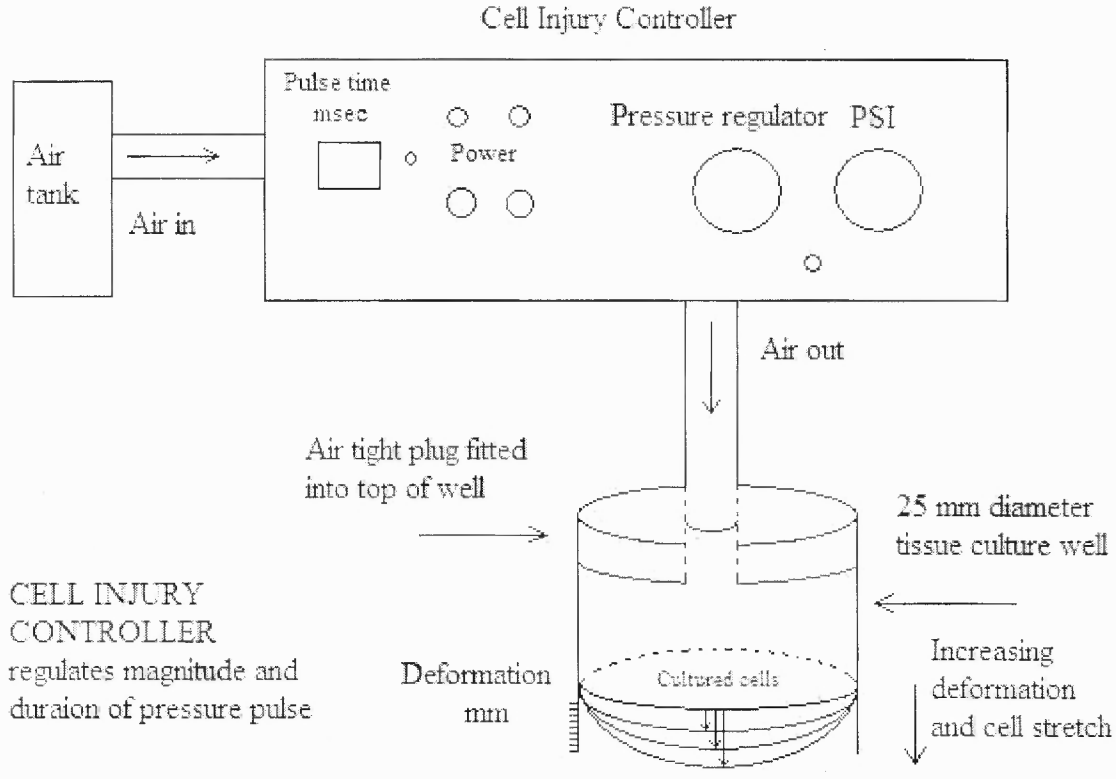


Figure 1.2 94A Cell Injury Controller (Commonwealth Biotechnology, Richmond, VA) [19].

From biomechanical studies is known that the tissue strain can be compressive, shear, or tensile (stretch) and after TBI all three components may occur. This model considers the stretch as being the main constituent of injury. It is also known that stretch is not uniform across a deformed silastic membrane. However, the present study showed that the deformation of the flexible membrane cultured with rat astrocytes was proportional to the amplitude and the duration of the pulse. The cell injury was also

proportional to the deformation of the membrane and it was evaluated qualitatively using microscopy and quantitatively through assessing the propidium iodide (PI) uptake and the lactate dehydrogenase (LDH) release.

The PI culture staining is a simple method to investigate cell injury. PI is a dye that penetrates only the membrane of injured cells and binds to their DNA. Fluorescence microscopy is used to observe and count the injured cells that appear red after staining. In this experiment, as expected, the degree of cell injury quantified by the number of PI stained cells was proportional to the amount of mechanical stretch.

LDH, another biochemical marker of cell injury, is an intracellular enzyme that is released by the injured cells. Present study confirmed that the LDH released was directly proportional with the degree of membrane deformation and cell stretch.

Ellis et al. *in vitro* model is simple and easy to reproduce in any laboratory. It was successfully evaluated using two biochemical markers, PI uptake and LDH release. The instrument offers another way of injuring various types of cell cultures and understanding TBI.

1.2.2.2 The Morrison III Model [14]. The following system was designed considering the previous models in which a clamped and cultured membrane was deformed in order to investigate the effect of deformation on the brain cells. The loading conditions chosen to produce mechanical damage to the cultured tissue were intended to simulate the nonimpact, inertial brain injury *in vivo*.

The device uses an O-ring to attach an elastic membrane (Specialty Mfg., Saginaw, MI), 0.38 mm thick, to a 60 mm stainless steel well that has an 18 mm diameter center hole. Various types of cells can be cultured on top of the membrane. The

previously mentioned well is clamped to a heated (37°C) brass plate (Minco, Minneapolis, MN) forming together a pressure chamber, which is evacuated to deform the membrane. Two solenoid-needle valve combinations allow for control of the rate flow of vacuum or exhaust and implicitly the membrane displacement. The deformation is measured with a laser displacement transducer (LDT) (Omron Electronics, Schaumburg, Il) that can be positioned using a 3-axis manipulator (Newport, Ervine, CA) situated under the brass plate. The entire system is controlled by a computer (ASO-1200 Keithley, Taunton, MA) and custom software (Matlab, The Math Works, Inc., Natick, MA) (Figure 1.3).

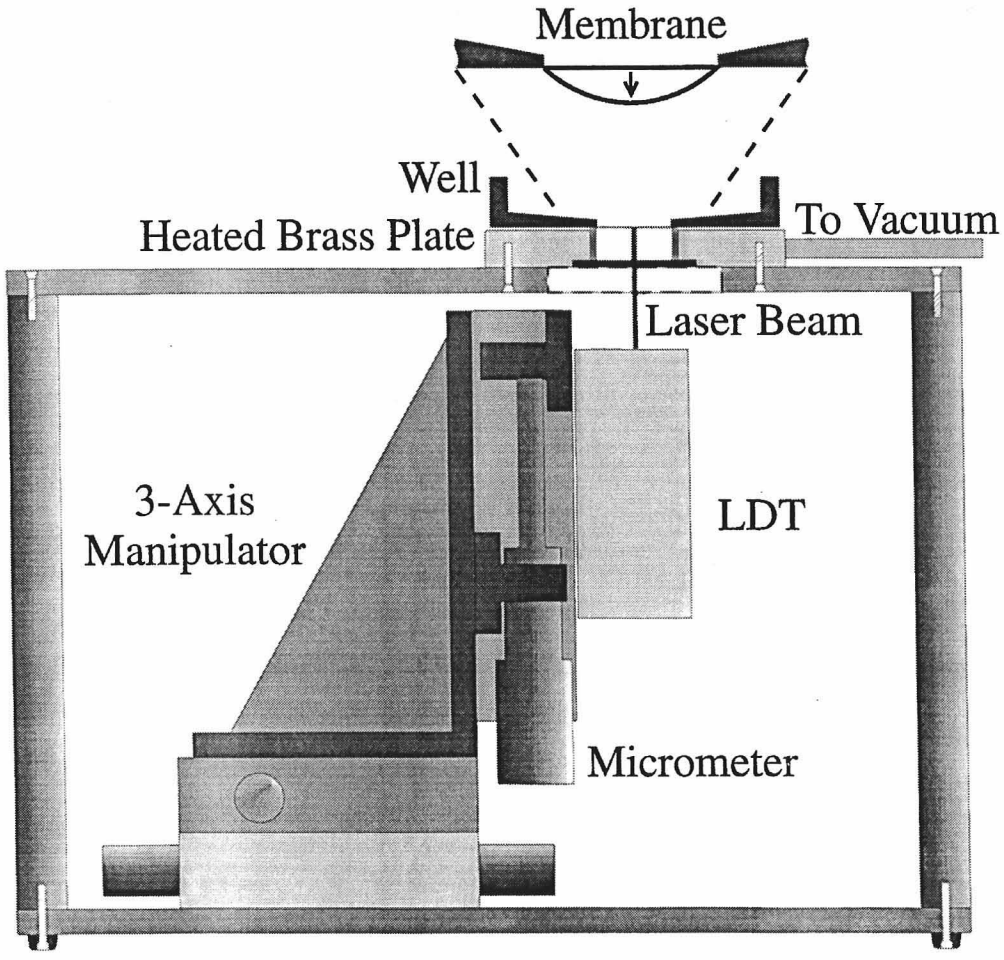


Figure 1.3 Morrison III B. et al. injury device [14].

Although an *in vitro* model does not elucidate the entire spectrum of injury mechanisms it becomes critical in understanding the complex aspects of TBI. In particular, the improvements associated with this model relate to the noncontact measurement of the membrane deflection, precise control of the deflection, and a wide range of strains (up to 0.65) and strain rates (up to 15 s^{-1}), values considered to be clinically relevant.

1.2.2.3 The Penn Model [3]. This *in vitro* model examines the deformation of mammalian CNS axons in a dynamic loading regime at specific strain rates and determines the threshold for primary axotomy. In order to do so, the system delivers uniaxial deformation intended to replicate the mechanical forces experienced by axons *in vivo* during TBI.

A N-Tera2 cl/D1 (NT2) cell line was differentiated and cultured. Neurons were isolated using trypsin, triturated, and replated on a deformable substrate coated with poly-D-lysine, fibronectin, and Matrigel. A 1.5 X 16 mm barrier created the "gap" at the center of membrane. Cells were allowed to attach for 24 hours before the barrier was removed. The cultures were maintained 3 weeks (maturing time of neurons) before an experiment, sufficient time for the axons to cross the gap and synapse in the other side.

The stretching device is made of an aluminum top with a quartz window, a stainless steel plate with a 1.5 X 18 mm slit, and an air pulse source. The cultures are introduced in the sealed chamber formed by the aluminum cover and the stage of the microscope. A solenoid (Parker General Valve, Elyria, Ohio) controls the air pulse that is allowed in the chamber through an air inlet and a dynamic pressure transducer (Entran model EPX-V01-25P-/16F-RF, Fairfield, NJ) measures the pressure inside. An analog–

to–digital board (Keithley, Metrabyte, Cleveland, OH) coupled with a computer (Capital Equipment Corporation, Billerica, MA) regulates the solenoid and the pressure transducer. The axons can be observed and photographed during the experiment through the quartz window (Figure 1.4).

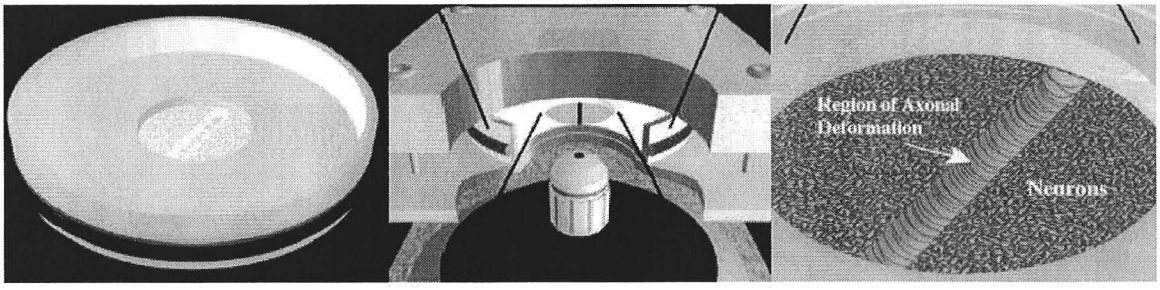


Figure 1.4 The Penn Model [3].

This *in vitro* system achieved strains of 0.58-0.77 (58%-77%) at rates of applied strain between 26 and 35 sec^{-1}, values considered within the range for traumatic injury. The response of the axons to the transient stretch that mimics the *in vivo* conditions had the form of axonal swellings containing accumulation of neurofilament proteins (axonal transport issue or disassembled cytoskeleton). Everything was observed in real time with the Nikon-inverted microscope (Optical Apparatus, Ardmore, PA). The axons were resilient to primary axotomy and demonstrated the ability to recover their original shape and orientation. Most important, the model offers similar patterns of traumatic brain injuries as observed clinically.

1.2.3 Comparison of NJIT Model with Previous Models for Studying TBI

The systems presented above as well as other systems not included here have tried to elucidate the intricate biomechanical factors that lead to axonal damage in TBI. The NJIT

uniaxial stretch device in particular can be considered a technical summation of the previous models, especially the Penn Model, trying to address some of its intrinsic drawbacks: low experimental yield, long investigation time, and difficult operation and handling.

The first issue our system deals with is the low productivity experienced by the other *in vitro* models. Typically, an *in vitro* system injures one culture at the time and requires numerous experiments and high level of expertise to gather the amount of tissue samples requested by the biomedical research. A 24 well injury plate made of PEEK (polyetheretherketone), which has basically the same dimensional specifications as a regular 24 well tissue culture plate, provides the same amount of data after one trial as any of previous models do after one week. The plastic PEEK plate is autoclavable and can be sterilized and reused. Compared with the stainless steel culture plate (Penn model), the PEEK culture plate is highly biocompatible, quality observed in the present research (Figure 1.5).

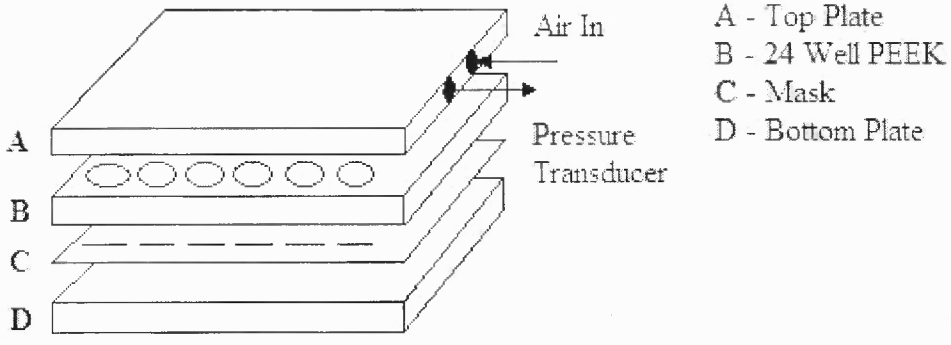


Figure 1.5 Schematic of 24 well injury plate and pressure chamber.

Secondly, the two important parameters that influence the pulse that produces the deformation in the injury chamber, the input air pressure and the time the solenoid valve

is open, have also been modified. The input pressure was increased from 50 psi to about 100 psi to allow the pressurization of a chamber that has a higher volume. The response time of the solenoid valve was also improved to 20 msec, with 2 -3 msec rise time. Short rise times that characterize the injury in dynamic regime due to rapid acceleration/deceleration can be obtained with this fast acting response. Furthermore, the NJIT model is fully automated requiring little experience for operation as opposed to the manually controlled Penn device.

Another improvement introduced by the NJIT model refers to the prevention of any pressure decrease inside the injury chamber during the experimentation. This can be achieved through a pressure reservoir that confers stability and consistency to the driving pressure pulse. It is controlled in closed loop mode through a proportional valve by custom computer software (Figure 1.6).

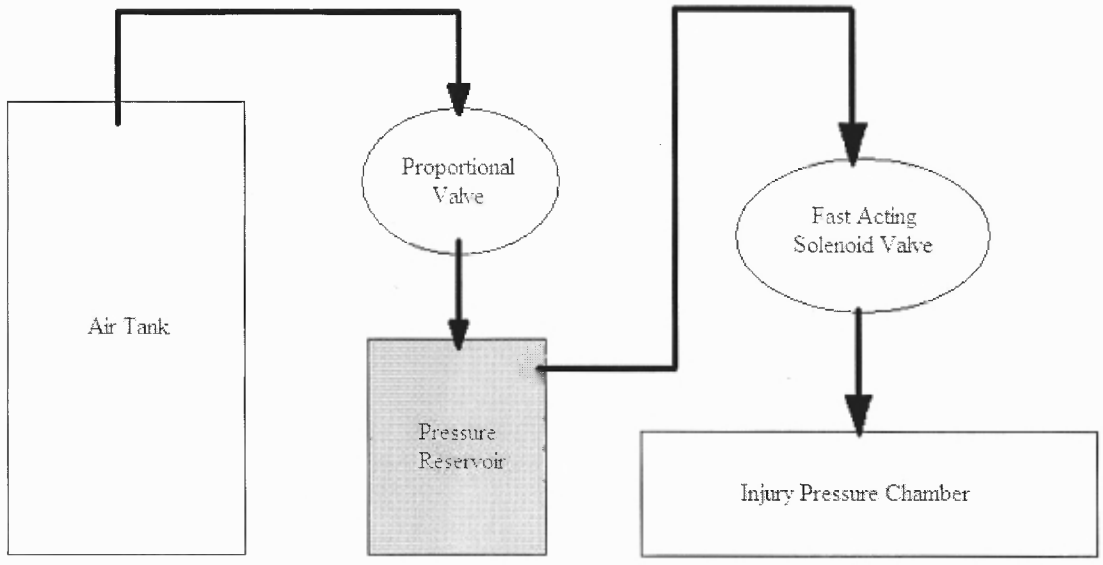


Figure 1.6 Schematic of the NJIT device air flow.

In theory, the NJIT *in vitro* device is technically superior to the other models presently used to injure in uniaxial regime various types of neuronal cultures. It is fully automated, easy to utilize, and very consistent. From a financial point of view, the system is highly efficient, reducing substantially the research time. To show its excellent qualities, viable cortical cell cultures will be mechanically stretched by the NJIT device and data will be analyzed prior and post injury.

CHAPTER 2

METHODS AND MATERIALS

2.1 Primary Cerebral Cortical Neuron Cell Culture and Staining Methodology

A cell isolation protocol depends on the type and properties (size, density, surface marker, etc.) of the target cell, in our case embryonic rat cortical neurons. It is the procedure that aims to maximize the yield of functionally viable, dissociated cells.

There are many factors that influence the outcome of such a procedure. Type of tissue and species of origin are considered "constants" because in general they are not a matter of choice. On the other hand, factors like age of animal, dissociation medium, enzymes used, concentration of enzymes, temperature, and incubation time are considered "variables" in the process and they are best defined empirically, through trial and error [20].

Primary cortical neurons from E17 rats were chosen for these *in vitro* experiments because adult cortical neurons do not survive the isolation procedure. E17 embryos were preferred to E16 or E15 since they were easier to harvest. The dissociation medium is customized to this particular type of cells and is based on previous successful experimentation with dorsal root ganglions in NJIT laboratories. The strongly digestive enzyme trypsin is commonly used for dissociation procedures. It was supplemented with DNase I (deoxyribonuclease I) to decrease the dissociation medium viscosity and reduce toxicity caused by the cell damage [20]. The enzyme concentration, temperature, and incubation time were determined through trial and error and compared with values found in scientific literature.

One of the objectives of the project is to prove that the high throughput 24 well plate made of PEEK is biocompatible and PEEK is appropriate material choice for neuronal cell cultures. The other component materials - the silicone sheet and the sealant used to attach the silicone membrane to the PEEK culture plate - have to be also biocompatible. In addition, all these material choices allow the 24 well plate to be reusable and autoclavable, without causing any contamination. To assess the biocompatibility of the material choices, the viability of multiple 24 well plate cultures has been studied using live/dead assays over ten-day period. Another cost efficient alternative (ABS plastic used to make a 24 well culture plate and the well dividers) has been examined. The mentioned components can be printed rapidly, reducing the time and cost of the experiment.

This is an entirely novel approach that did not exist before and requested long time to develop. All protocols and methods that follow are new and original.

2.1.1 Preparation of 24 Well Plate (PEEK)

2.1.1.1 Glue Silicone Sheet to 24 Well Plate (Monday).

1. Cut one silicone piece that has approximately 116 X 77 mm (0.005 inches thick) from a silicone sheet (Specialty Manufacturing, Saginaw, MI).
2. Wash silicone piece with diluted dish washing liquid soap in water, rinse excessively with purified DI water, let dry, and lay on clean sheet of paper.
3. Place top of fixture frame on top of silicone piece trying to avoid wrinkles and bubbles (Figure 2.1).
4. Attach bottom of fixture frame to top of fixture frame and silicone with 8 clamps (Figure 2.2).
5. Use little water and one drop of detergent to wet tensioning plastic block and assure tension of silicone piece is uniform.

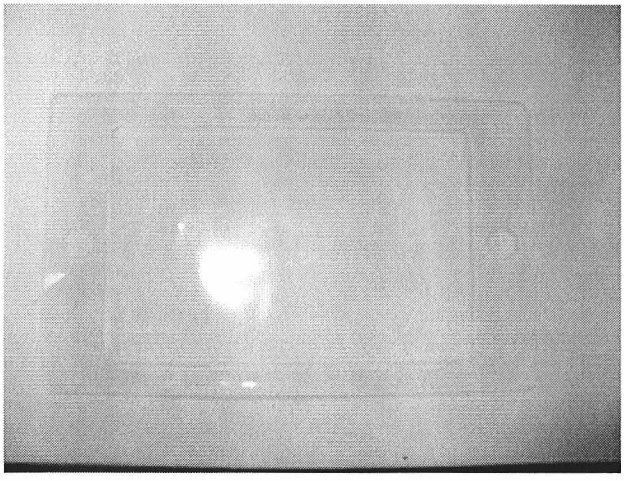


Figure 2.1 Top of fixture frame on silicone piece.

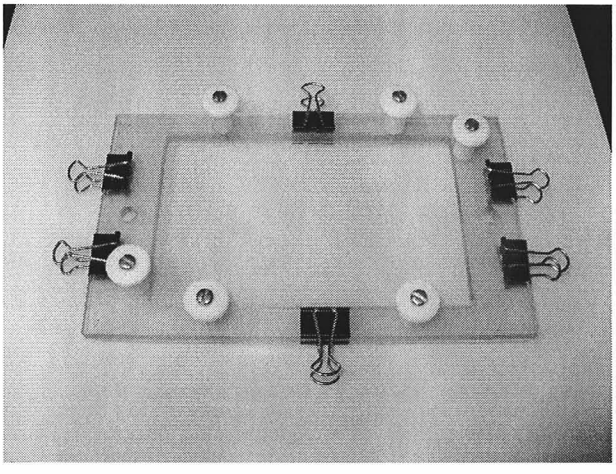


Figure 2.2 Top and bottom of fixture frame with silicone piece and clamps.

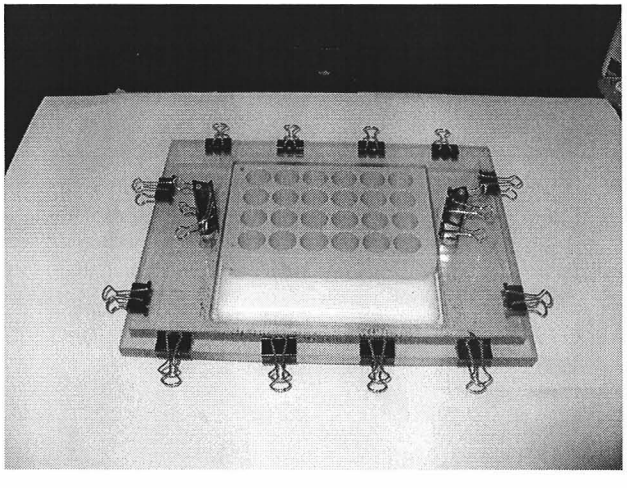


Figure 2.3 Fixture frame and 24 well culture plate (PEEK).

6. Tension slightly silicone piece by pulling fixture frame and silicone sheet over plastic block and fix it in position with another 4 clamps (Figure 2.3).

7. Coat bottom of 24 well plate with thin layer of glue (Dow Corning RTV Sealant 734) using roller. Minimize amount of glue inside 24 wells (Figure 2.4).

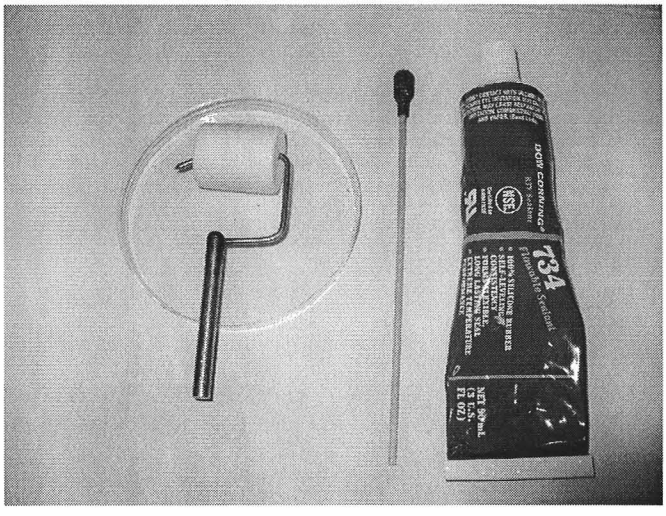


Figure 2.4 Roller, applicator, and sealant.

8. Place 24 well plate on pre-tensioned silicone sheet (Figure 2.3).

9. Place weight on top of 24 well plate.

10. Let it cure at room temperature for 24 hours before continuing with preparation.

2.1.1.2 Sterilize 24 Well Plate (Tuesday).

1. Remove weight from top of 24 well plate.

2. Trim silicone sheet edges with cutter.

3. Inspect if silicone sheet is attached properly (air bubbles).

4. Fill every well of plate with 2 ml DI water, wrap plate in aluminum foil, and place it in small autoclavable plastic box.

5. Autoclave box with 24 well plate for 120 minutes, long cycle for liquids in autoclave (Market Forge Sterilmatic).

6. Remove box from autoclave after cooling down, clean exterior with alcohol 70% and place in laminar flow hood.

7. Open box, remove aluminum foil, pipette out water from 24 well plate, and check for leaks.

8. Open box, remove aluminum foil, pipette out water from 24 wells plate, and check for leaks.

9. Rinse 24 wells with sterile DI water and let dry.

2.1.1.3 Prepare PLL Solution and Coat 24 Well Plate with Poly-L-Lysine (PLL).

1. Remove one PLL aliquot from freezer and let thaw.

2. Vortex aliquot, clean with 70% alcohol, and place in laminar flow hood.

3. Mix 500 µl PLL (100X stock) with 50 ml sterile DI water in 50 ml tube and vortex afterwards.

4. Add 1 ml PLL solution to each well and 2 ml to each control culture dish.

5. Leave PLL solution at least 1 to 2 hours.

6. Remove PLL solution with pipette and let dry for at least 1 to 2 hours.

7. Rinse 24 wells and culture dishes 3 times with sterile HBSS and let dry overnight. They can also be refrigerated for future use. No UV light to be used inside laminar flow hood with PLL coated plates.

2.1.2 Filters Preparation (Day Before Isolation)

1. Cut bottom of three 15 ml autoclavable plastic tubes with cutter.

2. Cut squares of three size meshes (Sefar) big enough to cover circular cross section of tubes.
   - Precision woven nylon mesh 376x376, .0012" opening.
   - Precision woven nylon mesh 307x307, .0020" opening.
   - Precision woven nylon mesh 168x168, .0039" opening.

3. Attach meshes to tubes using rubber O rings and mark tubes 1, 2, and 3 according to their mesh size.

4. Trim excess mesh (Figure 2.5).

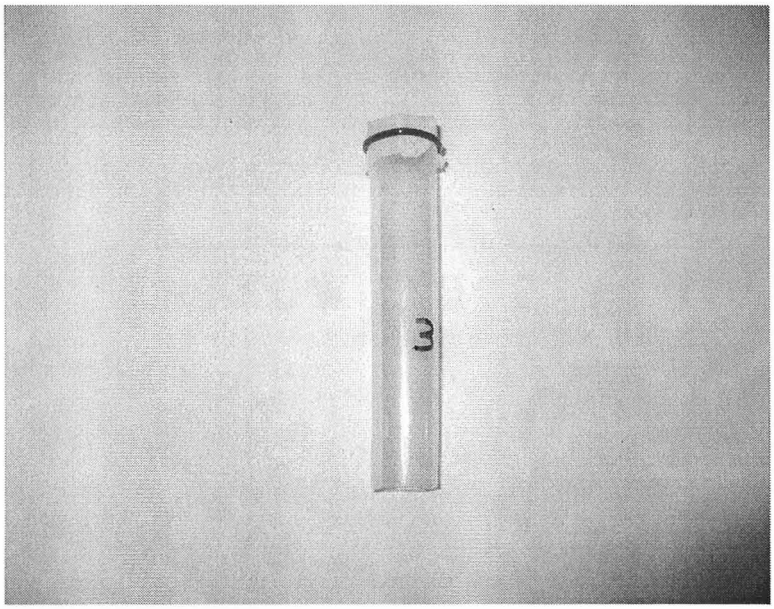


Figure 2.5 Custom-made filter size 3.

5. Autoclave custom filters in beaker covered with aluminum foil.

6. Place in laminar flow hood for dissociation.

2.1.3 Other Preparations (Isolation Day)

1. Fill up cooler with ice.

2. Fill two 50 ml tubes with HBSS and keep on ice.

3. Prepare one 50 ml tube with Neurobasal Medium (Invitrogen, Gaithersburg, MD) supplemented with 125 µl L-glutamine and 1 ml B-27 (Invitrogen), 1% fetal bovine serum (HyClone, Logan, UT), and 1% penicillin-streptomycin (Invitrogen).

4. Thaw one tube with 5 ml trypsin (0.25% + 1mM EDTA) supplemented with 1 mg/ml DNase I (Roche, NJ) and place in incubator at 37°C and 0 % CO2.

5. For isolation gather two sterile 25 ml tubes, two sterile 100 mm culture dishes, sterile dissection instruments, and one bottle of 70 % ethanol.

NOTE: As a result of cell damage, DNA may be present in the dissociation medium increasing toxicity, viscosity, and causing handling problems. Pure DNase is added to digest the DNA without causing any harm to the viable cells [20].

2.1.3.1 Autoclave (Sterilize) All Dissection Instruments (Day Before Isolation).

1. Clean instruments with DI water and rinse with 70% ethanol.
2. Autoclave instruments (instruments cycle) wrapped in aluminum foil in small autoclavable plastic box.

2.1.4 Harvest Procedure

2.1.4.1 Instruments (Figure 2.6) [21].

1. Two standard surgical scissors (straight and curved) (A)
2. One standard pattern forceps (B)
3. One tissue forceps (C)
4. One angled forceps (D)
5. One angled dissector scissors (E)
6. One Dumont forceps (size 4 & 5) (F)
7. Two scalpels (G)

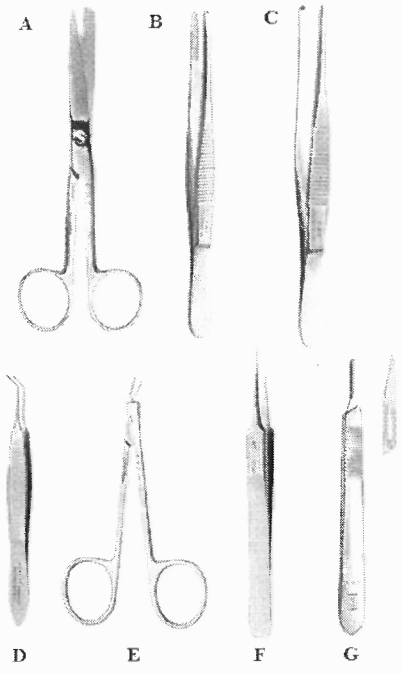


Figure 2.6 Instruments [21].

2.1.4.2 Harvest Procedure (Rutgers Animal Facility).

1. Get one time-pregnant E 17 (embryonic day 17) Sprauge-Dawley rat (Charles River, Wilmington, MA).

2. Euthanize animal using exposure to 100% CO2 for 5 minutes.

3. Transfer animal on a surgical table previously cleaned with 70% ethanol.

4. Lay body on table with abdomen up and rinse abdomen thoroughly with 70% ethanol.

5. Cut from lower abdomen to chest (diaphragm) to expose uterus. Instruments: curved surgical scissors, tissue forceps.

6. Puncture diaphragm with scissors.

7. Remove uterus and place in 100 mm culture dish. Cover dish immediately and place on ice. Instruments: straight surgical scissors, standard pattern forceps.

2.1.4.3 Harvest Procedure (Rutgers NJIT Laboratory).

8. Spray ethanol 70% on closed culture dish containing uterus and cortical isolation instruments box and place inside sterile laminar flow hood.

9. Remove fetuses from amniotic sacs and transfer to another 100 mm culture dish containing ice-cold HBSS. Keep dish on ice during isolation.

10. Transfer one fetus in culture dish containing ice-cold HBSS. Culture dish is fixed on microscope stage with double-side tape.

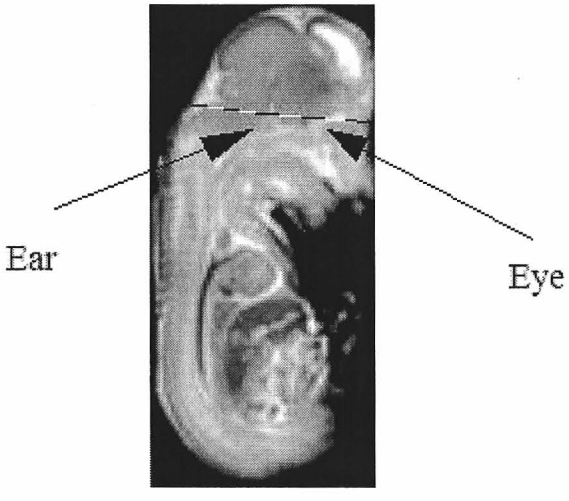


Figure 2.7 Embryo skull cut line [22].

11. Place embryo left side down. Fix embryo head down with one scalpel through left ear. Cut across line ear – eye half way through with second scalpel (Figure 2.7).

12. Open skull and gently drag out brain with second scalpel.

13. Remove embryo and place in 25 ml tube containing HBSS. Keep tube on ice. Embryos can be used for further DRG isolation.

2.1.5 Isolation Procedure

1. Place brain on sterile culture dish with hemispheres up (dorsal view) (Figure 2.8).

2. Detach / peel off meninges from two hemispheres with one Dumont forceps holding brain with second Dumont forceps in areas 1, 2 (Figure 2.8).

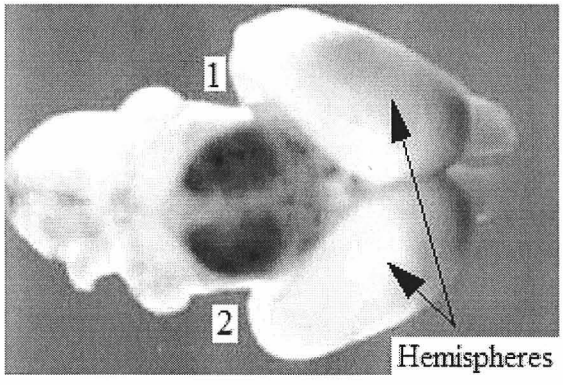


Figure 2.8 Brain dorsal view [22].

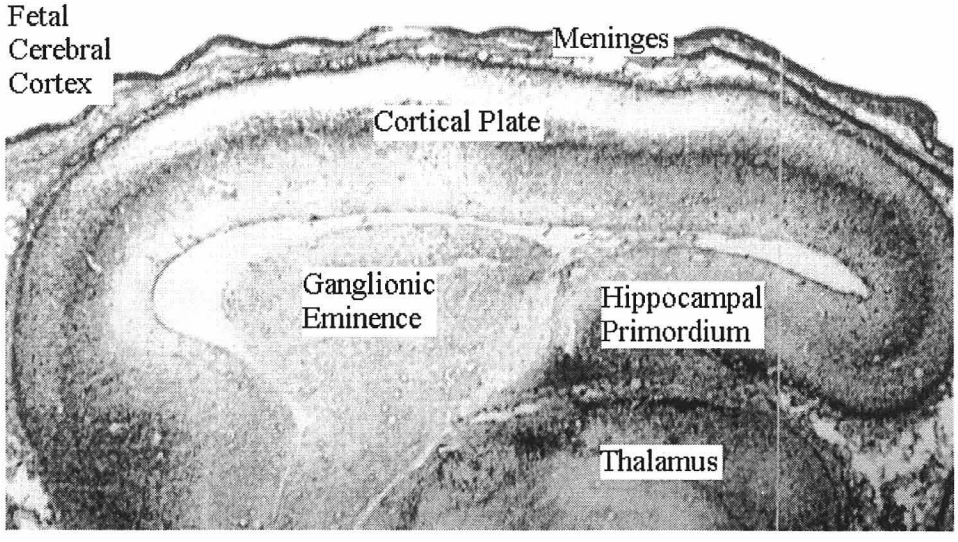


Figure 2.9 E-17 fetal cerebral cortex stained with anti-mouse Ig antiserum [23].

3. Open hemispheres such that lateral surfaces face down (Figure 2.9).

4. Remove cortical plates and place in 25 ml tube containing HBSS. Keep tube on ice.

5. 6-8 brains (12-16 hemispheres) are typically harvested. Expected yield is 3-5 million cells/hemisphere.

6. Dissociate immediately (recommended).

2.1.6 Dissociation Procedure (Chen NJIT Laboratory)

2.1.6.1 Preparation.

1. Place tube with cells in laminar flow hood and keep on ice.

2. Transfer 50 ml tube with Neurobasal Medium (Invitrogen, Gaithersburg, MD) supplemented with 125 µl L-glutamine and 1 ml B-27 (Invitrogen), 1% fetal bovine serum (HyClone, Logan, UT), and 1% penicillin-streptomycin (Invitrogen) and one tube with 5 ml trypsin (0.25% +1mM EDTA) supplemented with 1 mg/ml deoxyribonuclease I (DNase, Roche) to 37°C water bath.

2.1.6.2 Dissociation Procedure.

1. Remove initial HBSS as much as possible (careful not to disturb cortical tissue, do not use vacuum system) with small pipette.

2. Rinse two times with HBSS to remove debris.

3. Add pre-warmed (37°C) 5 ml trypsin (0.25% +1mM EDTA) supplemented with 1 mg/ml deoxyribonuclease I (DNase, Roche) to cells.

4. Incubate cells and trypsin solution 10 to 20 minutes in incubator at 37°C and 0 % CO2.

5. Remove trypsin being careful not to disturb tissue/cells.

6. Rinse two times with supplemented Neurobasal Medium to inactivate trypsin.

7. Re-suspend in 10 ml supplemented complete Neurobasal Medium and triturate gently (about 10 times) with pipette to break up tissue clumps.

8. Filter cell suspension using three custom-made filters.

9. Centrifuge cells at 1000 rpm (#3 setting) for 3 minutes.

10. Remove supernatant.

11. Re-suspend cells in 1 ml supplemented Neurobasal Medium and triturate gently few times.

2.1.7 Cell Counting

2.1.7.1 Cell Counting Notes. The cell concentration of a suspension of cells is important in order to perform appropriate cell culturing and viability measurements. The dissociation procedure also requires precise quantification of results. The most common method of cell counting involves the use of a counting chamber, called hemocytometer. The other supplies used for the procedure are:

1. Improved Neubauer Brightline Hemocytometer (Hausser Scientific).
2. Micropipettor.
3. Microscope 10X (Nikon TMS).
4. Counter.
5. HyQ trypan blue solution (HyClone, Logan, UT) - vital stain.

The hemocytometer slide has a highly polished semisilvered counting area (counting grid). A coverslip is placed over the 0.1 mm deep counting chamber (Figure 2.10). The cell suspension is introduced in the capillary space between the slide and the coverslip using a pipette through one of the V-shaped wells. The center grid square is 1x1 mm^2.

2.1.7.2 Cell Counting Procedure.

1. Carefully clean counting chamber surface and coverslip with 70% isopropanol and let dry.
2. Wet the edges of coverslip and press coverslip down onto slide.
3. Take 50 µl aliquot of well mixed dissociated cell suspension and mix with 450 µl

trypan blue solution.

4. Pipette up and down gently to mix well.

5. Add 10 µl of mixture to each V-shaped well of hemocytometer.

6. Place hemocytometer on stage of 10X microscope (Nikon TMS). Focus and adjust contrast to see clearly counting grid and dispersed cells.

7. Count cells in five squares from each grid (center and four corners) using counter.

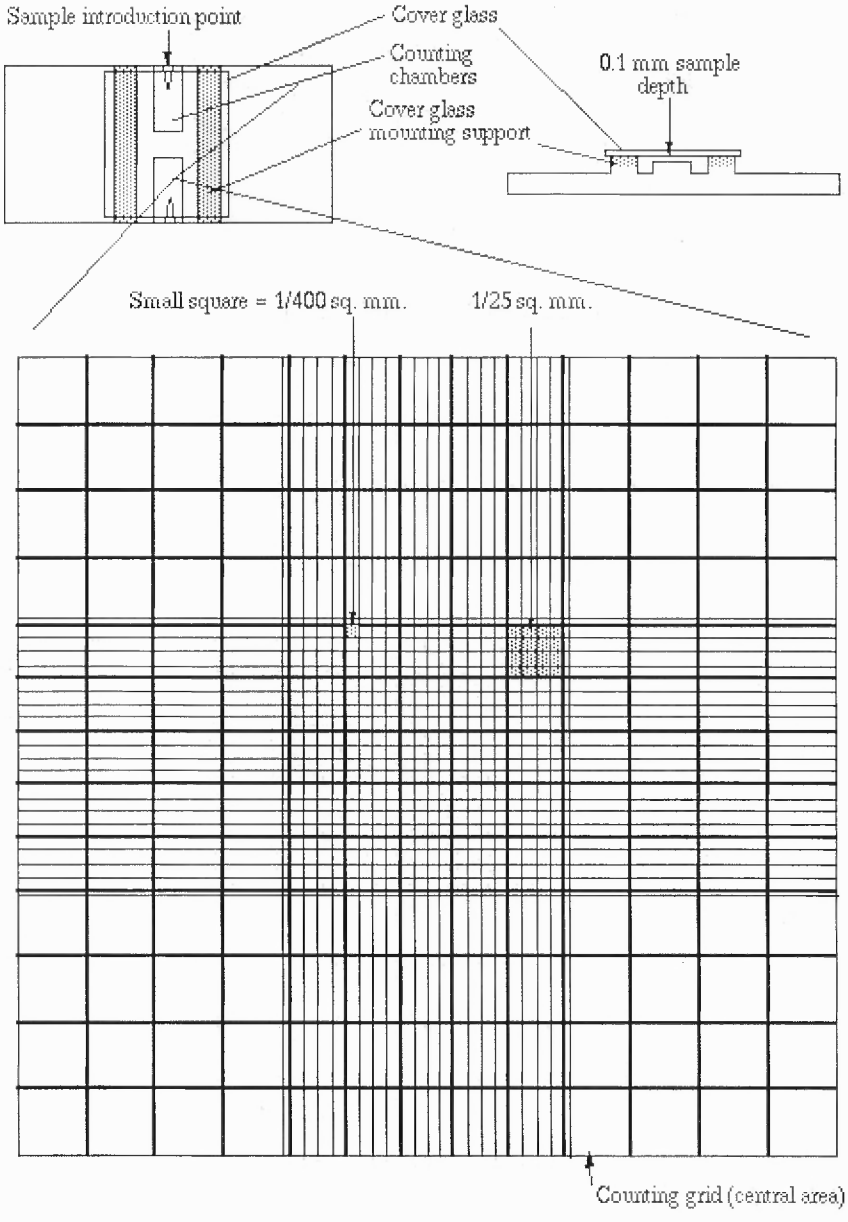


Figure 2.10 Hemocytometer with the counting grid [24].

2.1.7.3 Calculation. The cell concentration of the sample is calculated using formula:

c = n / v (cells/ml)

where c (cells/ml) is cell concentration, n is the cell count, and v (ml) is the volume counted. For the center square grid the volume is 0.0001 (ml).

Volume =1(mm) *1(mm) *0.1 (mm)

= 0.1(mm^3)

= 0.0001(ml)

The previous formula becomes:

c = n * 10000

2.1.7.4 Example.

Volume of cells = 10 (ml)

Count 1 = 443 (cells)

Count 2 = 483 (cells)

Average cell count = (Count 1 + Count 2) / 2

= 926 / 2 = 463 (cells)

Cell concentration = 463 x 10000

= 4,630,000 (cells/ml)

Total yield = c x v (cells)

= 4,630,000 (cells/ml) x 10 (ml)

= 46,300,000 (cells)

2.1.7.5 Example of Calculation of Number of Cells Needed per Cell Density.

Plating cell density = 200,000 (cells/cm^2)

Plating volume = 250 (µl/cm^2)

= 0.250 (ml/cm^2)

Well surface area = 2 (cm^2)

Well plating volume = 0.250 (ml/cm^2) * 2 (cm^2)

= 0.5 (ml)

Plating density/well = 400,000 (cells/2cm^2) in 0.5 (ml/2cm^2).

Cell concentration/well = 800,000 (cells/ml).

Plating factor pf = 800,000 (cells/ml) / 4,630,000 (cells/ml)

= 0.1728.

Total volume of cell suspension for plating is noted Vt

Volume of cell suspension for plating the PEEK is noted Vp

Volume of cell suspension for plating the control dishes is noted Vc

Vt = Vp + Vc

Vp = 24 (wells) * 0.5 (ml)

= 12 (ml)

Vc = 2 (control dishes) * 2 (ml)

= 4 (ml)

Vt = 12 (ml) + 4 (ml)

= 16 (ml)

The amount of cells necessary for this particular plating is noted V

V = Vt * pf

= 16 (ml) x 0.1728

= 2.7648 (ml)

Amount of media added = 16 (ml) – 2.7648 (ml)

= 13.2352 (ml)

2.1.8 Cell Plating

1. Mix (triturate) gently cell solution.
2. Dilute cell solution to desired density (200,000 cells / cm^2), volume = 250 ($\mu l/cm^2$). Density range: 180,000 – 200,000 (cells/cm^2).
3. Plate cells.
4. Incubate cell cultures (37°C, 5%CO2).
5. Change media after 24 hours and then every other day thereafter.
6. Verify cell density by counting cells in microscopic field of view and dividing by area of field of view.

Notes: Cultures should have minimal clustering and glial contamination (Figure 2.11).

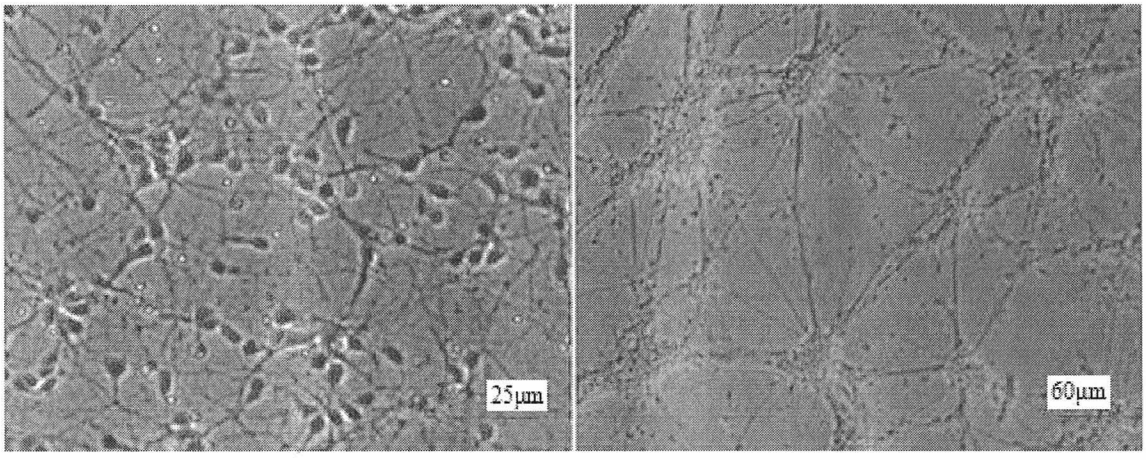


Figure 2.11 Low neuronal clustering (left) and high neuronal clustering (right).

2.1.9 Cell / DNA Staining

In order to view, qualify, and quantify cell cultures, various dyes, staining techniques, and microscopy are often utilized. For this particular experiment, Propidium Iodide and Acridine Orange were the two dyes chosen to stain the neuronal cell cultures. The viability assessment is performed after preliminary cell culture staining.

2.1.9.1 Cell Staining Procedure.

1. Make PI and AO stock solution of 5mg/ml (1000X) concentration. Sterile DI water can be used.
2. Store stock solutions in dark at 4°C. Wrap tubes in aluminum foil to protect from light.
3. Make PI and AO 5 µg/ml staining solutions by combining 10 µl PI stock solution, 10 µl AO stock solution, and 10 ml HBSS.
4. Work with light off in laminar flow hood.
5. Remove media from cell culture without letting it dry.
6. Rinse once with HBSS.
7. Add 1 ml staining solution to 35 mm culture dish and 0.5 ml staining solution to well.
8. Incubate cultures for 5-10 minutes at 37°C, 5%CO2.
9. Remove staining solution and rinse two times with HBSS.
10. Cover cell cultures with aluminum foil and take them to fluorescence microscope room for further investigation.

2.1.10 Viability Assessment (at 10 days *in vitro*)

The viability assessment is performed to determine the number of viable, living cells (Nv) in a cell solution (Nt).

Number of viable cells is noted Nv

Number of non-viable cells is noted Nn

Total number of cells is noted Nt

Nv = Nt – Nn

Viability (%) = Nv / Nt

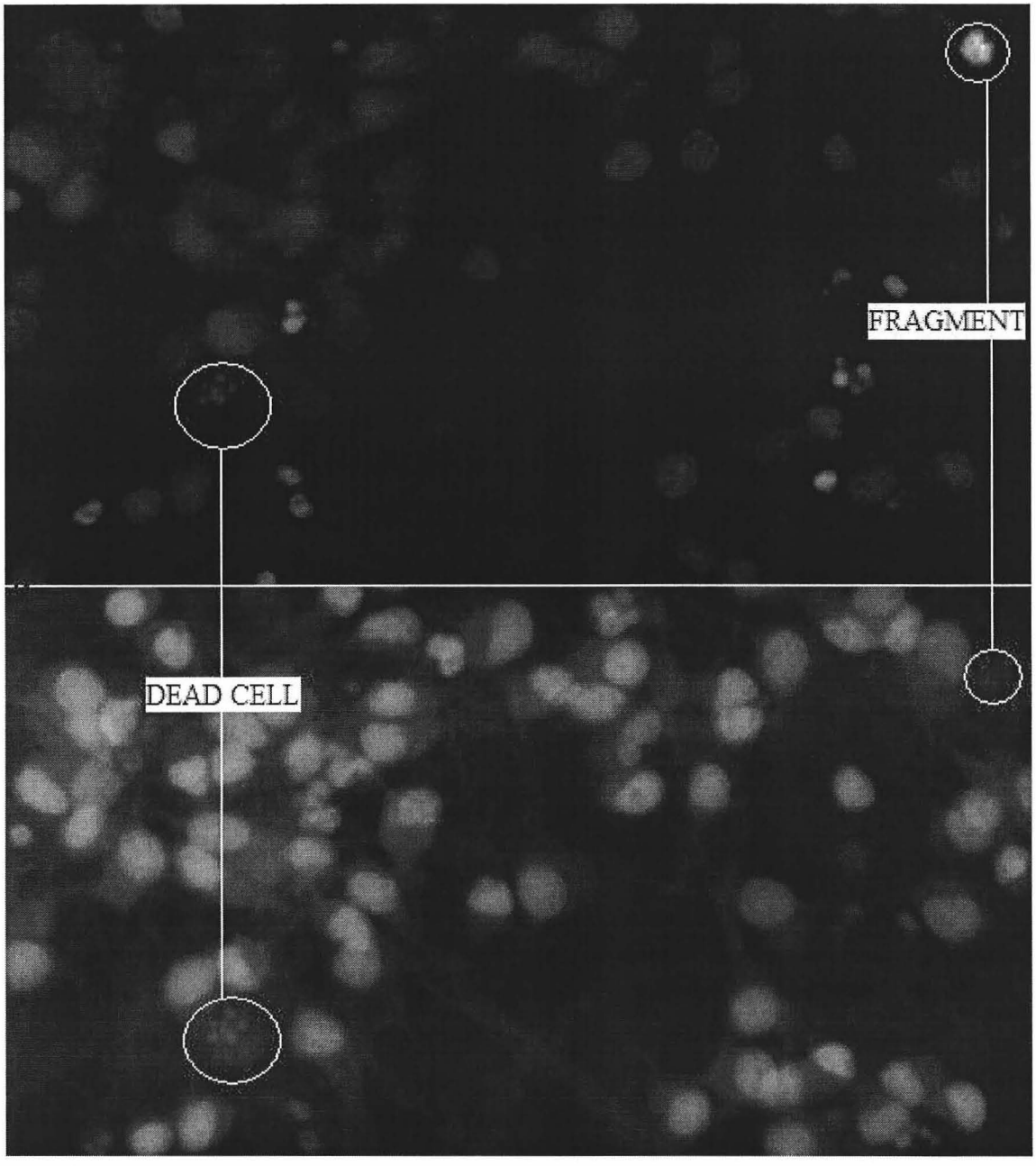


Figure 2.12 Dead cell (left, top and bottom images) and fragment (right, top image).

The differentiation between dead cells and fragments is really important for the viability calculations accuracy. Fragments are not counted and they do not influence the viability. The bottom picture (Figure 2.12) includes dead and live cells after staining with Acridine Orange. Top picture highlights just dead cells after staining with Propidium

Iodide. The cell underlined (left side of both pictures) is a cell that undergoes apoptosis. The typical pattern of a disintegrating nucleus can be easily observed. The fragment (right side of top picture) is noticed just in the top picture and is not considered in the viability counts.

2.1.11 Cell Free Zone (Axon Gap)

To selectively injure axons in uniaxial regime, a 1.5 mm cell free zone has been created on the silicone membrane of all or only the chosen wells of the 24 well culture plate. The barriers (dividers) that create the gap were made of ABS thermoplastic (acrylonitrile butadiene styrene). The ABS samples (Figure 2.13) had also to be tested in order to determine if they were biocompatible.

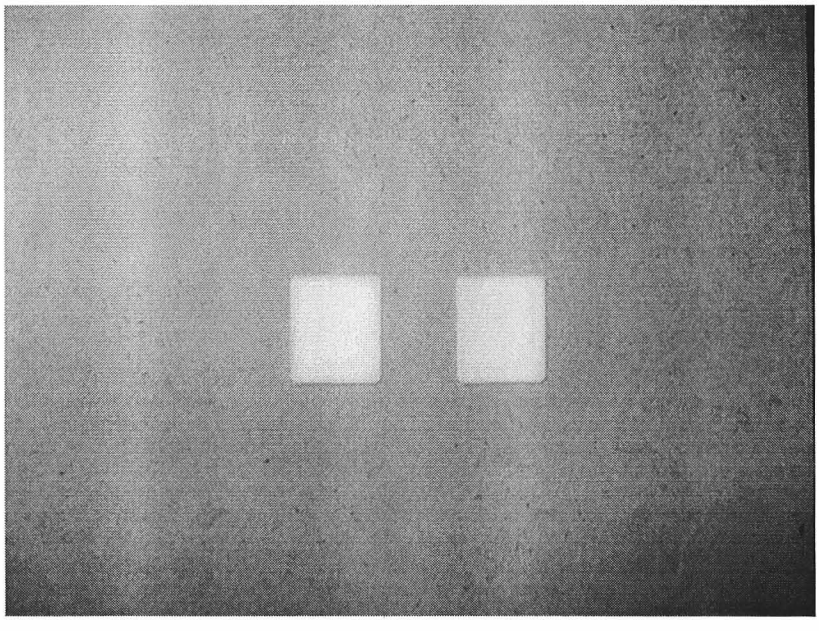


Figure 2.13 Two ABS dividers.

2.1.11.1 Cell Free Zone Procedure.

1. ABS sample dividers are sterilized (autoclaved). Other methods can be used (ultrasonics and 100% ethanol).

2. Dividers are inserted in all / selected wells of previously prepared 24 well culture plate (silicone membrane attached and PLL coated) (Figure 2.14).

3. Add (plate) cells to either side of barriers.

4. Let cells attach for approximately 4 hours. Incubate cell cultures (37°C, 5%CO2).

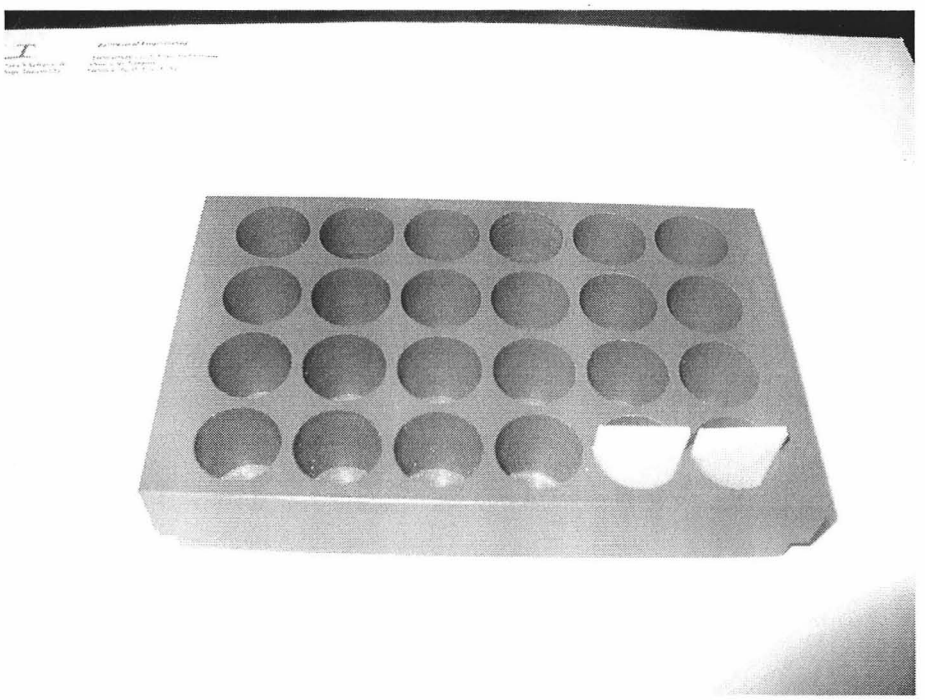


Figure 2.14 Two dividers inserted in a prepared 24 well culture plate.

5. Remove dividers.

6. Incubate cell cultures (37°C, 5%CO2).

7. Change media after 24 hours and every other day thereafter.

CHAPTER 3

RESULTS

3.1 The Attachment of the Silicone Membrane to the 24 Well Culture Plate

The attachment of the silicone membrane to the high throughput culture plate is a critical aspect in the cell culturing and injuring experiments. To prevent leaks, different glues and gluing techniques have been evaluated. The culture plate had the membrane glued to its base using Dow Corning RTV Sealant 734, and it was tested for leaks before plating. Upon careful examination, it was concluded that uneven sealant application, air bubbles, and membrane wrinkles were the main causes of leaking.

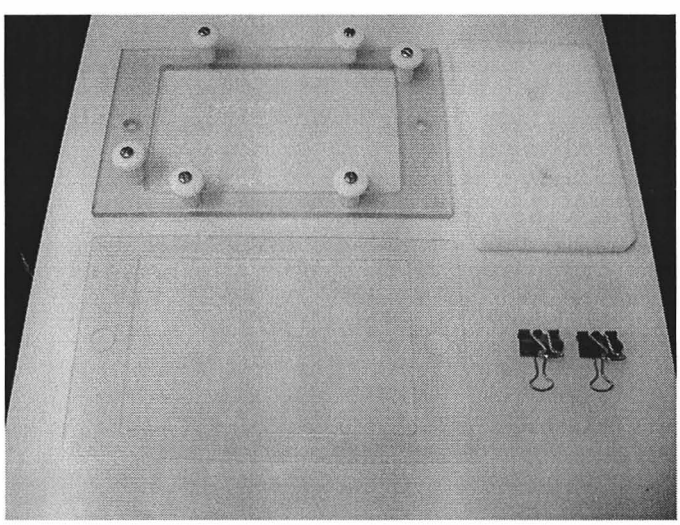


Figure 3.1 Frames, HDPE stretching block (upper right corner), and clips.

Two RTV silicone glues, Dow Corning RTV Sealant 734 and Dow Corning RTV Sealant 732, were tested to attach the silicone membrane to the culture plate. They had different viscosities and adhesive properties.

The Dow Corning RTV Sealant 732 had good adhesive properties and little glue accumulation in the wells of the plate. However, it was difficult to apply as a thin layer due to its higher viscosity. The air bubbles had a higher incidence in this case.

The Dow Corning RTV Sealant 734 (flowable version) offered the best results. A thin glue layer provided a good attachment of the membrane to the PEEK plate, with a minimum amount of glue oozing in the wells. The sealant was spread evenly over the bottom of the culture plate using a custom made roller. In the case when silicone sealant leaked into the wells, an applicator was utilized to remove the excess glue.

To reduce the membrane wrinkles and obtain a membrane that is uniformly stretched, a simple method was utilized. The silicone membrane was laid on a clean sheet of paper and all the wrinkles were brushed out. The top of the fixture frame was carefully placed on top of the membrane. If wrinkles were still present, the procedure was repeated. Soap, water, and the same brushing technique were utilized to eliminate the bubbles between the membrane and the stretching High Density Poly Ethylene (HDPE) block.

An additional measure was taken to check for leaks. 1 ml DI water was added to each well and the plate was autoclaved for 120 minutes at 128°C. The outcome was 99% positive. During the entire experiment only 6 wells out of a total of 480 wells (20 plates, 24 wells/plate) experienced leaks.

3.2 Primary Cortical Neuron Cell Culture Viability

Viability assays are used to determine the percentage of a cell suspension that is viable. The methods involved in measuring the viability of cells *in vitro* can be grouped in [25]:

1. Reproductive assays (clonogenic assays) are precise and time consuming methods used to find the number of cells capable of proliferation *in vitro*.

2. Metabolic assays involve cell metabolic activity. Tetrazolium salts are added to viable cells and transformed by the cellular mechanism in formazan dyes that are measured spectrophotometrically.

3. Direct proliferation assays measure DNA synthesis to determine cell growth.

4. Permeability assays are based on the principle of cell staining. In the dye exclusion staining for example, viable cells with intact membranes exclude the dye while damaged cells don't. The methods can be manual (trypan blue, hemocytometer) or mechanical (propidium iodide, flow cytometer). These methods, as opposed to the first three presented above, are simple, rapid, not expensive, and have been utilized to evaluate cell suspension concentration in the present paper.

In order to determine cell culture viability before and after injury at 10 DIV, dye uptake staining techniques (permeability assays) and fluorescence microscopy methods were employed. Propidium Iodide (PI) and Acridine Orange (AO) are utilized to stain the cell cultures and Nikon Eclipse TE 2000-2 microscope equipped with a () camera and () software to examine them (Figure 3.2).

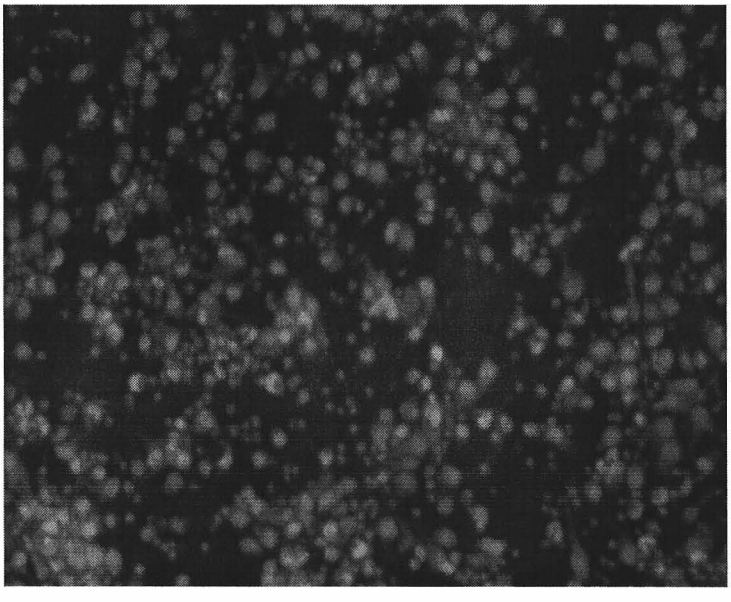


Figure 3.2 AO-stained viable cells (green) and PI-stained non-viable cells and fragments (red).

Propidium Iodide is a dye that absorbs the light in blue-green spectrum at 493 nm and fluorescence red at 630 nm. It readily enters and stains non-viable cells, but cannot cross the membrane of viable cells. For this reason it has been widely used for staining the DNA of fixed and unfixed cells [26].

Acridine Orange Hemi (Zinc Chloride) Salt is a fluorescent PH indicator that readily passes through a cell's membrane and labels all cells dead or alive. It is used to differentially stain the DNA and RNA of unfixed cells or as a viability stain. The AO-DNA complex is excited at 480 nm and emits at 510 nm as compared to the AO-RNA complex, which absorbs light at 440-470 nm and emits at 510 nm [27].

Pictures were taken in two different positions for each culture plate well. For every location, a picture was recorded for the total number of cells (viable and non-viable, AO stained) and a second picture, just for the non-viable cells (dead, PI stained). The pictures were analyzed and the cells counted with Image J (NIH Image), a Java image processing software (Figure 3.3).

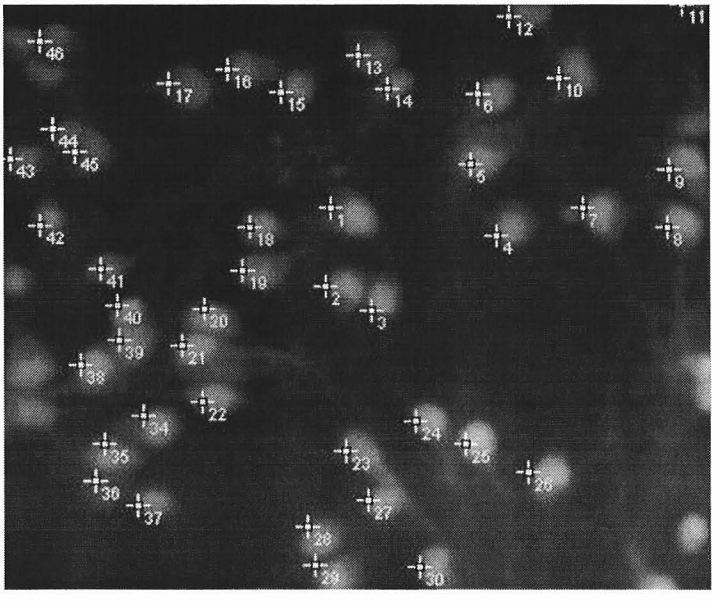


Figure 3.3 Counting cells using Image J.

The viability of the cell cultures was computed knowing the total number of cells Nt and the number of non-viable (dead) cells Nn, using the formula:

$$\text{Viability} = (Nt - Nn) / Nt.$$

3.2.1 Viability Assessment Before Injury

3.2.1.1 Experiment 1 at 10 DIV.

Cell plating density = 200,000 (cells / cm^2).

Average viability (%) = 97.84 (%).

Table 3.1 Viability Table for Experiment 1 Before Injury

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0101	317	4	313	0.9873817	98.74
0102	320	9	311	0.9718750	97.19
0201	464	10	454	0.9784483	97.84
0202	471	8	463	0.9830149	98.30
0301	472	8	464	0.9830508	98.31
0302	480	11	469	0.9770833	97.71
0401	404	10	394	0.9752475	97.52
0402	400	4	396	0.9900000	99.00
0501	424	12	412	0.9716981	97.17
0502	394	10	384	0.9746193	97.46
0601	480	6	474	0.9875000	98.75
0602	467	4	463	0.9914347	99.14
0701	420	4	416	0.9904762	99.05
0702	478	5	473	0.9895397	98.95
0801	344	14	330	0.9593023	95.93
0802	387	12	375	0.9689922	96.90
0901 handling issue	x	x	x	x	x
0902 handling issue	x	x	x	x	x
1001	314	14	300	0.9554140	95.54
1002	356	8	348	0.9775281	97.75
1101	319	12	307	0.9623824	96.24
1102	361	5	356	0.9861496	98.61
1201	417	6	411	0.9856115	98.56
1202	328	7	321	0.9786585	97.87
1301	397	13	384	0.9672544	96.73
1302	398	9	389	0.9773869	97.74

Table 3.1 Viability Table for Experiment 1 Before Injury (Continued)

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
1401	376	11	365	0.9707447	97.07
1402	396	7	389	0.9823232	98.23
1501	357	6	351	0.9831933	98.32
1502	343	8	335	0.9766764	97.67
1601	351	9	342	0.9743590	97.44
1602	417	14	403	0.9664269	96.64
1701	378	12	366	0.9682540	96.83
1702	381	8	373	0.9790026	97.90
1801	404	8	396	0.9801980	98.02
1802	358	6	352	0.9832402	98.32
1901	428	5	423	0.9883178	98.83
1902	432	6	426	0.9861111	98.61
2001	441	7	434	0.9841270	98.41
2002	443	9	434	0.9796840	97.97
2101	445	7	438	0.9842697	98.43
2102	434	10	424	0.9769585	97.70
2201	440	11	429	0.9750000	97.50
2202	435	12	423	0.9724138	97.24
2301	431	6	425	0.9860789	98.61
2302	409	9	400	0.9779951	97.80
2401	443	7	436	0.9841986	98.42
2402	438	11	427	0.9748858	97.49

3.2.1.2 Experiment 2 at 10 DIV.

Cell plating density = 200,000 (cells / cm^2).

Average viability (%) = 97.85 (%).

Table 3.2 Viability Table for Experiment 2 Before Injury

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0101	344	7	337	0.9796512	97.97
0102	488	9	479	0.9815574	98.16
0201	368	9	359	0.9755435	97.55
0202	464	9	455	0.9806034	98.06
0301	443	7	436	0.9841986	98.42
0302	368	8	360	0.9782609	97.83

Table 3.2 Viability Table for Experiment 2 Before Injury (Continued)

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0401	444	7	437	0.9842342	98.42
0402	409	9	400	0.9779951	97.80
0501	401	6	395	0.9850374	98.50
0502	407	7	400	0.9828010	98.28
0601	416	10	406	0.9759615	97.60
0602	419	8	411	0.9809069	98.09
0701	301	8	293	0.9734219	97.34
0702	311	7	304	0.9774920	97.75
0801	407	11	396	0.9729730	97.30
0802	387	9	378	0.9767442	97.67
0901	456	7	449	0.9846491	98.46
0902	384	5	379	0.9869792	98.70
1001	386	5	381	0.9870466	98.70
1002	439	7	432	0.9840547	98.41
1101	481	5	476	0.9896050	98.96
1102	468	6	462	0.9871795	98.72
1201	412	10	402	0.9757282	97.57
1202	420	12	408	0.9714286	97.14
1301	268	10	258	0.9626866	96.27
1302	387	12	375	0.9689922	96.90
1401	400	12	388	0.9700000	97.00
1402	403	8	395	0.9801489	98.01
1501	417	9	408	0.9784173	97.84
1502	389	6	383	0.9845758	98.46
1601	478	8	470	0.9832636	98.33
1602	451	9	442	0.9800443	98.00
1701	368	7	361	0.9809783	98.10
1702	481	10	471	0.9792100	97.92
1801	344	10	334	0.9709302	97.09
1802	317	12	305	0.9621451	96.21
1901	276	4	272	0.9855072	98.55
1902	269	4	265	0.9851301	98.51
2001	448	13	435	0.9709821	97.10
2002	469	14	455	0.9701493	97.01
2101	389	10	379	0.9742931	97.43
2102	486	17	469	0.9650206	96.50
2201	453	6	447	0.9867550	98.68
2202	431	7	424	0.9837587	98.38
2301	296	8	288	0.9729730	97.30
2302	330	5	325	0.9848485	98.48
2401	291	7	284	0.9759450	97.59

Table 3.2 Viability Table for Experiment 2 Before Injury (Continued)

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
2402	321	8	313	0.9750779	97.51

3.2.1.3 Experiment 3 at 10 DIV.

Cell plating density = 200,000 (cells / cm^2).

Average viability (%) = 97.20 (%).

Half of the culture plate was used for cell free zone experimenting.

Table 3.3 Viability Table for Experiment 3 Before Injury

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0101	468	9	459	0.9807692	98.08
0102	452	10	442	0.9778761	97.79
0201	483	12	471	0.9751553	97.52
0202	492	15	477	0.9695122	96.95
0301	495	12	483	0.9757576	97.58
0302	421	15	406	0.9643705	96.44
0401	367	11	356	0.9700272	97.00
0402	403	12	391	0.9702233	97.02
0501	398	7	391	0.9824121	98.24
0502	475	14	461	0.9705263	97.05
0601	501	13	488	0.9740519	97.41
0602	506	11	495	0.9782609	97.83
0701	498	16	482	0.9678715	96.79
0702	466	10	456	0.9785408	97.85
0801	395	13	382	0.9670886	96.71
0802	458	15	443	0.9672489	96.72
0901	431	16	415	0.9628770	96.29
0902	408	16	392	0.9607843	96.08
1001	414	10	404	0.9758454	97.58
1002	470	14	456	0.9702128	97.02
1101	451	15	436	0.9667406	96.67
1102	445	11	434	0.9752809	97.53
1201	398	9	389	0.9773869	97.74
1202	464	14	450	0.9698276	96.98

3.2.2 Viability Assessment After Injury

Cell cultures were injured at 10 DIV using the NJIT injury device and viability was evaluated 24 hours after the injury. The two important parameters, which determine the membrane deformation and implicitly the injury, are the rise time of the pressure pulse (rate of stretch) and the peak pressure. The approximate values for this experiment were 1-2 psi chamber pressure and approximately 20 ms (Figure 3.4).

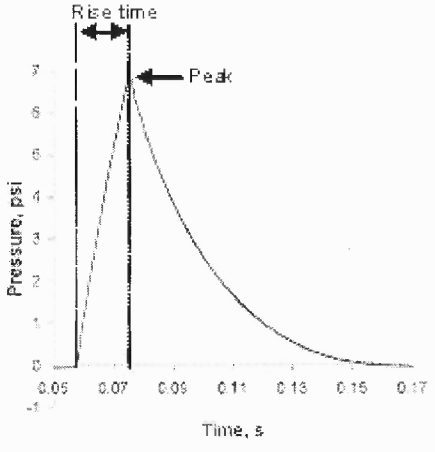


Figure 3.4 Value of the pressure pulse from Penn Device recorded by Dr. Bryan Pfister.

The same methodology (staining, taking pictures, and counting the cells) was employed for after injury experiments as for before injury experiments.

3.2.2.1 Experiment 4 – Calibration - 24 Hours After Injury at 10 DIV.

Cell plating density = 200,000 (cells / cm²).

Average viability (%) = 66.81 (%).

Table 3.4 Viability Table for Experiment 4 After Injury

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0101	450	140	310	0.688888889	68.89
0201	481	71	410	0.852390852	85.24

Table 3.4 Viability Table for Experiment 4 After Injury (Continued)

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0301	458	132	326	0.711790393	71.18
0401	482	193	289	0.599585062	59.96
0501	420	208	212	0.504761905	50.48
0601	459	158	301	0.655773420	65.58
0701	397	145	252	0.634760705	63.48
0801	513	160	353	0.688109162	68.81
0901	474	190	284	0.599156118	59.92
1001	492	191	301	0.611788618	61.18
1101	356	169	187	0.525280899	52.53
1201	483	171	312	0.645962733	64.60
1301	440	119	321	0.729545455	72.95
1401	483	151	332	0.687370600	68.74
1501	533	135	398	0.746716698	74.67
1601	451	103	348	0.771618625	77.16
1701	438	157	281	0.641552511	64.16
1801	417	121	296	0.709832134	70.98
1901	469	166	303	0.646055437	64.61
2001	501	133	368	0.734530938	73.45
2101	509	173	336	0.660117878	66.01
2201	399	123	276	0.691729323	69.17
2301	369	113	256	0.693766938	69.38
2401	405	161	244	0.602469136	60.25

3.2.2.2 Experiment 5 - 24 Hours After Injury at 10 DIV.

Cell plating density = 200,000 (cells / cm^2).

Average viability (%) = 86.74 (%).

Table 3.5 Viability Table for Experiment 5 After Injury

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0101	560	78	482	0.860714286	86.07
0201	460	63	397	0.863043478	86.30
0301	348	40	308	0.885057471	88.51
0401	509	81	428	0.840864440	84.09
0501	486	48	438	0.901234568	90.12
0601	461	33	428	0.928416486	92.84

Table 3.5 Viability Table for Experiment 5 After Injury (Continued)

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0701	336	41	295	0.877976190	87.80
0801	480	66	414	0.862500000	86.25
0901	499	78	421	0.843687375	84.37
1001	392	89	303	0.772959184	77.30
1101	508	83	425	0.836614173	83.66
1201	495	32	463	0.935353535	93.54

3.2.2.3 Experiment 6 - 24 Hours After Injury at 10 DIV.

Cell plating density = 200,000 (cells / cm^2).

Average viability (%) = 83.55 (%).

Table 3.6 Viability Table for Experiment 6 After Injury

Well Number / Measurement Number	Total Cell Number Nt	Dead Cell Number Nn	Live Cell Number Nt-Nn	Cell Viability (Nt-Nn)/Nt	Cell Viability (%)
0101	471	59	412	0.874734607	87.47
0201	324	38	286	0.882716049	88.27
0301	281	79	202	0.718861210	71.89
0401	404	103	301	0.745049505	74.50
0501	507	111	396	0.781065089	78.11
0601	491	87	404	0.822810591	82.28
0701	549	53	496	0.903460838	90.35
0801	371	43	328	0.884097035	88.41
0901	407	53	354	0.869778870	86.98
1001	431	83	348	0.807424594	80.74
1101	488	85	403	0.825819672	82.58
1201	530	141	389	0.733962264	73.40
1301	436	38	398	0.912844037	91.28
1401	512	94	418	0.816406250	81.64
1501	486	103	383	0.788065844	78.81
1601	394	28	366	0.928934010	92.89
1701	347	49	298	0.858789625	85.88
1801	412	48	364	0.883495146	88.35
C1	445	17	428	0.961797753	96.18
C2	421	12	409	0.971496437	97.15
C3	431	14	417	0.967517401	96.75

3.3 Cell Free Zone

At cellular level, TBI translates in uniaxial stretch of the axons aligned diagonally in the piece of brain tissue examined. In order to reproduce the axon stretching *in vitro* and to injure the axons only, a 1.5 mm cell free zone (axon gap) was created on the silicone membrane of the culture plate. The barrier that creates the gap prevents neurons from attaching in the gap region.

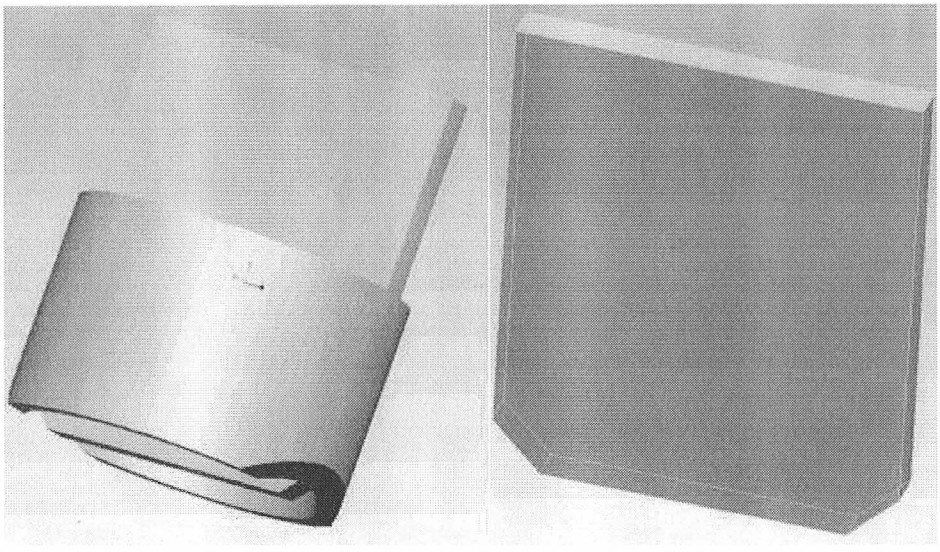


Figure 3.5 ProE design of two divider models.

Pro/ENGINEER (ProE) modeling software (Parametric Technology Corporation) was used to design few divider models. Other CAD/CAM software packages can be also utilized. The barrier designs were loaded on a 3D printing machine Dimension 768 SST (soluble support material), which produced a number of samples made of ABS thermoplastic (acrylonitrile butadiene styrene) (Figure 3.5).

After the culture plate was previously prepared (coated with PLL), the dividers were autoclaved and inserted in the selected wells. Some of the ABS dividers deformed in the autoclaving process. The second set of ABS dividers was successfully sterilized

using 100% ethanol and ultrasonics. The barriers were left in the culture plate for about four hours to allow the cells plated on either side to attach. After the barriers were removed, the axons began to cross the gap, reaching the neurons in the other side after 10 DIV, and integrated with them (Figure 3.6).

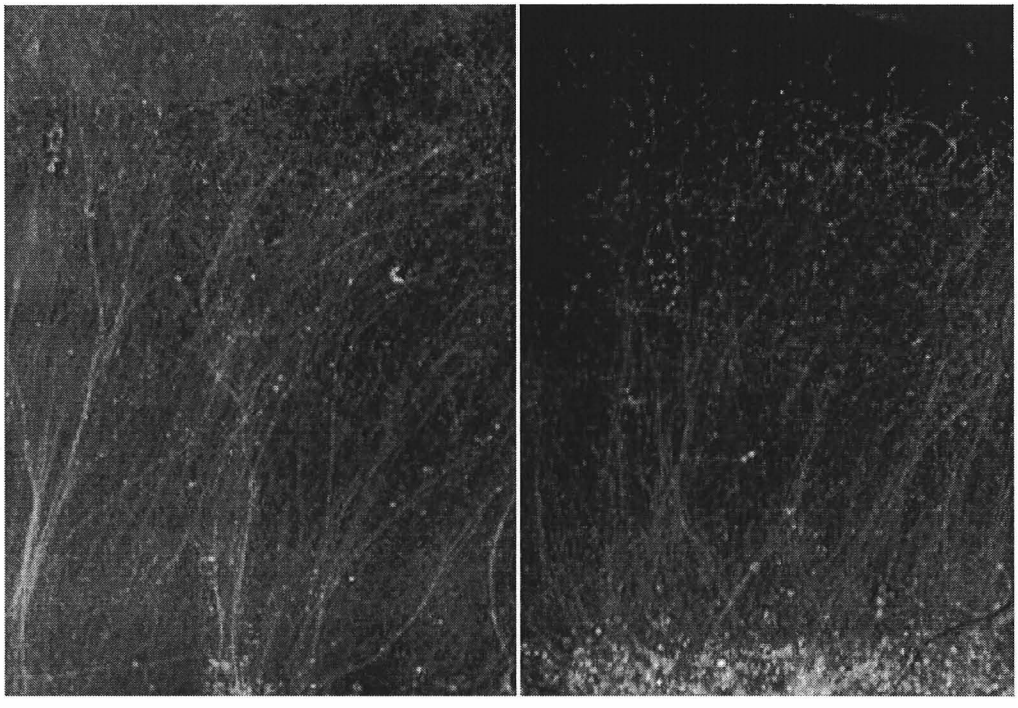


Figure 3.6 Axons crossing the gap in a 10 DIV (days *in vitro*) culture.

To complete the design, a metallic mask (Aluminum) was created to allow the selective deformation of the silicone membrane of the wells that had the axon gap (Figure 1.5). The mask is placed underneath the culture plate in the injury device.

The ABS samples utilized to create the axon gap did not cause toxicity. The cells plated in the wells where the dividers were present looked normal. It also has to be determined in future studies if cell culture plates made of ABS plastic are appropriate for neuronal cultures.

3.4 Biocompatibility of Used Materials

Evaluation of the biocompatibility of materials utilized in the experiments presented above has been done *in vitro*, using neuronal cell cultures. The primary cortical neurons from E17 rats have been cultured in custom made culture plates made of PEEK. Biocompatibility of the PEEK plate, the silicone membrane bottom, the silicone glue, and the ABS dividers was remarkable. The cell cultures were healthy at 10 DIV, displaying a good level of cellular development. The neurons grew strong axons able reach the neighboring neurons and synapse with them. The cell adhesion to the silicone membrane, previously coated with PLL, was also very good. The viability assessment confirmed all these observations. In all performed experiments viability was over 97%.

CHAPTER 4

DISCUSSION

TBI is a serious medical condition that requires thorough investigation. The most important goal of this project was to establish that the new and original NJIT injury device, designed to investigate TBI, was operational and highly productive.

A multiwell culture plate, made of PEEK, was created to culture and injure simultaneously, a high number of neurons. PEEK proved to be the right material for this specific application. It is highly biocompatible, autoclavable, and can be reused after sterilization without causing contamination. The preparation of the plate began with the attachment of the elastic silicone membrane to its bottom. The flowable version of Dow Corning RTV Sealant 732 was chosen for this task, due to the right balance between its viscosity and adhesive properties. The viability assessment before injury confirmed the fact that the high throughput culture plate was biocompatible and appropriate for cell culturing.

As stated before, healthy cell cultures were necessary for the evaluation of the NJIT injury device. Assembled 24 well injury plates were cultured with primary cortical neurons (E17 rat embryos) and tested for cell viability at 10 DIV. The neuronal cell culture development was achieved for the first time in NJIT laboratories. This complex procedure requested elaborate work and the creation of a large set of protocols. Every step of the process – harvest, isolation, dissociation, plating, staining, and the other preparatory steps – had to be designed independently, for this particular type of cell. Optimization was attained through trial and error. The cell culture viability before and

after injury was estimated using live/dead assays. The values ranged between 97.20% and 97.85% before injury and significantly lower after injury, demonstrating that the injury system was functional (Table 4.1).

Table 4.1 Average Viability Table Before and After Injury

Experiment Number	1	2	3	4	5	6
Average Viability Before Injury (%)	97.84	97.85	97.20	n/a	n/a	n/a
Average Viability After Injury (%)	n/a	n/a	n/a	66.81	86.74	83.55

The main purpose for the first three experiments was to show that rat cortical neurons could be maintained healthy for at least 10 DIV, after plating in the new injury plate. The high viability numbers of over 97% supported the idea that the methodology, the injury plate, and the materials involved in the process were appropriate for this goal.

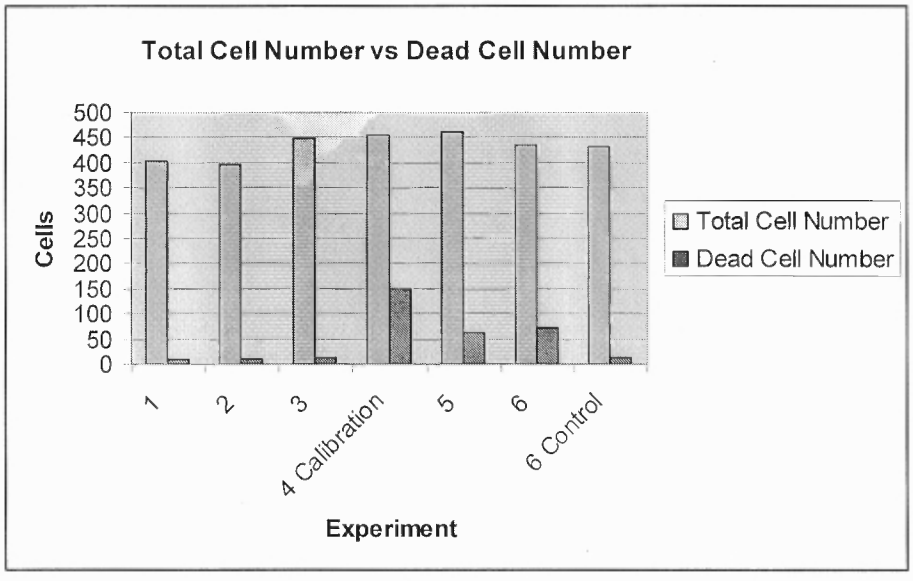


Figure 4.1 Total Cell Number vs. Dead Cell Number.

The following three experiments aimed to prove that the NJIT device could be used to injure neuronal cultures. First culture injury (experiment number 4) was a

calibration experiment. That explains the high number of dead cells and the very low cell culture viability (Figure 4.1).

Lower values for the pressure pulse were used in experiments number 5 and 6 and proportionally, higher viability numbers (around 85%) were recorded. Furthermore, a number of 3 cells were masked (covered with tape) and used as controls in experiment 6. The average numbers of live and dead cells, as well as the viability were expectedly very close to the values measured in first 3 experiments (Table 4.2).

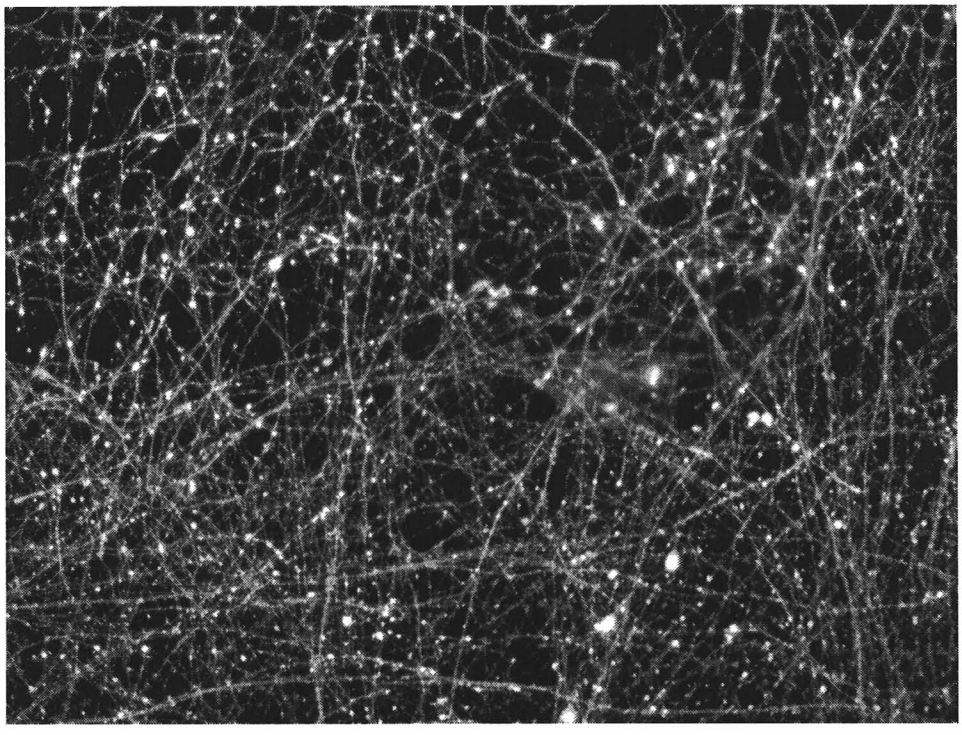
Table 4.2 Data Summary for the 6 Experiments

Experiment	Average Total Cell Number	Average Dead Cell Number	Average Live Cell Number	Average Viability (%)	STD
1	404.17	8.57	395.61	97.84	0.84
2	395.52	8.42	387.10	97.85	0.67
3	448.29	12.50	435.79	97.20	0.58
4 Calibration	453.29	149.29	304.00	66.81	7.58
5	461.17	61.00	400.17	86.74	4.38
6	435.61	71.94	363.67	83.55	6.33
6 Control	432.33	14.33	418.00	96.69	0.49

The standard deviation numbers calculated for the cell culture viability in experiments 4, 5, and 6 could be explained by the fact that not all the wells of the culture plate were uniformly injured. The tension of the silicone membrane is typically higher in the corner wells. In order to minimize the tension differences among the wells of the plate, the shape of the tensioning device was changed from rectangular to square. However, after comparing the viability numbers of the corner wells with the rest of the wells, no injury pattern could be established.

Additionally, in experiments 5 and 6 multiple wells were prepared to make the cell free zone. The axons grew and crossed the gap created by special dividers inserted in

the middle of the chosen wells. The images of these axons after injury could represent multiple foci of swellings along the axons, phenomenon cited in literature (Picture 4.2) [3]. Further studies are needed to investigate the nature of these axonal swellings.



Picture 4.2 Axons After Injury.

In summary, the present results suggest that the new set of protocols developed to culture rat cortical neurons in NJIT laboratories is valid. It offers a reliable work platform that is supported by present successful experimentation. Ultimately, the NJIT injury system is functional, providing the means to resolve the various aspects of TBI.

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